UNIVERSITÀ DEGLI STUDI FIRENZE

DOTTORATO DI RICERCA TOSCANO IN NEUROSCIENZE

CICLO XXXIV

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Adenosine A_{2B} receptors and carbonic anhydrase: new therapeutic targets for cerebral ischemia

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Introduction.

Chapter I: Cerebral ischemia

Stroke is a cerebrovascular disease affecting blood supply to the brain. With an incidence of approximately 240 per 100.000 and a mortality of about 30% (Grysiewicz et al., 2008), brain ischemia is the second leading cause of death worldwide (Mozaffarian et al., 2015) and the third cause of disability (Murray & Holmes, 2011). The prevalence of stroke is approximately 27% for individuals older than 80 years of age, and 13% for individuals 60 to 79 years of age (Grysiewicz et al., 2008). Since ageing and growth of the population is expected to increase in the coming years, with subsequent social and economic cost due to post-stroke care, stroke is nowadays considered a global burden and a major health problem.

Several neurological complications and clinical manifestations may occur following stroke, depending on the brain region affected, degree and duration of the ischemic event. Deficits after cerebral ischemia can include depression, partial paralysis, difficulties in coordination, memory, and language.

Stroke can be classified into two main types: ischemic and hemorrhagic.

Hemorrhagic stroke accounts for about 20% of the total stroke cases (Sudlow & Warlow, 1997; Virani et al., 2021), it can be subarachnoid or intracerebral and is caused by the rupture of a cerebral blood vessel or an aneurysm within the blood vessel, resulting in an accumulation of blood and compression of brain tissue (Levine, 2004; Sacco et al., 1989).

Ischemic stroke accounts for 80% of all stroke cases (Sudlow & Warlow, 1997; Virani et al., 2021) and is caused by occlusion of a major cerebral artery by a thrombus or an embolism, which leads to loss of cerebral blood flow, a condition of hypoxia and glucose deprivation (oxygen, glucose deprivation: OGD) and subsequently tissue damage in the affected region (Gibson, 2013).

Ischemic brain injury results from a complex sequence of pathophysiological events that evolve over time and space (Figure 1).

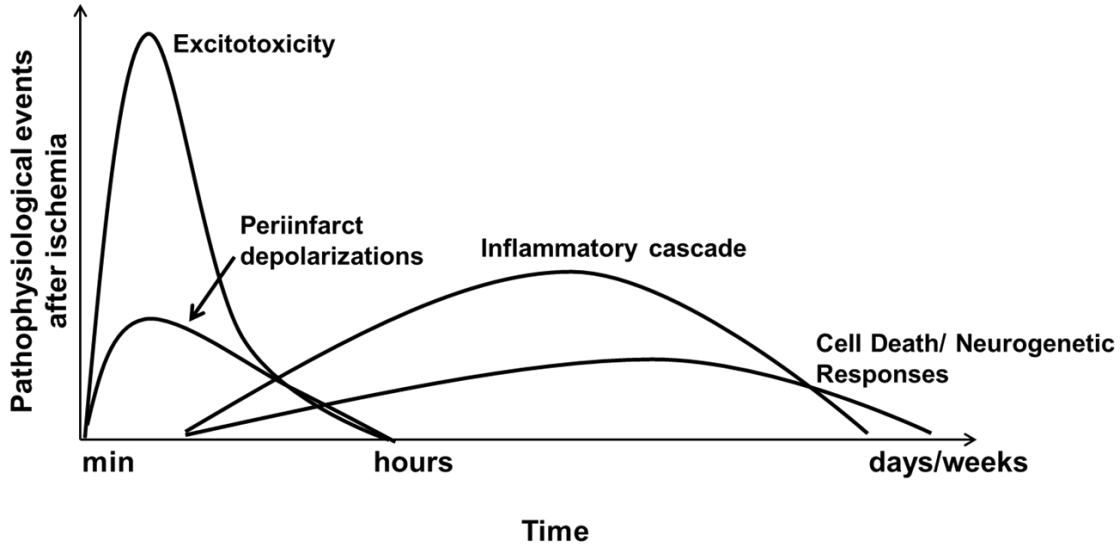

Figure 1. Cascade of pathogenetic mechanisms after ischemia. Primary mechanisms of excitotoxicity lead to acute cell death. Depolarization spreads in the peri-infarct areas. In the following several hours, activation of resident immune cells, i.e. microglia and a cascade of production of inflammation mediators occurs. Cell death/neurogenetic responses progress along days/weeks after ischemia. Modified from (Dirnagl et al., 1999).

The major pathogenic mechanisms of this cascade include acute excitotoxicity and peri-infarct depolarizations followed by activation of resident immune cells, i.e microglia, and production or activation of inflammation mediators (Dirnagl et al., 1999). Pro-inflammatory cytokines, that upregulate cell adhesion molecules, exert an important role in promoting neutrophil infiltration and accumulation in brain parenchyma (Huang et al., 2006; Stoll et al., 1998). Although the activation of immune cells after cerebral ischemia may be neuroprotective and contributes to cerebral tissue repair, protected neuroinflammation is now recognized as the predominant mechanism of secondary brain injury progression (Ma et al., 2017).

Following a focal stroke, the ischemic lesion can be divided in two distinct regions (Figure 2). The center of the ischemic area is termed ischemic *core* and corresponds to the central perfusion territory of the affected artery, in which blood flow reduction is more severe (~15% of control), and leads to rapid energy failure and cell death (Hossmann, 1994).

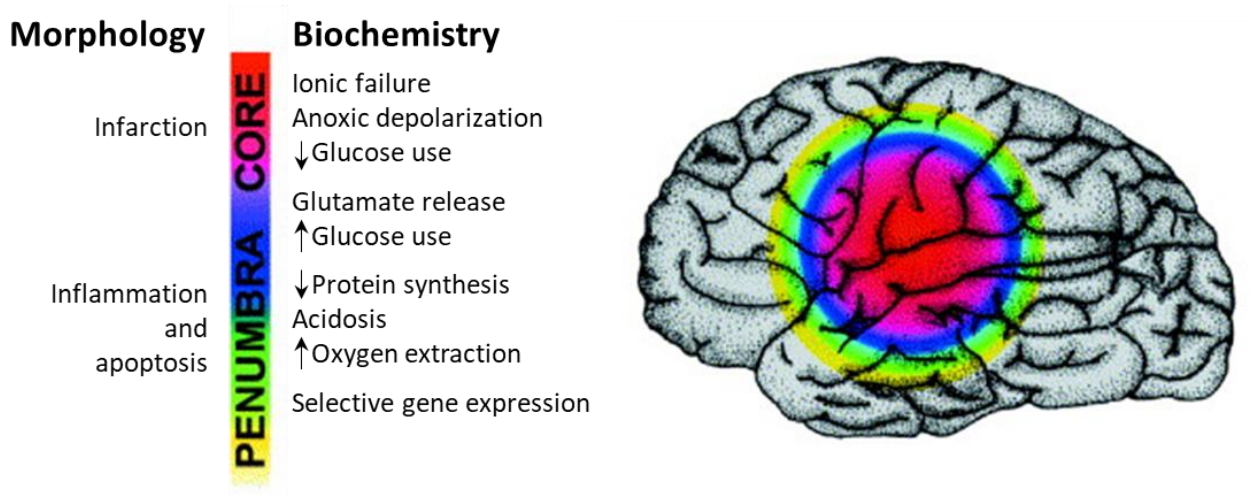

Figure 2. The red area on the figure marks the infarcted *core*, a brain region of low perfusion in which cells have lost their membrane potential. The ischemic *penumbra*, marked by the remaining colors, is potentially salvageable if an appropriate treatment is administered within a specified therapeutic window in which intermediate perfusion prevails and identified as the salvageable brain area. Modified from (Dirnagl et al., 1999).

The area surrounding the ischemic *core* is termed ischemic *penumbra* and represents the most salvageable area, where the initial fall in cerebral blood flow is graded, and electrical functions are lost but energy metabolites and ion homeostasis are not significantly altered (Astrup et al., 1981). It is estimated that initially the area of *penumbra* corresponds to 50% of the tissue, but if reperfusion does

not occur the *penumbra* will progress into infarct between 4 and 8 hours after stroke onset (Dirnagl et al., 1999; Hossmann, 2006) (Figure 3). The main goal of a neuroprotective pharmacological treatment is therefore to recover the area of ischemic *penumbra* from an irreversible neurodegeneration.

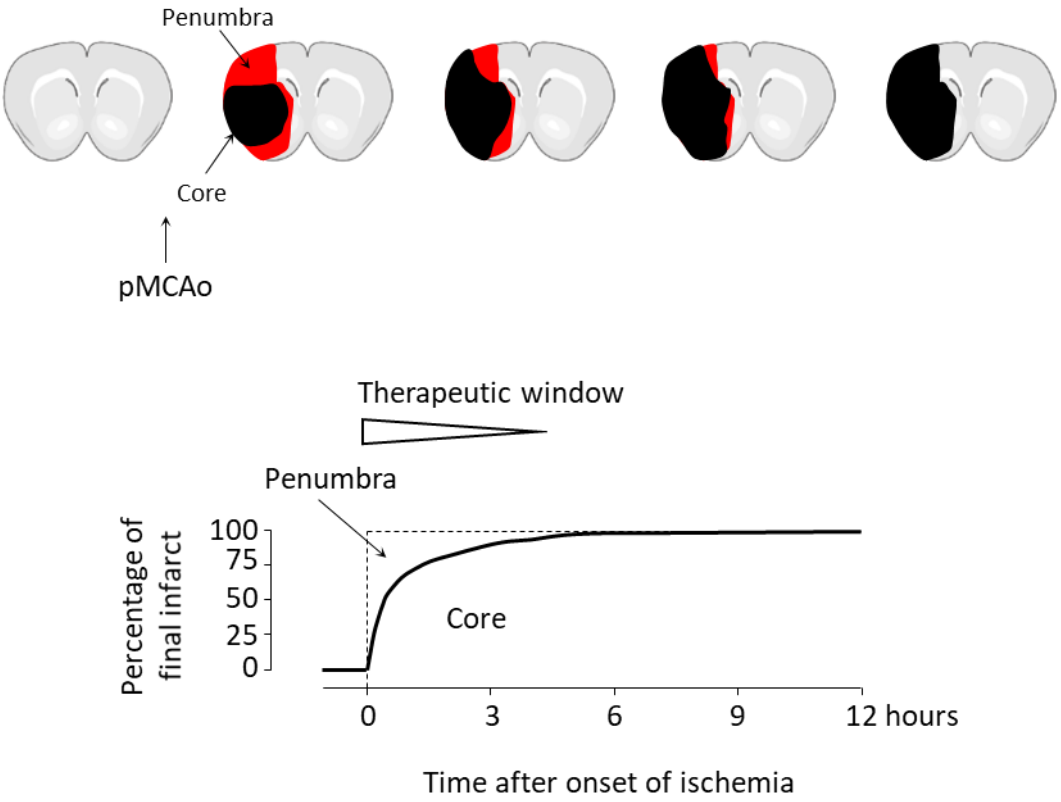

Figure 3. Schematic representation of infarct evolution during permanent middle cerebral artery occlusion (pMCAo). *Core* and *penumbra* are differentiated by biochemical and magnetic resonance imaging according to established viability thresholds of brain ischemia. With ongoing ischemia, the *core* expands into the *penumbra* until—within ~3 hours—infarct reaches its maximum volume. This time is the therapeutic window during which infarct expansion, and hence final infarct volume, can be alleviated. Modified from (Hossmann et al., 2012).

Despite advances in the understanding of the pathophysiology of cerebral ischemia, therapeutic strategies for acute ischemic stroke still remain very limited. Indeed, in the last years basic research yielded numerous pharmacologic agents leading to the identification of more than 1000 molecules with brain-protective effects in animal models of brain ischemia and to the implementation of more than 250 clinical trials (Thauerer et al., 2012). However, drugs have failed to be efficacious during clinical trials and the only successful pharmacological treatment approved to date is tissue plasminogen activator (t-PA), a specific enzyme that enhances the endogenous formation of plasmin from plasminogen, thus enabling restoration of blood flow by dissolving the intravascular clot of fibrin, an essential protein in blood clotting (MacRae, 2011). The main issue of this treatment is that in order

to perform this function t-PA must be necessarily administered in the first 4-4.5 hours after symptoms onset (Rabinstein, 2020). Because of its short therapeutic window and the hemorrhagic complications that its use may cause, the number of patients who might receive treatment and therefore potentially benefit is very restricted in clinical settings (Chen et al., 2014). Consequently, besides preventive stroke therapy with drugs like aspirin and other antiplatelets and anticoagulants (Albers et al., 2011; Macrez et al., 2011), search for successful treatment of acute ischemic stroke still remains one of the major challenges in clinical medicine. In the last years a particular attention has been given to the search of a suitable adjuvant therapy to t-PA, especially to neuroprotective strategy, unfortunately without success so far.

1.1 Pathobiology of brain ischemia

Cerebral ischemia triggers a series of functional and biochemical changes. Initially the decrease in blood flow affected neurological function, and subsequently metabolic activity is suppressed in order to maintain the structural integrity of the cells (Hossmann, 1994). This leads to activation of several signaling pathways that may be neuroprotective or harmful towards the brain. These events that include excitotoxicity, ionic imbalances, oxidative stress, inflammatory reactions and inflammatory cascade, occur simultaneously and depend on the intensity and duration of the ischemic insult.

1.1.1 Changes in cellular homeostasis

Although human brain account only for a few percentage of body weight (~2%), it has a high metabolic rate and requires about 20% of total oxygen (Magistretti & Pellerin, 1996). In contrast to other organs, brain uses almost only glucose for metabolism and has minimal storage capacities of energy substrates, thus it requires a constant blood supply containing an adequate glucose and oxygen concentration to maintain its function and structural integrity (Markus, 2004). All these characteristics render brain tissue highly vulnerable to ischemia, such that even transient loss of oxygen and glucose to the cerebral tissue may culminate in cellular death (Woodruff et al., 2011).

As a consequence of the reduction of cerebral blood flow and subsequent oxygen depletion during ischemia, mitochondrial oxidative phosphorylation is interrupted, thus resulting in a rapid decline in cellular level of ATP (Katsura et al., 1994; Martin et al., 1994; Rama & Garcia Rodriguez, 2012). Low blood flow values caused by ischemia induce energy failure, thus an increase of extracellular potassium ions and increase of sodium ions ensue into cells with subsequent plasma membrane depolarization (Rama & Garcia Rodriguez, 2012). Furthermore, reduction in ATP level further stimulates the glycolytic metabolism of residual glucose, which leads to protons and lactate accumulation and thus to rapid

intracellular acidification and further reduction in cellular ATP production (Martin et al., 1994). Indeed, it has been observed that the lactate content during ischemia rises to ~20 mM (normal conditions ~15-20 mM), leading to a pronounced acidosis (Katsura et al., 1999). The accumulation of lactic acid in ischemic brain greatly disturbs the normal H^+ homeostasis, and it has been suggested that excessive lactic acidosis facilitates ischemic mechanisms leading to cerebral infarction (Plum, 1983). Several studies have underlined the importance of acidosis to the evolution of ischemic brain injury, since pH regulates several cellular processes (Busa & Nuccitelli, 1984) and modulates the activity of many enzymes and ion channels (Heales, 1999).

pH in the brain is strictly regulated by active (ion pump transport) and passive (ion channel transport, intracellular buffer solution) mechanisms, which maintain brain pH at approximately 7.2. (Casey et al., 2010). The main mechanisms underlining pH regulation are summarized in Figure 4.

The ischemic insult cause phosphocreatine stores depletion, intracellular CO_2 accumulation and ATP and glucose reduction, leading to excessive production of H^+, and thus pH may fall to 6.5.

Brain pH alteration after ischemic damage have been well documented by Back and co-workers in a model of permanent middle cerebral artery occlusion (MCAo), in which areas of acidification and alkalization can be detected in ischemic *penumbra* surrounding the markedly acid *core* (Back et al., 2000).

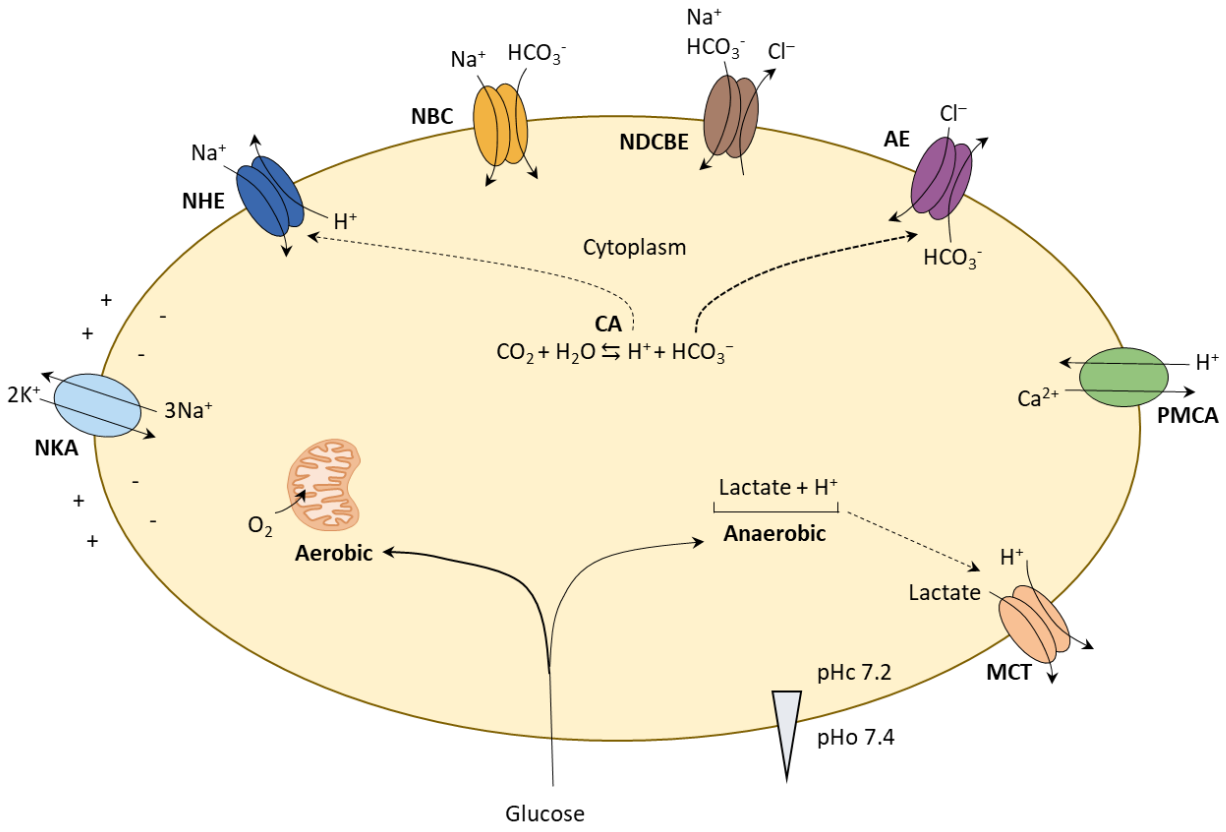

Figure 4. Cytoplasmatic pH regulation. The cytoplasm tends to acidify owing to the activities of various metabolic pathways, such as the ATP production in the cytoplasm by glycolysis that generates lactate (the anaerobic component in the figure) and the oxidative phosphorylation in mitochondria that produces CO_2 (the aerobic component shown in the figure). The predominant pH-regulatory transporters that are responsible for alkalinization are the plasma membrane Na^+ –H^+ exchangers (NHEs) and Na^+–HCO_3^-– co-transporters (NBCs). A limited number of cell types also alkalinize their cytosol through the actions of Na^+-dependent Cl^-–HCO_3^- – exchangers (NDCBEs). These transporters use the energy stored in the inwardly directed electrochemical Na^+ gradient that is established by Na^+–K^+-ATPase pumps (NKAs) to drive solute transport. These alkalinizing mechanisms are counterbalanced by the actions of plasma membrane Cl^-–HCO_3^- – or anion exchangers (AEs), which acidify the cell. Plasma membrane Ca^{2+}-ATPases (PMCAs), which exchange cytosolic Ca^{2+} for extracellular H^+, also acidify the cytosol in response to stimuli that elevate intracellular Ca^{2+}. In tissues undergoing anaerobic metabolism, alkalinization is favored by monocarboxylate–H^+ co-transporters (MCTs). CA, carbonic anhydrase; pHc, cytosolic pH; pHo, extracellular pH. Modified from (Casey et al., 2010).

1.1.2 Glutamate-mediated excitotoxicity

Glutamate is the predominant excitatory transmitter in the mammalian central nervous system (CNS) and acts at a range of different receptor types. Under physiological conditions, the extracellular concentration of glutamate is maintained in the micromolar range by neuronal and glial uptake systems (Bralet et al., 1992). Under ischemic conditions, energy failure leads to depolarization of cell membrane and subsequent Ca^{2+} entry in the cell through voltage-gated channels. Thus, glutamate is massively released (initially mediated by vesicular release from nerve terminals, and later by reverse transport from astrocytes), reaching millimolar concentrations in the extracellular space (Giffard & Swanson, 2005; Mena et al., 2000). This accumulation of extracellular glutamate overstimulates N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate-type glutamate receptors, promoting an increased permeability to Na^+, K^+ and Ca^{2+} ions which further depolarize the cell membrane. It is thought that most of the neuronal degeneration is due to the intense activation of NMDA receptors. Moreover, through metabotropic receptors, glutamate cause the mobilization of Ca^{2+} from intracellular stores. The excessive influx of Na^+ and Ca^{2+} together with water results in cytotoxic edema (Furukawa et al., 1997), and the increased intracellular concentration of Ca^{2+} leads to the activation of proteases, lipases, phospholipases and endonucleases that determine the rupture of the plasma membrane, of the cytoskeleton and DNA fragmentation, bringing to neuronal degeneration. Moreover, an influx of Ca^{2+} via NMDA receptor can determine mitochondrial dysfunction, caspase-3 activation through the action of calpain, and production of nitric oxide (NO) and reactive oxygen species (ROS) leading to neuronal death (Dobrek & Thor, 2011).

The cells in the ischemic *core* undergo to anoxic depolarization (AD) and are destined to die, instead those in the *penumbra* may re-polarize (Hossmann, 1994; Ohta et al., 2001). AD is a robust neuronal depolarization demonstrated both *in vivo* and *in vitro* (Pugliese et al., 2006; Tanaka et al., 1997), which gives rise to recurrent peri-infarct depolarization (Figure 1), a series of spontaneous depolarization waves that propagate from the ischemic *core* toward the *penumbra* area during the first 3–4 h post-stroke (Back et al., 1994; Nallet et al., 1999; Nedergaard et al., 1986; Röther et al., 1996; Strong et al., 1996). It has been observed that the infarcted area extends with the number of depolarizations (Mies et al., 1993), thus drugs able to reduce the number of depolarizations are also able to limit the extension of the infarct (Lu et al., 2005; Ohta et al., 2001).

The prevention of glutamate-mediated excitotoxicity was a central therapeutic target in numerous clinical trials. Unfortunately, drugs failed to show efficacy, neither by inhibiting glutamate receptors or modulating downstream signaling pathways such as calcium influx (Lai et al., 2014).

1.1.3 Oxidative and nitrative stress

In cerebral ischemia high levels of intracellular Ca^{2+}, Na^{+} and ADP cause mitochondria to produce deleterious levels of ROS. The brain is prone to oxidative stress, having relatively low levels of endogenous antioxidants (Halliwell, 2006). Indeed, the brain has low to moderate catalase and glutathione activity, which eliminate the hydrogen peroxidase (H_2O_2) reducing oxidation (Cooper & Kristal, 1997). Moreover, the nervous tissue has some peculiarities that render the brain particularly vulnerable to the action of free radicals: it is indeed rich in lipids and unsaturated fatty acids, which may react with ROS to form peroxyl radicals that cause the lipid oxidation of the neuronal membrane (Porter, 1984).

During reperfusion ROS production is even accelerated due to cytotoxic events such as lipid peroxidation, protein oxidation and fragmentation of DNA (Crack & Taylor, 2005). Furthermore, lipid membrane peroxidation by ROS generates toxic aldehydes that damage a large number of ion channels, transporters, and cytoskeleton proteins.

It has been demonstrated how the application of antioxidant enzymes can be effective in contrasting oxidative stress (Huang et al., 2001). For instance, superoxide dismutase (SOD) plays a protective role in ischemia (Noshita et al., 2001; Sugawara et al., 2002) and SOD1 overexpression attenuates apoptosis (Saito et al., 2004). Also, melatonin is known for its neuroprotective effect in models of ischemia, due to its antioxidant properties and capability in eliminating free radicals (Pei et al., 2002; Tan et al., 2000).

During ischemia, an increased generation of NO, a free radical synthesized from L-arginine, occurs. NO is produced by three types of nitric oxide synthases (NOS), and ischemia causes an increase in NOS type I and III activity in neurons and vascular endothelium, respectively. At a later stage, elevated inducible NOS type II (iNOS) activity occurs in glia and infiltrating neutrophils. Thus, free radicals are regarded as an important therapeutic target for improving the stroke outcome, and several antioxidant compounds including ebselen (Yamagata et al., 2008), and resveratrol (Ozkan et al., 2009), have been demonstrated to reduce stroke-related brain damage in animal models.

The NO has both neuroprotective and neurotoxic properties. Indeed, NO reacts efficiently with superoxide to produce peroxynitrite ($ONOO^-$), a potent radical. $ONOO^-$ irreversibly inhibits mitochondrial function and in addition, being pro-oxidant, it damages lipids, proteins and DNA and increases glutamate release determining neuronal cell death and blood-brain barrier (BBB) (Beckman, 1996). Conversely, the activation of NOS in the endothelial cells is neuroprotective because NO production acts relaxing the adjacent smooth muscle cells leading to vasodilation and thus increasing blood flow in the affected cerebral region (Bolaños & Almeida, 1999).

Radicals also activate matrix metalloproteases (MMPs), which degrade collagen and laminins in the basal lamina, thus disrupting the integrity of the vascular wall and increasing BBB permeability (Doyle et al., 2008).

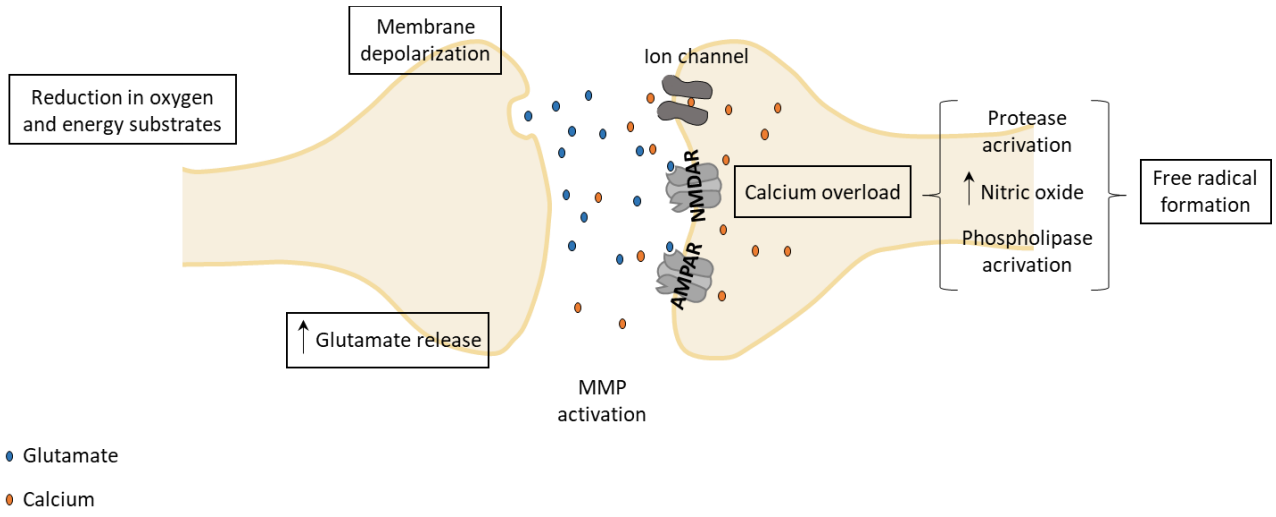

Figure 5. Damaging events and mechanisms involved in ischemic stroke. Summary of neurochemical changes that trigger the initiation of ischemic cascade. AMPAR: AMPA receptors; NMDAR: NMDA receptors; MMP: metalloproteinase. Modified from (Pullagurla et al., 2015).

1.1.4 Stress signaling in response to cerebral ischemia

Among the pathophysiological events that occur after cerebral ischemia, it is worth to mention the activation of mitogen-activated protein kinase (MAPK) cascade (Nozaki et al., 2001; Takagi et al., 2000)

triggered by free radicals generated following oxidative stress (Cao et al., 2005). MAPKs comprise three subfamilies: the extracellular-signal regulated protein kinases (ERK), c-jun N-terminal kinases (JNK) and p38 (Koistinaho & Koistinaho, 2002; Sugino et al., 2000), which are signal transduction pathways that serve several different functions at the cellular level. In particular, following focal cerebral ischemia in the rat, activation of both ERK and p38 MAPK up to 24 hours after ischemia was reported (Irving et al., 2000).

Most evidence indicates that ERK activation is associated with the regulation of cell proliferation and differentiation (Tibbles & Woodgett, 1999), is involved in the short- and long-term plasticity at glutamatergic synapses (Hartmann et al., 2001) and exerts neuroprotection mediated by neurotrophins and in particular by the brain-derived neurotrophic factor (BDNF) (Han & Holtzman, 2000).

On the contrary, p38 and JNK kinases act as mediators of cellular stress in ischemia (Alessandrini et al., 1999; Irving & Bamford, 2002), and play a role in neuronal damage and angiogenic response in cerebral ischemia (Lennmyr et al., 2002). Therefore, their inhibition has a neuroprotective role in ischemic stroke (Barone et al., 2001; Borsello et al., 2003). Concerning p38 MAPK, data agree on protection by p38 inhibition against focal ischemia induced infarct, neurological deficit and expression of inflammatory cytokines (Barone et al., 2001; Barone & Feuerstein, 1999). Several studies have shown that all MAPKs are activated twenty-four hours after focal brain ischemia in the rat (Irving et al., 2000): p38 and ERK in neurons and in microglia (Irving et al., 2000; Melani et al., 2009; Piao et al., 2003; Takagi et al., 2000), JNK in neurons and in oligodendrocytes (Melani et al., 2009).

1.1.5 Neuroinflammation

Inflammation plays a key role in ischemic brain injury progression, exacerbating cerebral tissue damage. A few hours after stroke onset, a switch from the acute damage to an inflammatory damage occurs. Brain ischemia leads to release of several damage-associated molecular pattern molecules from damaged cells, such as the chromatin-associated protein termed high mobility group protein B1, heat shock proteins, ATP, S100 proteins, heparan sulphate, DNA, and RNA. These damage-associated molecules trigger an inflammatory response, characterized by the activation of microglia with subsequent production of cytokines and chemokines (G. Y. Chen & Nuñez, 2010; Gelderblom et al., 2009). Indeed, in the ischemic brain cytokine expression is upregulated (Liu et al., 1994; Sairanen et al., 2001): interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) can aggravate the ischemic damage, whereas IL-6, IL-10 and transforming growth factor β (TGF-β) may have a neuroprotective role (Allan & Rothwell, 2001). The Ca^{2+} accumulation that occurs following the overactivation of NMDA receptor

results in the activation of phospholipase A_2 and of the linked arachidonic acid pathway (Stanimirovic & Satoh, 2000), whose metabolites are potent mediators of the post-ischemic cerebral inflammation (Sánchez-Moreno et al., 2004).

Microglial cells represent the resident phagocytic immune cells in the CNS (Kreutzberg, 1996). Following ischemia microglia gets rapidly activated (within minutes) (Denes et al., 2007; Lai & Todd, 2006), thus undergoes to proliferation, chemotaxis, morphological changes and release of several substances, both cytotoxic and cytoprotective (Wood, 1995). Microglia expansion peaks at 2-3 days after the ischemic insult and last for weeks thereafter (up to 16 after two hours MCAo in rats) (Eric Thomas, 1992; Lalancette-Hébert et al., 2007; Ekdahl et al., 2009). Once activated, microglia release several pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, as well as other potential cytotoxic molecules including NO, ROS and prostanoids (Lucas et al., 2006), thus leading to cell death and tissue damage. Microglia can also produce other cytokines related to inflammation, such as IL-10 and TGF-β, which are neuroprotective (Spera et al., 1998; Zhu et al., 2002). Increased production of pro-inflammatory and lower levels of the anti-inflammatory IL-10 are related to larger infarctions and poorer clinical outcome (Lakhan et al., 2013).

Astrocytes, like microglia, can express different kinds of inflammatory mediators such as cytokines, chemokines, and the iNOS (Dong & Benveniste, 2001). Following ischemia astrocytes become activated, they increase the expression of the glial fibrillary acidic protein (GFAP) and participate to the cerebral inflammation by expressing the major histocompatibility complex and by stimulating the expression of cytokines such as IL-2 (Dong & Benveniste, 2001).

Cytokines produced by glial cells in turn upregulate the expression of cell adhesion molecules (CAMs) (Lakhan et al., 2013; Sughrue et al., 2004), which causes the influx of peripheral leukocytes (neutrophils, lymphocytes and monocytes). The interaction between leukocytes and the vascular endothelium is mediated by three main groups of CAMs: the selectins, the intracellular adhesion, (ICAM-1,2) and vascular cell adhesion (VCAM-1) molecules and the integrins (DeGraba, 1998; Emsley & Tyrrell, 2002). After ischemia and reperfusion, the expression of selectins, especially E- and P-, (Huang et al., 2000; Okada et al., 1994) and of the ICAM and VCAM is upregulated (Shyu et al., 1997; Zhang et al., 1995) and it has been shown that the blockade of adhesion molecules leads to a reduction of neurological damage (Clark et al., 1995; Clark et al., 1997; Iadecola & Anrather, 2011). After 4-6 hours from ischemia onset, the circulating leukocytes adhere to the vascular endothelium and infiltrate in the ischemic brain tissue where they contribute to the accumulation of pro-inflammatory mediators.

Neutrophils are the first leukocyte to infiltrate the ischemic lesion, peak at 48 hours after the ischemic event and then decrease rapidly with time (Jin et al., 2010). They exacerbate brain damage following

the release of deleterious substances or of pro-inflammatory mediators, such as iNOS and MMPs stored in neutrophil vesicles (Hallenbeck, 1996; Jin et al., 2013), thus exacerbating oxidative stress and BBB damage (Justicia et al., 2003; Kolaczkowska & Kubes, 2013). Therefore, the inhibition of neutrophil infiltration can significantly reduce the infarct volume after ischemia (Bowes et al., 1995; Chopp et al., 1996; Clark et al., 1995; Connolly et al., 1996; Garau et al., 2005; Yenari et al., 1998).

Following permanent MCAo in rats, lymphocytes represent the second most abundant leukocyte subtype, after neutrophils, infiltrated in the ischemic brain (Stevens et al., 2002). $CD4^+$, $CD8^+$ and γδ T cells are deleterious in stroke by producing pro-inflammatory cytokines (e.g. IF-γ and IL-17), whereas Treg cells have a beneficial role by producing anti-inflammatory cytokines (e.g. IL-10) (Nadareishvili et al., 2004; Yilmaz et al., 2006; Liesz et al., 2009).

Other molecules upregulated in the brain in response to injury are the MMPs (Montaner et al., 2001). MMPs are a family of proteolytic enzymes that are responsible for remodeling the extracellular matrix and that can degrade all its constituents. Neurons, astrocytes, microglia, and endothelial cells have all been shown to express MMPs after injury. Stroke is associated with the disruption of the BBB leading to edema and hemorrhage, and experimental studies have shown that these events result from the expression and activation of MMPs (Asahi et al., 2001). Among the MMPs, the MMP-2 and MMP-9 are implicated in cerebral ischemia. Elevated MMP-9 levels were found in brain tissue and in serum from patients with acute ischemic stroke and in animal models of stroke beginning at 12 hours after permanent MCAo (Lakhan et al., 2013). The described post-ischemic neuroinflammatory changes are summarized in Figure 6.

Given that inflammation is a key contributor to ischemic injury (Anrather & Iadecola, 2016), it has been one of the most targeted mechanisms in acute neuroprotectant studies, alongside excitotoxicity and oxidative stress (Chamorro et al., 2016). Anti-inflammatory therapeutic approaches have been investigated in preclinical studies, which have demonstrated that inhibition of brain leukocyte infiltration using agents directly blocking adhesion molecules (e.g., CD11b/CD18, ICAM-1, P-selectin) and neutrophil inhibitory factor inhibition reduces infarct size, edema, and neurological deficits in transient MCAo models, even administrated up to 12–24h after ischemia, but the benefits do not extend to permanent stroke models (Chopp et al., 1994; Ruehl et al., 2002; Zhang et al., 1995). Clinically, several drugs targeting leukocyte recruitment in brain parenchyma have been investigated for the treatment of ischemic stroke, such as antibody to ICAM-1 (Enlimomab, R6.5), antibody to the CD11b/CD18 (Hu23F2G or LeukArrest), and recombinant neutrophil inhibitory factor (Becker, 2002; Emsley et al., 2005; Jin et al., 2013; Krams et al., 2003). However, all these agents have failed in clinical trials.

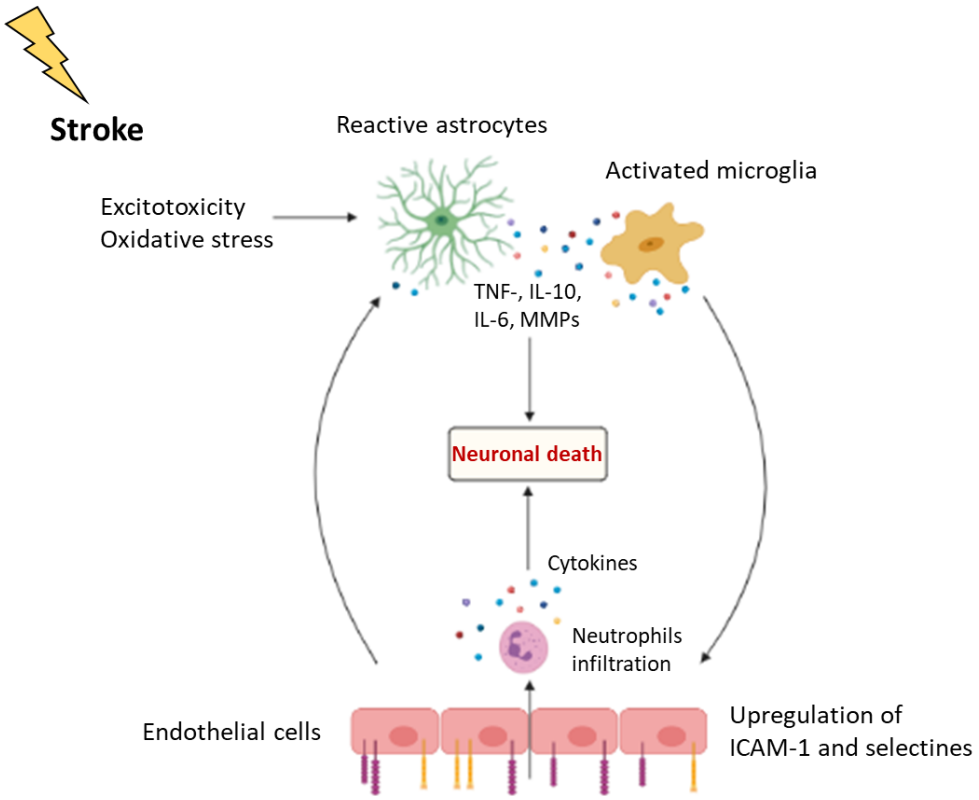

Figure 6. Post-ischemic inflammatory response. Excitotoxicity and oxidative stress caused by the initial ischemic event activate microglia and astrocytes which react by secreting cytokines, chemokines and matrix metalloproteases (MMPs). These inflammatory mediators lead to an upregulation of cell adhesion molecules on endothelial cells, allowing blood derived inflammatory cells, mainly neutrophils, to infiltrate the ischemic brain area. Neutrophils themselves also secrete cytokines which cause a further activation of glial cells. These processes all result in neuronal cell death and enhance the damage to the ischemic brain. Modified from (Lakhan et al., 2009).

1.1.6 Cellular death

After cerebral ischemia, brain cells that are compromised by excitotoxicity, Ca^{2+} overload, ROS or by mitochondrial and DNA damage can die by necrosis or apoptosis. Necrosis represents the main cell death mechanism in the acute phase, whereas apoptosis occurs mainly in milder injury, particularly within the ischemic *penumbra*. Cells that undergo necrosis appear characterized by cell swelling, formation of cytoplasmic blebs, swollen or ruptured mitochondria, rupture of cytoplasmic organelles, and eventually loss of the cell membrane integrity (Kerr et al., 1972; Majno & Joris, 1995). This leads to the release into the surrounding tissue of the cytoplasmic contents, which represent a chemotactic signal for recruitment of inflammatory cells in the damaged area (Elmore, 2007).

Apoptosis is a strictly controlled cell death process crucial for tissue homeostasis characterized by no loos of intracellular content. The enzymes involved in apoptosis are the caspases, whose gene is highly

expressed during the infarction (Dirnagl et al., 1999). To date, two main apoptotic pathways are known: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway.

Post-ischemic apoptosis occurs via intrinsic (or mitochondrial) activation, that is initiated by high levels of glutamate, intracellular Ca^{2+}, ROS, and DNA damage. Caspases become activated when cytochrome C, released from mitochondria, leads to the activation of caspase-9, that in turn activates, among others, the caspase-3 which initiates the cell death by apoptosis (Carbonell & Rama, 2007).

Chapter II: Role of Carbonic Anhydrase Inhibitors in cerebral ischemia

Discovered in 1933, the carbonic anhydrases (CAs, EC 4.2.1.1) are a family of ubiquitous metalloenzymes present in most organisms all over the phylogenetic tree. To date, eight CA classes are known: α-, β-, γ-, δ-, ζ-, η-, θ-, and ι-CAs (Mishra et al., 2020), the last three recently discovered (Del Prete et al., 2014; Jensen et al., 2019; Kikutani et al., 2016).

CAs present in animals belong to α-class (Aspatwar et al., 2014; Supuran, 2007), plants and algae have α-, β-, γ-, δ- θ- and ι-classes; fungi encode for α-and β-CAs; protozoa for α-, β- and/or η-CAs; bacteria for α-, β-, γ- and ι-CA classes (Capasso & Supuran, 2015). Vertebrates encode only α-CAs, and a large number of α-CA isoforms have been described: 15 in humans and other primates, and 16 in other mammals, with different catalytic activity, subcellular localization, tissue distribution and expression levels. α-CAs can be classified into four groups based on the subcellular localization: there are five cytosolic forms (CA I, CA II, CA III, CA VII and CA XIII), five membrane-bound enzymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial forms CA (Va and CA Vb), and a secreted CA isozymes (CA VI). Three cytosolic a-catalytic forms are also known, which are denominated CA related proteins (CARP): CARP VIII, CARP X, and CARP XI. CA distribuition is summarized in Table 1.

Isoenzymes	Catalytic activity	Subcellular Localization	Tissue/Organ Localization
CA I	Low	Cytosol	Erythrocytes, GI tract, eye
CA II	High	Cytosol	Erythrocytes, eye, GI tract, bone osteoclasts, kidney, lung, testis, brain
CA III	Very low	Cytosol	Skeletal muscle, adipocytes
CA IV	Medium	Membrane-bound	Kidney, lung, pancreas, brain capillaries, colon, heart muscle, eye
CA VA	Low	Mitochondria	Liver
CA VB	High	Mitochondria	Heart and skeletal muscle, pancreas, kidney, spinal cord, GI tract
CA VI	Low	Secreted (saliva, milk)	Salivary and mammary glands
CA VII	High	Cytosol	CNS
CA VIII	Acatalytic	Cytosol	CNS
CA IX	High	Transmembrane	Tumors, GI mucosa
C AX	Acatalytic	Cytosol	CNS
CA XI	Acatalytic	Cytosol	CNS
CA XII	Low	Transmembrane	Renal, intestinal, reproductive epithelia, eye, tumors, CNS
CA XIII	Low	Cytosol	Kidney, brain, lung, gut, reproductive tract
CA XIV	Low	Transmembrane	Kidney, brain, liver, eye
CA XV	Low	Membrane-bound	Kidney

Table 1. Distribution, localization, and catalytic activity of human carbonic anhydrase (CA) isoforms. GI= gastro-intestinal. Modified from (Mishra et al., 2020).

2.1 Carbonic anhydrase structure and catalytic mechanism

The role of CAs is to catalyze the reversible hydratation of carbon dioxide into a bicarbonate ion and a proton ($CO_2+H_2O \leftrightarrows HCO_3^- + H^+$) by ensuing a two-step reaction process (except the three acatalytic isoforms), thus playing a pivotal role in pH regulation and several metabolic pathways such as lipogenesis, gluconeogenesis and urogenesis (Hewett-Emmett, 2000; Supuran & Scozzafava, 2002).

The basic catalytic mechanism of CA was established from studies of bovine CA and human CAs I and II (Silverman & Lindskog, 1988). The active site of α-CAs contains a zinc ion, which is essential for catalysis, coordinated by three histidine resudues and a water molecule/hidroxide ion (Supuran, 2008). The three-dimensional fold of the main CA mammalian isoform (in this specific case the human (h) isoform hCA II) is shown in Figure 7, with the hydrophobic, hydrophilic and proton transfer regions highlighted (Figure 7A), whereas the zinc coordination and the amino acid residues crucial for catalysis and inhibition are shown in detail in Figure 7B (Mishra et al., 2020; Supuran, 2008, 2018a). Indeed, the active site architecture of CAs is unique, with half of the cavity being lined with hydrophobic and the opposite half with hydrophilic amino acid residues, as observed from Figure 7. The metal ion is placed at the bottom of this cavity, and the water molecule coordinated to it plays a crucial role in the catalytic process, being activated by the zinc ion for the nucleophilic attack on the various substrates on which the CAs act, but the physiological one seems to be only CO_2, which is hydrated to bicarbonate and protons (Mishra et al., 2020; Supuran, 2008, 2018a).

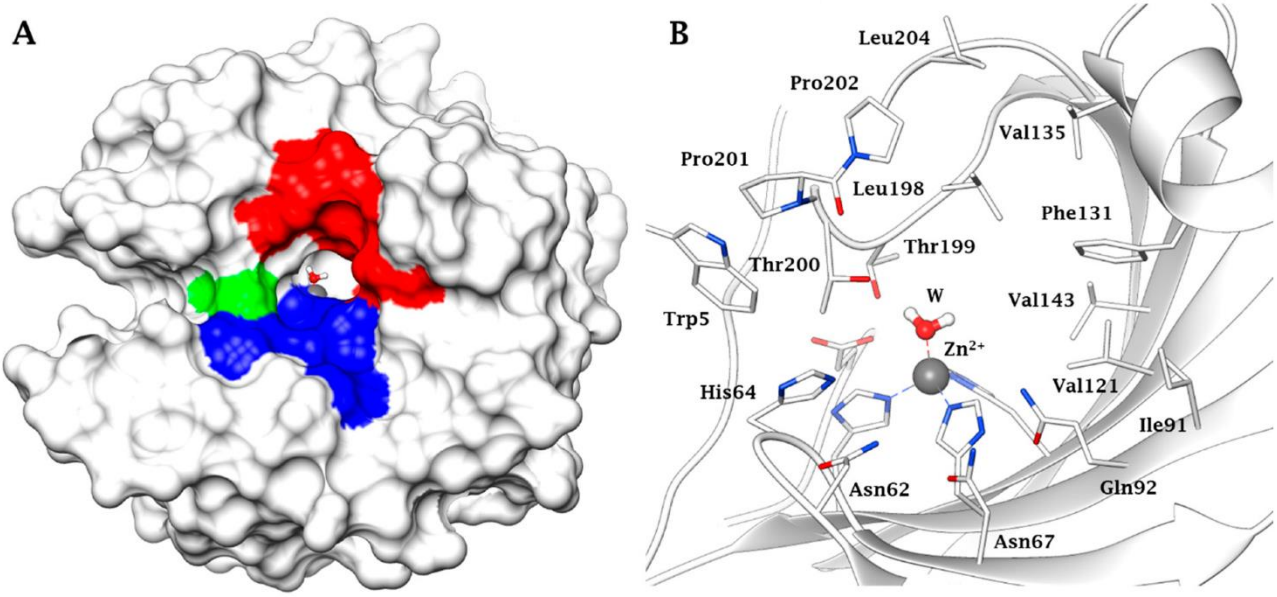

Figure 7. **(A)** Surface representation of human (h) isoform carbonic anhydrase (hCA II) (pdb 3KKX). The hydrophobic half of the active site is colored in red (Ile91, Val121, Phe131, Val135, Val143, Pro201, Leu204), the hydrophilic one in blue (Asn62, Asn67, Glu69, Gln92, His94). His64, the proton shuttle residue, is in green. **(B)** Active site view of hCA II. The zinc ion, represented as grey sphere, is tetrahedrally coordinated to residues His94, His96 and His119 and to a water molecule/hydroxide ion as fourth ligand. From (Bulli et al., 2021).

2.2 Carbonic anhydrase isoforms in the CNS

The CNS has the highest number of CA isoforms (at least 9), although their functions are still not completely understood (Supuran, 2015; Thiry et al., 2007). The cytosolic and ubiquitous CA I is expressed in the motor neurons in human spinal cord (Liu et al., 2016). CA II, the physiologically dominant isoform, is expressed in the choroid plexus, oligodendrocytes, myelinated tracts, astrocytes and myelin sheaths in the vertebrates' brain (Thiry et al., 2007). CA III, despite its low catalytic activity, is present in the choroid plexus (Thiry et al., 2007), and the membrane-associated CA IV is located on the luminal surface of cerebral capillaries, thus associated with the BBB, and is also expressed in layers III and VI in the cortex, hippocampus and thalamus (Halmi et al., 2006). The presence of the mitochondrial CA VA in the nervous tissue was also demonstrated by immunocytochemical experiments, with the enzyme being expressed in astrocytes and neurons (Supuran, 2018b; Thiry et al., 2007). There is instead a lack of studies on CA VB in the brain, although it was recently detected in rat middle cerebral arteries (Rasmussen & Boedtkjer, 2018). The isoforms CA VII and VIII are present in relatively high levels throughout the cortex, hippocampus and thalamus, although CA VIII is acatalytic, whereas CA VII shows a good activity for the physiological CO_2 hydration reaction (Supuran, 2008). CA X and XI, both acatalytic, are expressed in the myelin sheath, and in the neural cell body and astrocytes, respectively, in a relatively limited region of the brain. CAXII is present in normal tissues, and a high level of this isoform was reported in the choroid plexus (Supuran, 2015; Thiry et al., 2007). CA XIII has not been identified in the brain to date, whereas CA XIV is expressed in nuclei and nerve tracts associated with pontine, medullary and hippocampal functions (Thiry et al., 2007), and was shown also to be located on the plasma membrane of some neurons and on axons of both mouse and human brain.

Several physiological functions are attributed to cerebral CAs, such as fluid and ion compartmentation and formation of cerebrospinal fluid (Maren, 1967), pH regulation (Maren, 1967), and generation of bicarbonate for biosynthetic reactions such as lipogenesis, neoglucogenesis, ureagenesis and pyrimidine synthesis (Chegwidden & Carter, 2000).

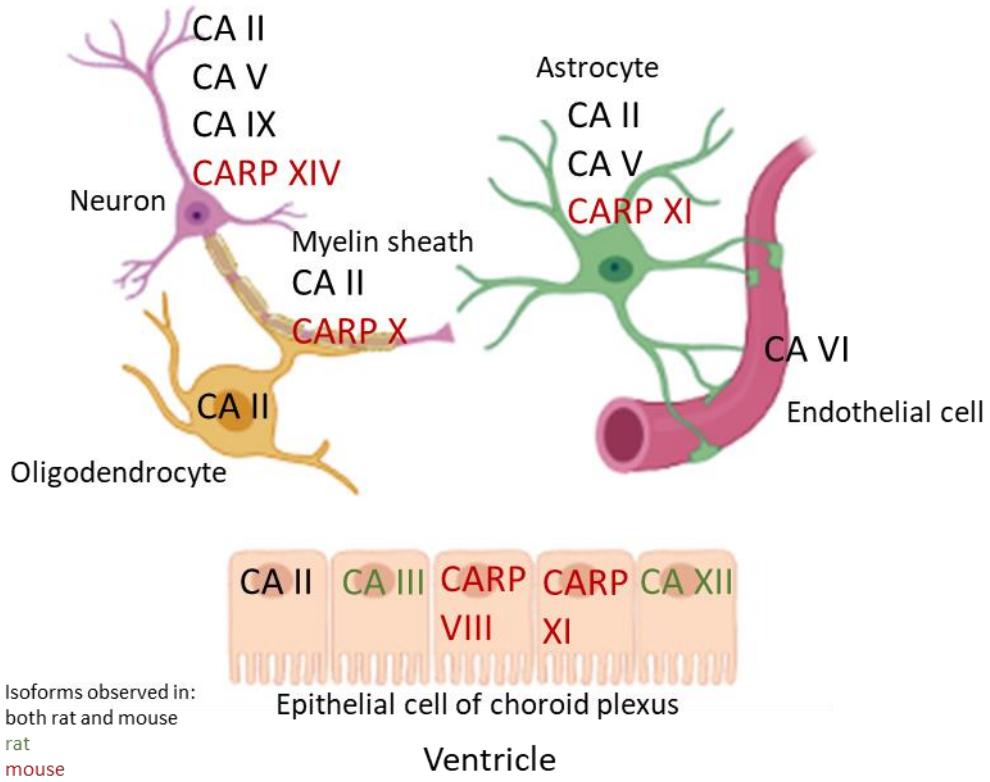

Figure 8. Localization of CA isozymes in the CNS. Epithelial cells of the choroid plexus possess CA II, III, XII and CARP VIII and XI. CA IV is located in endothelial cells of blood vessels. Astrocytes expressed both CA V and CARP XI. CARP X is present in the myelin sheath. CA II is also expressed in oligodendrocytes. Neurons contain CA V, XIV, and CARP XI. The presence of CA III and CA XII was demonstrated in the rat (colored in green) and CARP X, XI, and CA XIV were observed in the mouse (colored in red). For all the other isozymes, experiments were done both in the rat and the mouse (colored in black). Modified from (Thiry et al., 2007). Created in Biorender (https://biorender.com/).

2.3 Carbonic anhydrase inhibitors and their therapeutic applications

The particular, rather large, type of active site shown in Figure 7 probably is responsible for the fact that these enzymes are inhibited by many classes of very diverse inhibitors, with over 20 CAIs that have received FDA (Food and Drug Administration) approval or are in clinical trials for drug therapies (Mincione et al., 2007; Pinard et al., 2013). The classical ones are the primary sulfonamides and their isosteres, such as the sulfamides and the sulfamates. They coordinate to the zinc ion as anions, in deprotonated form and some of them show low nanomolar affinity for the various CA isoforms present in vertebrates, including humans (Mishra et al., 2020; Supuran, 2008, 2018a). In the last decade, a variety of new chemotypes with CA inhibitory activity and with new inhibition mechanisms were discovered, some of which are independent of the metal ion found within the enzyme active site (Mishra et al., 2020; Nocentini et al., 2021; Supuran, 2016b). They include the anchoring to the zinc-coordinated water (for phenols, polyphenols, polyamines, sulfocoumarins, thioxocoumarins) (Mishra

et al., 2020; Nocentini et al., 2021; Supuran, 2016b); the occlusion of the active site entrance, for coumarins and their derivatives (Mishra et al., 2020; Nocentini et al., 2021; Supuran, 2016b); and even compounds which bind outside the active site cavity, such as some benzoic acid derivatives (Supuran, 2016b). The inhibition mechanisms with some of these compounds are shown in Figure 9, as determined by X-ray crystallography (Mishra et al., 2020; Nocentini et al., 2021; Supuran, 2016b).

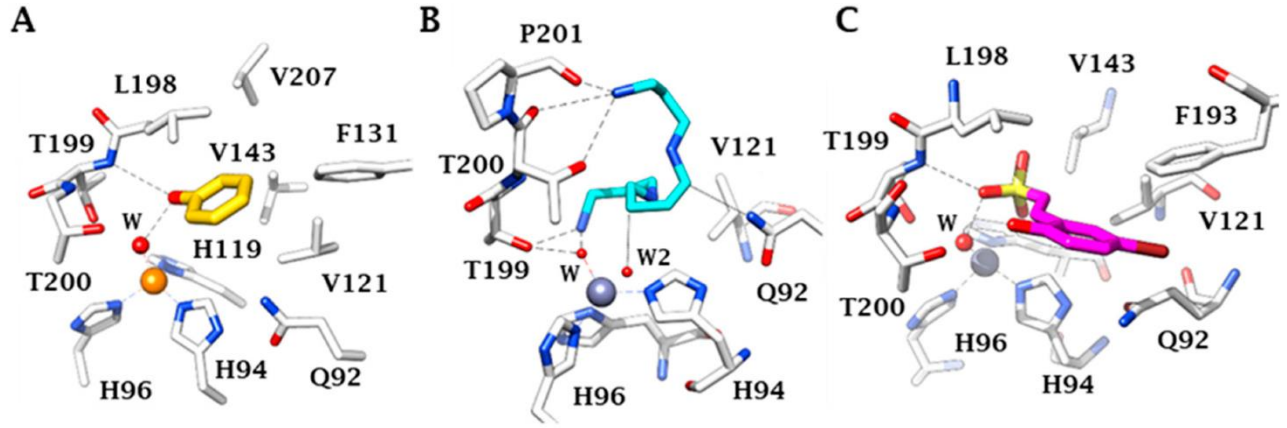

Figure 9. X-ray crystal structure for the adducts of hCA II with phenol **(A)**, spermine **(B)** and hydrolysed sulfocoumarin **(C)**, new CA inhibitory chemotypes which bind by anchoring to the zinc-coordinated water molecule. The metal ion is shown as a gold or gray sphere with its three histidine ligands and the coordinated water molecule. Amino acid residues involved in the binding of the inhibitors are also highlighted. Phenol is shown in yellow, spermine in blue and the hydrolysed sulfocoumarin in magenta. From (Bullli et al., 2021).

The abundance of CA isoforms expressed in humans and their contribution in several human diseases (Table 2) have rendered CA an ideal candidate for the design of inhibitors for the management of various diseases, including those affecting the CNS (Supuran, 2008).

The sulfonamides were recognized to act as potent CAIs already in the 1950, and the first diuretic based on this class of pharmacologic agents, acetazolamide (5-acetamido-1,3,4- thiadiazole-2-sulfonamide, ACTZ), started to be clinically used in 1956, with this drug still being used nowadays (Supuran, 2008). To date, CAIs are clinically used as diuretics (Carta & Supuran, 2013), anti-epileptics agents (Aggarwal et al., 2013; Berrino and Carta, 2019; Thiry et al., 2007), anti-glaucoma drugs (Carta et al., 2012), against rheumatoid arthritis (Silvia Bua et al., 2017; Margheri et al., 2016), and in idiopathic intracranial hypertension (Supuran, 2018a; Uldall et al., 2017). Furthermore, combination therapies of a CAI were claimed recently in two patents (Angeli & Supuran, 2018) as a therapeutic option for obstructive sleep apnea, and a CA IX/CA XII inhibitor is running a phase II clinical trial for cancer therapy (Lou et al., 2011). Moreover, several CAIs have been under investigation for controlling pathological conditions like high altitude sickness (Burtscher et al., 2016), obesity (Gordon & Price, 1999) and osteoporosis (Chang et al., 2010).

Isoform	Pathologies
CA I	Retinal/central edema
CA II	Glaucoma, edema, epilepsy, altitude sickness
CA III	Oxidative stress
CA IV	Glaucoma, retinitis pigmentosa, stroke
CA VA/VB	Obesity
CA VI	Cariogenesis
CA VII	Epilepsy
CA VIII	Neurodegeneration
CA IX	Cancer
CA XII	Cancer, glaucoma
CA XIII	Sterility
CA XIV	Epilepsy, retinopathy

Table 2. Carbonic anhydrase isoforms as drug targets for various pathologies. Modified from (Mishra et al., 2020).

Possible pharmacologic applications of CAIs targeting CNS isoforms include neuropathic pain (Asiedu et al., 2010; Supuran, 2016), diabetes-induced BBB disruption (Price et al., 2017; Salameh et al., 2016), migraine (Silberstein, 2017), and amyloid beta-induced mitochondrial dysfunction typical of Alzheimer's disease (Angiulli et al., 2018; Fossati et al., 2016; Provensi et al., 2019).

Recently, a relationship between brain hypoxia, a key feature of cerebral ischemia, and CA has been highlighted (Neri & Supuran, 2011). It has been reported that CA II-deficient mice are more resistant to hypoxia-induced neuronal damage (Velíšek et al., 1995), and that blocking CA leads to a reduced neuronal apoptosis via pH stabilization (Kniep et al., 2006). Moreover, hypoxic conditions elicit the overexpression of two CA isoforms (IX and XII), through the hypoxia inducible factor (Stiehl et al., 2006; Wykoff et al., 2000). All these findings led to the hypothesis of a possible CA relevance in brain ischemia, with CA inhibition contributing to pH homeostasis (Pettersen et al., 2015; Supuran, 2018).

2.4 Role of CAIs in brain ischemia preclinical and clinical models

The first paper assessing the effect of CAIs in cerebral ischemia demonstrated that cats undergoing MCAo and treated with ACTZ (in five doses, for a total of 500 mg intramuscularly in the first two days after ischemia), 8 days after ischemia induction, generated a more severe neurological deficit, larger areas of infarction and more brain swelling with respect to untreated cats (Regli et al., 1971). In a

model of collagenase-induced striatal hemorrhage in rats ACTZ was tested, 50 mg/kg intraperitoneally (i.p.) administered, starting 3 h after inducing intracerebral hemorrhage. Despite reducing the spike of increased intracranial pressure by presumably reducing cerebrospinal fluid production, ACTZ did not improve behavioral function or did not affect lesion size up to 28 days thereafter (Williamson et al., 2019).

In contrast, several investigations support a potential therapeutic role of low doses of new CAIs in stroke (Di Cesare Mannelli et al., 2016). There are many classes of CAI, but the most investigated ones are the sulfonamides and the coumarins (Supuran, 2018).

Di Cesare Mannelli et al. (2016) evaluated the effect of several newly synthetized sulphonamide and coumarin CAIs in the permanent MCAo (pMCAo) model of cerebral ischemia in the rat. They found that repeated subcutaneous injections (5 and 20 min after surgery) of CAIs at the dose of 1 mg/kg were able to significantly reduce the neurological deficit 24 h after pMCAo, whereas the prototypical CAI, ACTZ, 30 mg/kg subcutaneously was ineffective in reducing the neurological deficit. In addition, it has been reported that ACTZ at the dose of 100 mg/kg injected into the femoral vein 30 min after transient (1.5 h) MCAo, 22 h thereafter, reduced the infarct volume in male Wistar rats (Han et al., 2020).

The properties of novel CAIs to improve neurological functionalities after cerebral ischemic insult were thus demonstrated, opening the way to a novel, innovative pharmacological treatment of this condition.

Despite few studies are present in literature about carbonic anhydrase and its inhibitors in stroke, previous investigations on the role of CA isoenzyme in the brain supported a potential therapeutic role of CA inhibition in stroke. Indeed, intracaudate injection of CA increases brain water content and neuronal death, whereas intracerebral injection of ACTZ (5 µL, 1 mM) reduces brain edema, neuronal death and neurological deficit 24 h after intracerebral hemorrhage in Sprague–Dawley rats (Guo et al., 2012).

Up until now, CAIs are not used in the management of cerebral ischemia. The only available clinical study exploring protection by CA inhibitors has been made in hemorrhagic stroke patients. ACTZ treatment (750 mg/day administered every 8 h), proved protective 72 h and 3 weeks after intracerebral hemorrhage improving neurological functionalities and decreasing the mortality rate in treated patients (Abbasi, Salimi, & Amani, 2017).

Chapter III: The adenosinergic system

Adenosine (Figure 10) is a nucleoside formed by a molecule of the purinergic nucleobase adenine bound to a ribose. It represents one of the most ubiquitous and conserved

neuromodulators/neurotransmitters in the CNS, usually with a cytoprotective function (Fredholm et al., 2007).

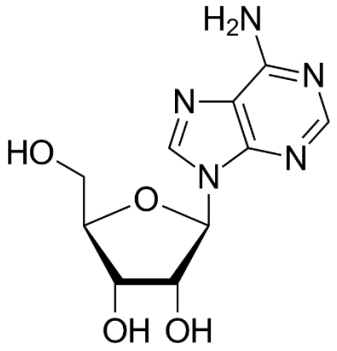

Figure 10. Adenosine molecular structure.

Adenosine is central in the regulation of several physiological functions in different cell type (neurons, platelets, mast cells, smooth muscle cells, myocytes), due to its role as modulator of neurotransmission, conduction, vasodilation, proliferation and cell death.

Extracellular adenosine levels in CNS are regulated by a complex machinery comprised of synthetic and degradative enzymes, as well as transporters. The relative relevance of each element depends on the cell metabolic state and, in the case of neuronal cells, their excitability status. Under basal conditions extracellular adenosine concentration is in the range of 25–250 nM (Dunwiddie & Masino, 2001), being sufficient for tonic activation of a substantial fraction of adenosine receptors. However, pathological conditions such as hypoxia, ischemia, and seizures result in markedly elevated extracellular concentrations of adenosine, largely due to an increase in the extracellular metabolism of adenine nucleotides to adenosine (Dunwiddie & Masino, 2001; Fredholm et al., 2005; Latini & Pedata, 2001). Accordingly, many adenosine-mediated effects are responsible of tissue protection during stress conditions, such as ischemia or hypoxia. Protective effects and tissue repair are exerted by adenosine by increasing the supply of oxygen, by triggering anti-inflammatory responses and by promoting angiogenic processes (Linden, 2005).

In the CNS, adenosine acts both at central and peripheral level. In 1929, Drury and Szent-Györgyi proved that systemic administration of adenosine in anesthetized animals induced transient bradycardia, vasodilation, decrease of blood pressure and intestinal motility (Drury & Szent-Györgyi, 1929). In the years 1970s Sattin and Rall showed a specific role of adenosine in the CNS, in the increase of cAMP formation in mammalian brain slices (Sattin & Rall, 1970). This effect was inhibited by the methylxanthines: caffeine and theophylline.

Later on, a depressant role of adenosine on the firing of cortical (Phillis et al., 1979) and cerebellar neurons (Kostopoulos et al., 1975) and on excitatory synaptic potential amplitude in cortical and

hippocampal slices (Dunwiddie & Hoffer, 1980; Kuroda et al., 1976; Okasa & Ozawa, 1980) was demonstrated. Moreover, it was shown the inhibitory role of adenosine on the acetylcholine release in different brain areas (Harms et al., 1978) and on the release of dopamine (Michaelis et al., 1979), noradrenaline (Harms et al., 1978), excitatory amino acids (Corradetti et al., 1984) and serotonin (Harms et al., 1979). All these effects were inhibited by methylxanthines, increased by inhibitors of the adenosine reuptake and correlated with changes in cAMP levels.

3.1 Adenosine synthesis

In the CNS, adenosine originates from neurons and from non-neuronal cells such as glial cells (Halassa et al., 2009), synthetized both intra- and extracellularly by dephosphorylation of AMP operated by 5'-nucleotidases (Zimmermann, 1992). Intracellularly, adenosine is generated from AMP by the cytosolic enzyme 5'-endonucleotidase, or through the hydrolysis of S-adenosil-homocysteine (SAH) by the enzyme SAH hydrolase (Figure 11). This reaction occurs mainly at cardiac level, where SAH represents an important source of intracellular adenosine in physiological conditions (Latini et al., 1996 a). At neuronal level, this synthesis pathway does not significantly contribute to the adenosine production either in normoxic or in hypoxic conditions (Latini et al., 1996 b; Pak et al., 1994).

Extracellularly, adenosine synthesis occurs by enzymatic conversion of released nucleotides (especially ATP) through coupled 5'-ectonucleotidase: CD39 that converts ATP/ADP to AMP and CD73 that hydrolyses AMP to adenosine. A further enzyme able to generate adenosine by sequential hydrolysis of ATP, is the alkaline phosphatase (Zimmermann, 2000). Another possible source of extracellular adenosine is represented by released cAMP. In the extracellular space, cAMP can be converted to 5'-AMP by ecto-phosphodiesterase, and then to adenosine by 5'-ectonucleotidase. Alternatively, cAMP can be converted into 5'-AMP within the cell and then released in the extracellular space, where it represents a further source of adenosine. This suggests that many neurotransmitters that act on metabotropic receptors whose signaling is linked to adenylate cyclase, by favoring the accumulation of cAMP, may regulate the adenosine levels and thus the inhibitory effects in the CNS (Serena Latini & Pedata, 2001). The intracellular adenosine was considered as the most important source of extracellular adenosine. Over the past decade however, it was increasingly recognized that the rapid (1 ms) extracellular conversion of locally released ATP by ecto-nucleotidases represents another important source of extracellular adenosine in tissue (Fredholm et al., 2007).

3.1.1 5'Endonucleotidases

Six 5'-endonucleotidases with different subcellular localization have been cloned in mammals. Three different cytosolic 5'-nucleotidases cN-I, cN-II, and cN-III have been described; among them, the AMP-selective 5'-nucleotidase (cN-I) mostly accounts for adenosine during enhanced oxygen demand or metabolic load. Two different isoforms of cN-I enzyme exist: cN-IA and cN-IB. cN-IA is able to hydrolyze several substrates but has a preference for AMP. It has been purified from a variety of different tissues including the brain. cN-IB is less characterized and has the lowest expression in the brain (Sala-Newby & Newby, 2001).

Adenosine can be formed intracellularly from AMP not under basal physiological normoxic conditions but only under different stress conditions (i.e. depolarization, ischemia/hypoxia, seizures) when AMP concentrations increases because of ATP breakdown (Sala-Newby et al., 2000). Only in these conditions, AMP concentration reaches a value in the range of cN-I affinity. In fact, the Km of cN-I for AMP (1.2–8.3 mM) is higher than the intracellular concentrations of AMP that have been evaluated under physiological conditions (in rat brain 30–40 μM) (Traut, 1994). Therefore, a massive production of adenosine can occur only in response to a condition of energy imbalance when ATP consumption exceeds the capacity of its own re-synthesis, with a consequent production of adenosine (Dunwiddie & Masino, 2001). Typically, in pathological conditions such as ischemia when the energy imbalance is massive, the resulting massive AMP production is the key mechanism that accounts for adenosine formation (Melani et al., 2006). In this condition, adenosine cannot be efficiently re-phosphorylated to ATP due to the lack of oxygen and glucose.

In addition, since ATP and ADP inhibit the 5'-endonucleotidase activity, a reduction in the intracellular concentration of ATP and ADP leads to the enzyme activation (Meghji, 1993).

3.1.2 5'Ectonucleotidases

Ecto-5'-nucleotidase (CD73 or 5'-NT) is the enzyme that catalyzes the terminal dephosphorylation of 5'–AMP. 5'-ectonucleotidases allow the transformation of the nucleotides released outside the cell into adenosine. These enzymes are homodimers anchored to the plasma membrane through a molecule of glycosyl–phosphatidylinositol. Their catalytic site faces the extracellular space where 5'-ectonucleotidases exert their enzymatic action. CD73 hydrolyses several nucleosides 5'–monophosphate such as: CMP, UMP, GMP, IMP and AMP. On the basis of the Vmax/Km ratio, AMP is the most efficient (Naito & Lowenstein, 1985) and physiological substrate (Zimmermann, 1992), with adenosine being the major nucleoside produced by CD73 (Hunsucker et al., 2005). In fact, CD73 is the

major enzyme responsible for the formation of extracellular adenosine from released adenine nucleotides (Zimmermann, 2000).

CD73 is ubiquitously expressed both in central and peripheral nervous system. In CNS it is expressed poorly in proximity of neurons in comparison to glial cells: astrocytes, oligodendrocytes and microglia (Kreutzberg & Barron, 1978; Resta et al., 1998; Schoen et al., 1993; Spychala, 2000; Zimmermann, 1992). Pathological conditions such as ischemia and hypoxia are major inducers of CD73 expression (Braun et al., 1997; Kitakaze et al., 1996; Ledoux et al., 2003). Moreover, the modulation of the adenosine formation is central since it impacts on neurotransmission processes and CD73 has been reported as the major regulator of the adenosinergic signaling in the mouse brain (Kulesskaya et al., 2013).

CD73 action is limited by ATP and ADP, that act as inhibitors with a Ki in the low micromolar range (Zimmermann, 1992). In fact, in striatal cholinergic synapses it has been shown that ATP, released in response to a high frequency stimulation, can be degraded to adenosine only at the end of the stimulation, when the concentrations of ATP and ADP decrease under the threshold of inhibition of the enzyme (James & Richardson, 1993).

3.2 Adenosine release

Adenosine concentrations are regulated by two classes of nucleoside transporters that mediate physiologic nucleoside transport across cellular membranes: equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs) (Baldwin et al., 2004; Gray et al., 2004). CNTs are evolutionarily conserved symporters that require an inwardly directed sodium-dependent, or proton-dependent, coupling (Gray et al., 2004; Young et al., 2013). In contrast, ENTs are uniporters capable of a bidirectional transport across the plasma membrane of both purines and pyrimidines, in a concentration-gradient dependent manner (Boswell-Casteel & Hays, 2017).

Both transporters have been cloned and classified as: ENT1-4, equilibrative transporters; CNT1-3, concentrative transporters (Anderson et al., 1996; Baldwin et al., 1999; Williams & Jarvis, 1991). The equilibrative transporters work bidirectionally in order to maintain the intracellular and extracellular concentrations of adenosine in a range of 25-250 nM (Dunwiddie & Masino, 2001). Inhibitors of adenosine equilibrative transporters such as dypiridamole might increase or decrease the efflux of adenosine differently according to the physiopathological conditions. Under *in vivo* physiological conditions, dypiridamole increases adenosine efflux from the striatum indicating that the equilibrative carrier transports adenosine into cells (Melani et al., 2012; Pazzagli et al., 1993). Conversely, under

conditions of energetic imbalance, dypiridamole decreases extracellular adenosine indicating that the equilibrative carrier transports adenosine out of the cells (Latini & Pedata, 2001).

It has been observed that tetrodotoxin (TTX) completely blocks adenosine release in brain slices electrically stimulated and that a reduction of extracellular Ca^{2+} concentration can cause a remarkable reduction of adenosine release (Pedata et al., 1990). Most recently, it was demonstrated that adenosine efflux occurring *in vivo* from the striatum under normoxic physiological conditions does not arises from extracellular degradation of nucleotides and is not inhibited by dypiridamole (Melani et al., 2012). Under these conditions, the efflux of adenosine is Ca^{2+}-sensitive (Melani et al., 2012) and is inhibited by TTX (Dobolyi et al., 2000; Pazzagli et al., 1993). Overall, observations *in vitro* and *in vivo* led to speculate that adenosine efflux under normoxic physiological conditions is consequent to electrical activity propagated along the typical modality of nervous cells that involves activity of voltage-gated Na^{+} channels. In fact, the TTX sensitivity and the Ca^{2+} dependency of adenosine release indicate that adenosine release occurs by an excitation-secretion modality which is typical of neurotransmitters. Observation that the presence of adenosine is associated with intracellular vesicles support the notion that adenosine is stored in vesicles and released by exocytosis in an excitation-secretion modality typical of neurotransmitters (Corti et al., 2013).

3.3 Adenosine catabolism

Adenosine can be catabolized both extra- and intracellularly (Figure 11). Extracellularly, adenosine is primarily converted to inosine by adenosine deaminase (ADA), though ecto-ADA expression is usually relatively low. Given the generally low expression of ecto-ADA, most extracellular adenosine is taken up by the ENTs and CNTs, and then catabolized. This occurs mainly through phosphorylation into AMP by adenosine kinase (AK), but also by conversion to inosine by intracellular ADA (Gomes et al., 2021).

Since the basal endogenous levels of adenosine are in the nanomolar range, it is likely that under physiological conditions the main degradation pathway is the phosphorylation operated by AK, while the action of extracellular ADA is important only when significant increases in adenosine concentration occur, such as during ischemia (Latini & Pedata, 2001).

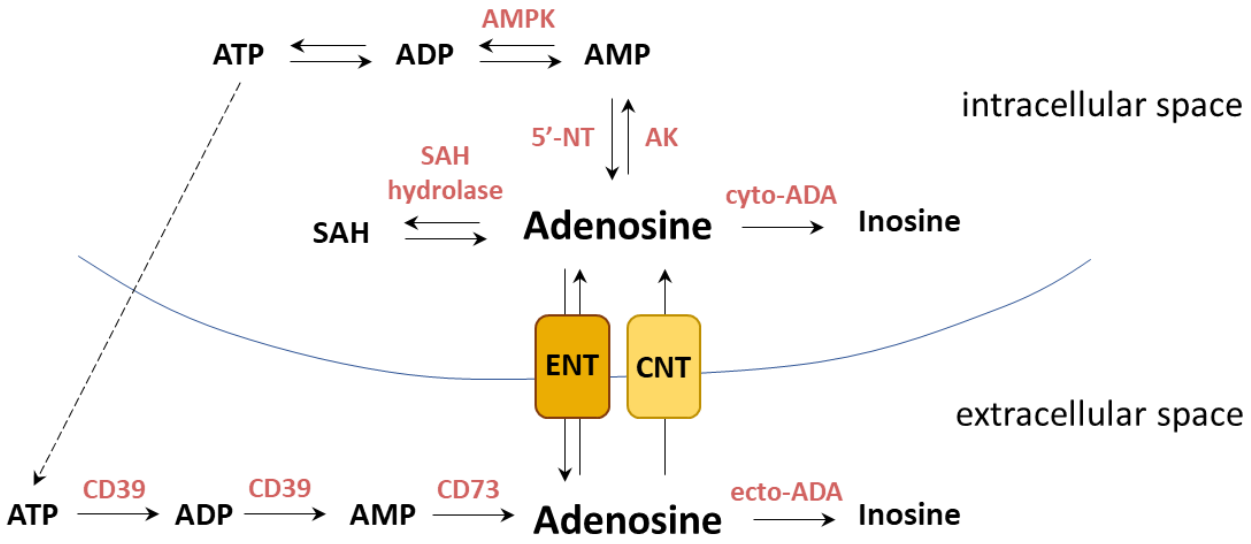

Figure 11. Adenosine synthesis, catabolism and transport. Adenosine is produced intracellularly by AMP dephosphorylation mediated by 5'NT or by SAH hydrolysis and then released from the cell through specific membrane transporters. Extracellularly, adenosine is synthetized through the hydrolysis of ATP and ADP mediated by ecto-nucleotidase. Adenosine is deaminated by ADA and converted to inosine. 5'-NT, 5'-nucleotidase; ADA, adenosine deaminase; AK, adenosine kinase; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP kinase; ATP, adenosine triphosphate; CNT, concentrative nucleoside transporter; CD73, Ecto-5'nucleotidase/cluster of differentiation 73; ENT, equilibrative nucleoside transporter; CD39, Ecto-nucleoside triphosphate diphosphydrolase/cluster of differentiation 39; SAH, S-adenosyl-homocysteine. Adapted from (Latini et al., 2001).

3.4 Adenosine receptors

Adenosine receptors were initially distinguished into two receptor classes: A_1 and A_2, on the basis of their excitatory or inhibitory actions on adenylyl cyclase (Calker et al., 1979). Subsequently, molecular cloning and pharmacological studies have identified four subtypes of adenosine receptors: A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm et al., 2001). Key features for their classification are their amino acid sequence, the coupled G-protein and the affinity for the endogenous ligand. All adenosine receptors have been cloned from rat, mouse and human tissues, and are metabotropic heteromeric G-protein coupled receptor (GPCR) (Fredholm et al., 2001). Typically, their structure is formed by a polypeptide chain characterized by 7 transmembrane hydrophobic domains with α helix structure (7TM, helices 1-7) of approximately 25 residues, followed by one short membrane-associated helix (helix 8). TM domains are closely associated by three extra-cellular loops and three intracellular loops (Cristalli et al., 2008). Biochemical experiments and computational approaches have revealed the importance of the TM3 and TM7 in binding the endogenous ligand and agonist molecules (Rivkees et al., 1995). All adenosine receptors present an extra-cellular amino terminus (N-terminus) and a cytosolic carboxy terminus (C-terminus), (Cristalli et al., 2008). The extracellular N-terminus contains one or more

glycosylation sites, while the intracellular C-terminus provides sites for phosphorylation and palmitoylation, thereby playing a role in receptor desensitization and internalization mechanisms (Borea et al., 2018).

A_1, A_{2A}, A_{2B} amino acid sequence is well conserved among mammalian species, except for A_3 receptors (Burnstock et al., 2011). This subtype presents a considerable structural variability among different species. For instance, almost 30% difference in the amino acid sequence is found between humans and rat (Linden, 1994).

Adenosine receptors transduce signals via G proteins. A_1 and A_3 receptors are negatively coupled to adenylyl cyclase through $G_{i/o}$ protein α-subunits, thus leading to adenylyl cyclase inhibition and decrease of intracellular cAMP levels. A_{2A} and A_{2B} receptors are positively coupled to adenylyl cyclase through G_s proteins, that activate the same enzyme increasing cAMP concentration in the cytosol. However, adenosine receptors have also been reported to couple to other G-proteins, modulating different second messenger systems. For instance, in addition to their effects on adenylate, adenosine A_1, A_{2B} and A_3 receptors can couple to G_{q11}, thus stimulating phospholipase C (PLC) (Abbracchio et al., 1995; Feoktistov & Biaggioni, 1998), while A_1 and A_3 receptors can also activate phospholipase D (PLD) (Fredholm et al., 2001). In addition, by a direct G protein-channel interaction or by second messenger systems, adenosine receptor stimulation also modulates several types of Ca^{2+} and K^+ channels (Figure 12).

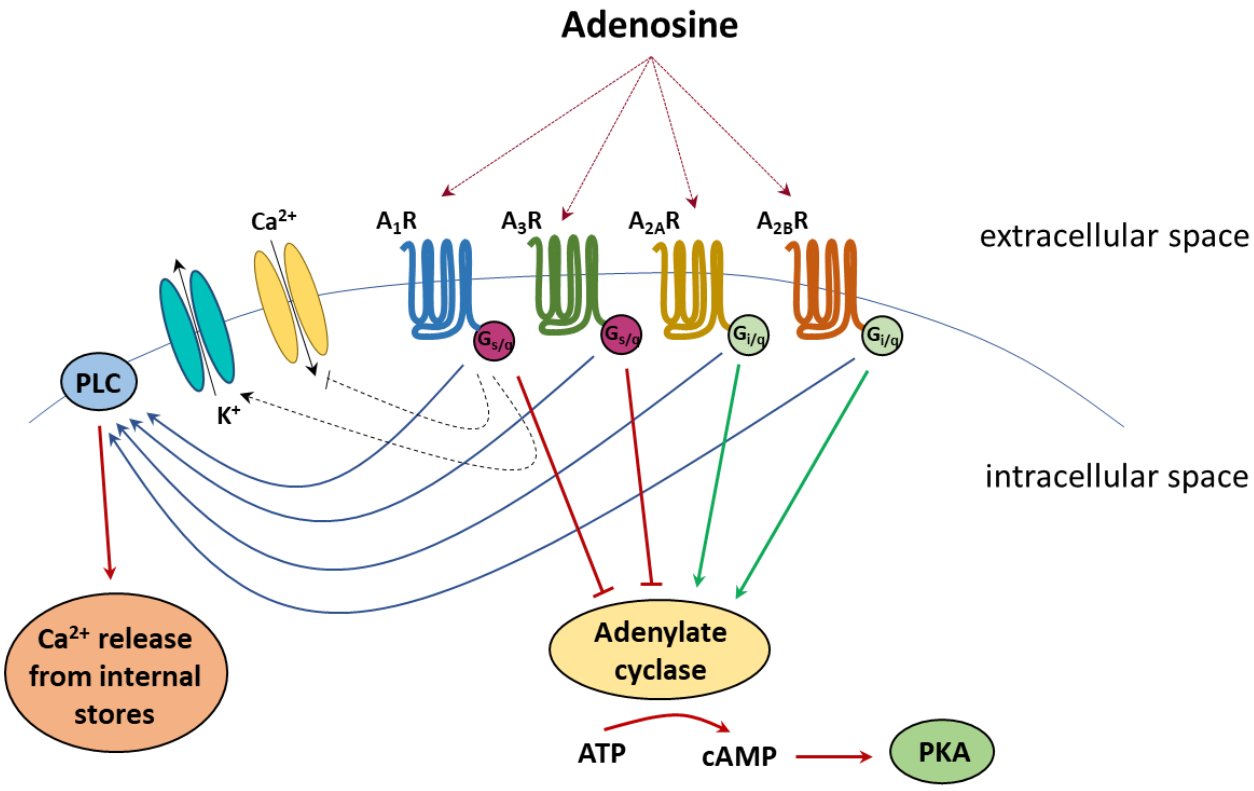

Figure 12. Adenosine receptors and signal transduction mechanisms. Adenosine receptors are G protein-coupled receptors. The A_1 and A_3 subtypes inhibit adenylate cyclase via G_i protein, while the A_{2A} and A_{2B} receptors mediate stimulation of the enzyme via G_s protein, stimulating cyclic AMP (cAMP) production and subsequent activation of protein kinase A (PKA). In addition, they can modulate, by G_o protein or G_q, phospholipase C (PLC) activity, inducing the production of diacylglycerol (DAG) and inositol-triphosphate (IP3) and an increase in Ca^{2+} release from intracellular deposits. Modified from (Gomes et al., 2021).

3.4.1 A_1 receptor: molecular structure, distribution and function

The A_1 receptor is a monomeric glycoprotein of 35-36 kDa formed by 326 amino acids. It is highly conserved, with an 87%-92% homology between different species, and it is coupled with the $G_{i/0}$ protein at the TM3 loop. The different effects of the stimulation of $G_{i/0}$ are mediated by both α subunit and βγ complex (Birnbaumer, 1992).

The highest levels of A_1 receptor expression are found in the CNS, suggesting a particularly important role of adenosine in brain functions. A_1 receptor is highly expressed in the cortex, cerebellum, hippocampus, and dorsal horn of spinal cord, whereas intermediate expression levels have been found in basal ganglia structures including the striatum (Dixon et al., 1996). mRNA encoding A_1 receptors is present in large striatal cholinergic interneurons (Dixon et al., 1996) and in neurons of the globus pallidus, subthalamic nucleus and thalamus. An abundant expression of adenosine A_1 receptor also occurs in the trigeminal ganglia, supporting a role of this receptor in pain regulation (Schindler et al., 2001). A_1 receptors are most abundant in neurons, both pre- and postsynaptically (Deckert & Jorgensen, 1988), but are also expressed in glial cells, such as in astrocytes (Cristóvão-Ferreira et al., 2013) and microglia (Boison et al., 2010). Under physiological conditions A_1 receptors mediate sedative-hypnotic, anti-epileptic and anti-nociceptive effects (Dunwiddie, 1985) by exerting a tonic inhibition of synaptic transmission both *in vitro* and *in vivo*. This has been demonstrated in several brain regions containing a high concentration of A_1 receptors such as the hippocampus, but also in striatum and olfactory cortex (Latini & Pedata, 2001; von Lubitz, 1999). The inhibitory effect of adenosine A_1 receptor stimulation has a pre- and postsynaptic component. At presynaptic level, the activation of A_1 receptor reduces Ca^{2+} influx through the preferential inhibition of N-type and, probably, Q-type channels (Wu & Saggau, 1994; Yawo & Chuhma, 1993), with a consequent decrease in transmitters release (Prince & Stevens, 1992). In fact, adenosine, by stimulation of A_1 receptor, has been found to inhibit the release of several neurotransmitters, including glutamate, acetylcholine, dopamine, noradrenaline and serotonin (Fredholm & Dunwiddie, 1988), leading to the inhibition of synaptic transmission (Ambrósio et al., 1997; Proctor & Dunwiddie, 1987). In particular, a powerful

suppression of glutamate release from presynaptic terminals has been described in the hippocampus (Burke & Nadler, 1988; Corradetti et al., 1984).

At postsynaptic level, A_1 receptors mediate a direct hyperpolarization of neurons via activation of G-protein-coupled inwardly rectifying potassium (GIRK) channels (K_{ir} 3.2 and 3.4 channels) (Takigawa & Alzheimer, 1999, 2002) and by a direct increase of Cl^- conductance (Greene & Haas, 1991) thus, stabilizing the membrane potential. Postsynaptically, A_1 receptor activation is associated with the inhibition of glutamate NMDA receptor mediated currents (de Mendonça et al., 1995). Moreover, A_1 receptor can also mediate the stimulation of PLC (Burnstock et al., 2011), and of PLD (Fredholm et al., 2001). An overview of A_1R intracellular signaling pathways is summarized in Figure 13.

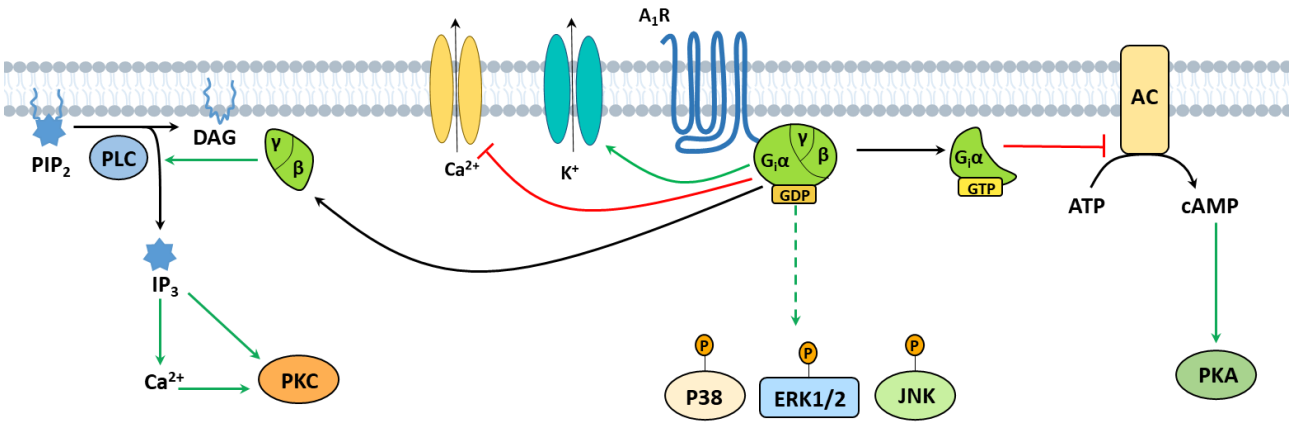

Figure 13. Overview of A_1 receptor (A_1R) intracellular signaling pathways. A_1R stimulation decreases adenylate cyclase (AC) activity and cAMP production, thus inhibiting protein kinase A (PKA), while activated phospholipase C (PLC) and Ca^{2+}, K^+ and Ca^{2+} channels are opened and closed, respectively, by A_1R enrollment. Mitogen-activated protein kinases p38, ERK1/2, and JNK1/2 phosphorylation are induced by A_1R activation.

A_1 receptors can be found also extrasynaptically on dendrites (Rivkees et al., 1995) and on the axonal fibers of the hippocampus (Swanson et al., 1995). Activation of A_1 receptors along the axon may be a powerful extra synaptic mechanism by which adenosine alters axonal electric transmission to inhibit neurotransmitter release (Swanson et al., 1995).

Out from CNS, high levels of A_1 receptor expression are found in adrenal glands, eye and atria. Intermediate levels are found in skeletal muscles, liver, kidney, adipose tissue, gastrointestinal smooth muscles and bronchi. Lung and pancreas present low level of A_1 receptor expression (Fredholm et al., 2001).

Pharmacology

Selective agonists for the A_1 receptor have been synthesized from adenosine molecule by modifications on its purine ring or its ribose. By substitution of adenosine in N6 position CPA (N6-

cyclopentyladenosine), a compound with greater affinity for A_1 receptor, has been obtained. Its derivative CCPA (2-chloro-N6-cyclopentyladenosine) presents a slightly higher affinity for A_1 receptor in human, rats and mouse tissues and is active at nanomolar concentrations. Further agonist for A_1 receptor analogues of adenosine are CADO (2-chloroadenosine), L-PIA ((R)-N-(1-methyl-2-phenylethyl) adenosine), CHA (N(6)-cyclohexyladenosine) and R-PIA (N6-R-phenylisopropyladenosine) (Jacobson et al., 1992). Allosteric enhancers for this receptor subtype are also available such as PD81,723 (2-amino-3-benzoylthiophene) and analogues. The discovery and development of pyridine-based non-nucleoside agonists of the adenosine receptors started from the publication of patents from Bayer describing pyridine derivatives endowed with agonist activity at the adenosine receptors. One of these compounds is the adenosine A_1 receptor agonist 2-amino-6-[[2-(4-chlorophenyl)-1,3-thiazol-4-yl] methylsulfanyl]-4-[4-(2-hydroxyethoxy) phenyl] pyridine3,5-dicarbonitrile, also named BAY68-4986 or Capadenoson. Capadenoson, currently classified as an adenosine A_1 receptor partial agonist, has undergone two phase II clinical trials, initially in patients with atrial fibrillation and subsequently in patients with stable angina (Tendera et al., 2012). Structural modifications at the exocyclic amine group of Capadenoson led to the development of the adenosine A_1 receptor agonist 2-(((2-(4-chlorophenyl)-1,3-thiazol-4-ylmethyl) sulfanyl)-4-(4-(2-hydroxyethoxy) phenyl)-6-(pyrrolidin-l-yl) pyridine-3,5-dicarbonitrile, also named Neladenoson. Like Capadenoson, Neladenoson is a selective partial agonist at the A_1 receptor (Meibom et al., 2017; Voors et al., 2017). Neladenoson showed in rat preclinical models cardioprotective effects analogous to Capadenoson, although with lower central effects. Methylxanthines are non-selective antagonists of adenosine receptors (Sattin & Rall, 1970). Antagonists with high affinity have been synthesized from theophylline, by substitution in position 8 with a cycloalkyl group (e.g. compounds 8-CPT, 8-cyclopentyltheophilline and 8-PT, 8-phenyltheophilline). By substitution of the methyl groups in 8-CPT with propyl groups DPCPX (8-Cyclopentyl-1,3-dipropylxanthine), a selective antagonist for A_1 receptor with high affinity, has been synthetized (Bruns et al., 1987). The substitution of the propyl group in position 3 of DPCPX with a more complex group led to the synthesis of BW-A884U (3-(4-Amino)phenethyl-1-propyl-8-cyclopentylxanthine) (Patel et al., 1988), a compound more selective than DPCPX. Other derivatives as KFM 19 (8-(3-oxocyiclopentane)-1,3-dipropylxanthine), KW-3902 (Rolofylline; 8-(Hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-1,3-dipropyl-1H-purine-2,6-dione), KF 15372 (8-dicyclopropylmethyl-1,3-dipropylxanthine) and ENBX (1,3-dipropyl-8-endonorbornyl- xanthine), poorly soluble in water, were obtained from theophylline performing substitutions in position 8 (Jacobson et al., 1992).

3.4.2 A_{2A} receptor: molecular structure, distribution and function

A_{2A} receptor is a glycoprotein of approximately 45 kDa with a highly conserved amino acid sequence, with a homology of 90% between different species. Its intracellular section is particularly extended (412 amino acids in 4 loops) and it seems to be involved in several responses A_{2A}-mediated (Cristalli et al., 2008). The receptor is associated to a G_s protein at TM2 and TM3 loops (Moreau & Huber, 1999), thus it stimulates adenylate cyclase. A_{2A} receptors are expressed throughout all the CNS (Dixon et al., 1996), principally in the basal ganglia: in the caudate-putamen, nucleus accumbens and olfactory tubercle (Jarvis et al., 1989; Rosin et al., 1998). In particular, this receptor subtype is expressed on striatopallidal GABAergic-enkephalin neurons (where it co-localizes with dopamine D_2 receptors, but not on GABAergic-dynorphin striatal neurons (Fredholm & Svenningsson, 2003). High levels of adenosine A_{2A} receptor mRNA have been found in striatum (Schiffmann et al., 1991; Svenningsson et al., 1997), while lower levels have been found in hippocampus and cortex (Dixon et al., 1996; Svenningsson et al., 1997). Besides postsynaptically, A_{2A} receptors are also located presynaptically on different GABAergic, cholinergic, glutamatergic neuron types (Hettinger et al., 2001; Rosin et al., 2003). They are also expressed on astrocytes (Biber et al., 1999; Lee et al., 2003), microglia (Pedata et al., 2014), oligodendrocytes (Stevens et al., 2002), blood cells and vasculature (Phillis, 2004).

Contrary to the A_1 receptor, which inhibits synaptic transmission in the CNS, A_{2A} receptor activation mediates excitatory actions (Latini et al., 1996; Pedata et al., 1984; Sebastião & Ribeiro, 1996; Spignoli et al., 1984). Indeed, the A_{2A} receptor G_s-mediated signaling increases cAMP level, a second messenger that activates protein kinase A (PKA) (Gubitz et al., 1996). PKA phosphorylates the Ca^{2+} channels of P type and thus, directly modulates the mechanisms of vesicle release. Vesicle exocytosis can also be regulated by βγ subunits of the G_s protein, which can promote the phosphorylation, mediated by PKC, of Ca^{2+} channels of N-type (Gonçalves et al., 1997; Gubitz et al., 1996) (Figure 14).

Presynaptically, A_{2A} receptor stimulation triggers the release of several neurotransmitters, including glutamate (Cunha & Ribeiro, 2000; Lopes et al., 2002), acetylcholine (Rebola et al., 2002), dopamine (Navarro et al., 2009), 5-HT (M. Okada et al., 2001), and GABA (Cunha & Ribeiro, 2000). In addition, presynaptic A_{2A} receptors play a critical role in suppressing cannabinoid type 1 receptor- (CB_1), and A_1 receptor-mediated inhibition (Ciruela et al., 2006). Postsynaptically, A_{2A} receptors modulate the activation of NMDA receptors (Rebola et al., 2008; Wirkner et al., 2004), CB_1 receptors (Andersson et al., 2005), metabotropic glutamate receptor 5 mGluR5 (Ferré et al., 2002), dopamine D_2 receptors (S. Ferre et al., 2008). Postsynaptic A_{2A} receptors are also required for the maintenance of long-term potentiation (LTP) (Fontinha et al., 2008), a form of synaptic plasticity associated with memory, central for learning process that is increased by A_{2A} receptor activation (Almeida et al., 2003). Moreover, A_{2A}

receptor activity is known to facilitate the effects of BDNF upon synaptic transmission and plasticity (Diógenes et al., 2004).

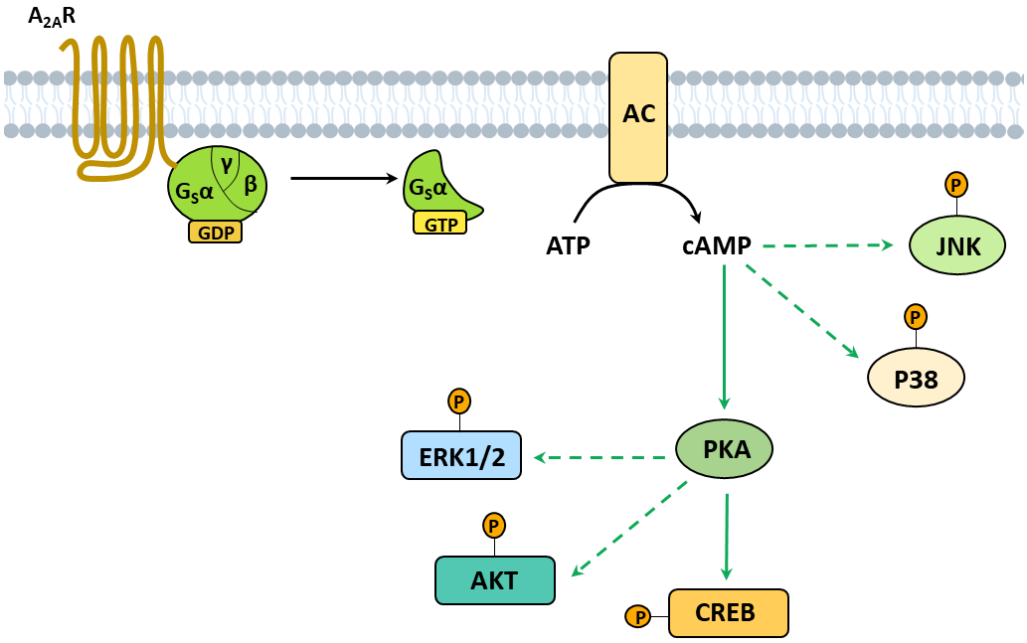

Figure 14. Overview of A_{2A} receptor ($A_{2A}R$) intracellular signaling pathways. $A_{2A}R$ stimulation increases adenylate cyclase (AC) activity, cAMP production, protein kinase A (PKA), and cAMP-responsive element-binding protein (CREB) phosphorylation. AKT and mitogen-activated protein kinases p38, ERK1/2 and JNK1/2 are activated following by $A_{2A}R$ recruitment. Modified from (Borea et al., 2018).

In recent years, particular interest has been dedicated to study receptor dimerization, either in homomeric and heteromeric structures, since this phenomenon seems to frequently occur in numerous cell types and can modify the pharmacological profile of receptors and their functional role. A_1 receptor and A_{2A} receptor heteromers are located presynaptically in glutamatergic terminals of the striatum (Ciruela et al., 2006), exerting opposite effects on the modulation of glutamate release through a "concentration-dependent switch" mechanism, by which low adenosine concentrations inhibit while high concentrations stimulate glutamate release (Ciruela et al., 2006).

Moreover, various lines of evidence indicate that such an interaction occurs postsynaptically in the striatum between A_{2A} adenosine and D_2 dopamine receptors, and that this heterodimerization inhibits D_2 receptor functions (Ferre et al., 1991). The association between A_{2A} and D_2 receptors results in an antagonistic interaction which provided a rationale for evaluating A_{2A}-selective antagonists in Parkinson's disease. It was suggested that A_{2A} receptor antagonists not only provide symptomatic relief but also decelerate dopaminergic neuron degeneration in patients (Xu et al., 2005). On July 2019, A_{2A} receptor antagonists have been approved in the United States by FDA in the therapy of Parkinson's disease (fda.gov) (Takahashi et al., 2018).

A_{2A} receptors are also highly present in spleen, thymus, immune cells both on cells of innate (macrophages, mast cells, monocytes, dendritic cells, and granulocytes) and on cells of adaptive (lymphocytes) immunity (Antonioli et al., 2014; Haskó et al., 2008). Lower levels are also found in the heart, lung and blood vessels (Fredholm et al., 2001).

Pharmacology

Most of A_{2A} receptors agonists have been obtained from the adenosine molecule by substitution of the purine ring in the position 2 or by different types of substitutions at the ribose ring. By the replacement of an alchilamide group in position 5' of the ribose ring, the agonists NECA (5'-N-ethylcarboxamidoadenosine) and CGS21680 have been synthetized. NECA was long considered to be a selective A_2 receptor agonist, but it has been largely demonstrated that it is an unselective agonist to all P1 receptors, with a slightly preference for A_{2A} subtype. However, based on evidence that 2-substitution of NECA molecule increased selectivity, CGS21680 was developed as an A_{2A} selective agonist. This compound is less potent and selective in humans than in rats, but it has been replaced by another recently developed A_{2A} receptor agonist, ATL-146e (4-(3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl)-cyclohexanecarboxylic acid methyl ester), which is 50-fold more potent than CGS21680 for the human A_{2A} receptor. By substitution in position N6, the compound DPMA has been obtained (Bridges et al., 1988).

Many of A_{2A} receptor antagonists have been developed from the caffeine (1,3,7-trimethylxanthine) molecule. DPMX (1,3-dipropyl-7-methyl-xanthine) and 8-cicloesilcaffeine are low selective A_{2A} receptor antagonists (Williams & Jarvis, 1991). CSC (Jacobson et al., 1993) and KF17837 ((E)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxanthine) (Nonaka et al., 1994) are more selective but less easy to use since undergo to photoisomerization. Among the numerous A_{2A} receptor antagonists, the most selective so far are SCH58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolol[1,5-c]pyrimidine) and SCH442416 (2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine) and the structurally related ZM241385 (4-(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl amino]ethyl)phenol). SCH58261 does not derive from xanthine, as well as CP66713 (8-chloro-1-phenyl- [1,2,4] triazolo[4,3-a] quinoxalin-4-amine). However, also CP66713 presents a good selectivity for the A_{2A} receptor (Sarges et al., 1990).

3.4.3 A_{2B} receptor: molecular structure, distribution and function

The A_{2B} receptor is a glycoprotein of approximately 36-37 kDa, coupled to G_s protein, that stimulates adenylate cyclase, intracellular Ca^{2+} mobilization (Mirabet et al., 1997) and activates PKC and PLC (Abbracchio et al., 1995; Feoktistov & Biaggioni, 1998).

A_{2B} receptors are primarily expressed in peripheral tissues, in particular in the gastrointestinal tract, mainly in caecum, colon, urinary bladder, lung, blood vessels and adipose tissue (Fredholm et al., 2001). Nevertheless, A_{2B} receptors are known to be expressed both in the CNS and in the peripheral nervous system (PNS) (Dixon et al., 1996). At the central level, they are ubiquitously distributed in the brain, with a prevalent presence on hippocampal neurons (Kessey et al., 1997; Mogul et al., 1993) and on glial cells (Fiebich et al., 1996; Peakman & Hill, 1994).

Despite previous studies attributed poor physiological relevance to A_{2B} receptor, due to its low affinity for adenosine in comparison with the other adenosine receptors (Sun & Huang, 2016), increasing evidences indicate a role for this receptor in the modulation of inflammation and immune responses in several pathologies like cancer, diabetes, as well renal, lung and vascular diseases (Borea et al., 2018). Indeed, A_{2B} receptor expression is upregulated in injurious conditions such as hypoxia, inflammation, and cell stress throught hypoxia-inducible factor (HIF), since a functional binding site for HIF-1 is present in the A_{2B} receptor promoter (Eckle et al., 2014; Kong et al., 2006).

A_{2B} receptor signaling pathways involve AC activation through G_s proteins, leading to PKA phosphorylation and enrollment of different cAMP-dependent effectors. In addition, A_{2B} receptor can stimulate PLC through the G_q protein, resulting in Ca^{2+} mobilization, and can regulate ion channels through their βγ subunits. Moreover, this subtype acts as stimulator of MAPK activation in several cell models in both central and peripheral systems (Sun & Huang, 2016) (Figure 15).

A_{2B} receptors induce the release of excitatory amino acids and acetylcholine, while they reduce the release of γ-amino butyric acid (GABA) in rat cortex (Phillis et al., 1993). In addition, at hippocampal level, they modulate the LTP process (Kessey et al., 1997).

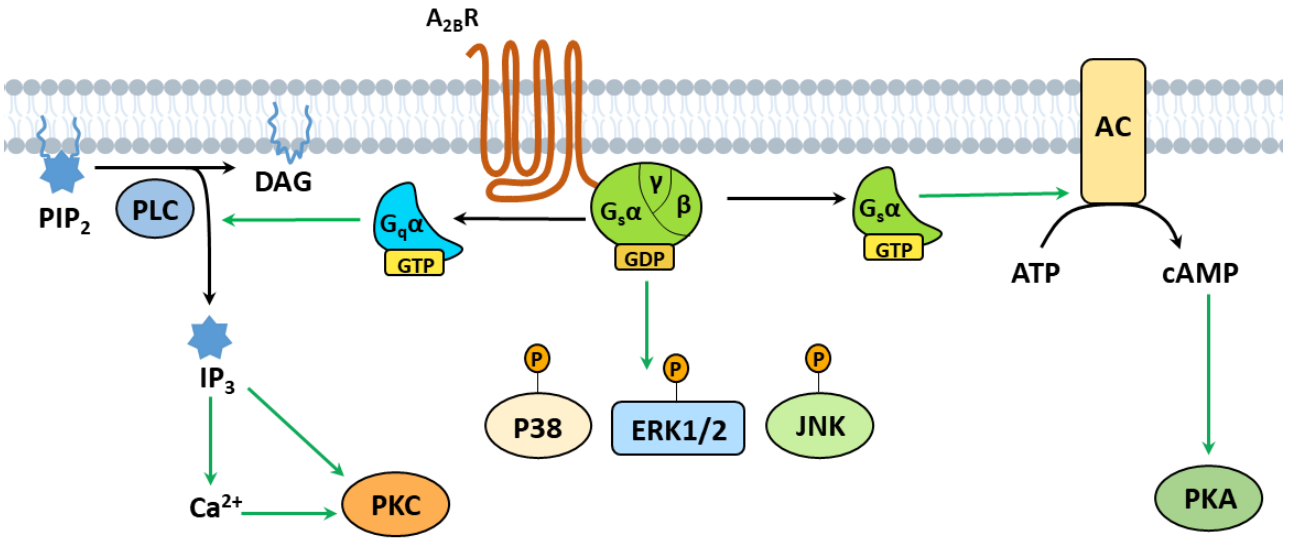

Figure 15. Overview of A_{2B} receptor ($A_{2B}R$) intracellular signaling pathways. $A_{2B}R$ stimulation increases adenylate cyclase (AC) activity, cAMP production, and protein kinase A (PKA) phosphorylation. $A_{2B}R$ enrollement activates phospholipase C (PLC) and increases Ca^{2+}. Mitogen-activated protein kinases p38, ERK1/2, and JNK1/2 phosphorylation are induced by $A_{2B}R$ activation.

Moreover, the signaling pathways generated by A_{2B} receptors stimulation are strongly influenced by the signaling of other receptors that affect the PLC-Ca^{2+}-PKC pathway. Indeed, in brain slices, it has been demonstrated that cAMP accumulation due to the activation of the A_{2B} receptor is markedly increased by drugs that stimulate PKC (Fredholm et al., 1987; Hollingsworth et al., 1985).

Pharmacology

A class of non-adenosine A_{2B} receptor agonists with affinity values in the low nanomolar range has been synthesised by Beukers and colleagues as pyridine derivatives (Beukers et al., 2004). Among them the most important are LUF5834 (2-amino-4-(4-hydroxyphenyl)-6-(1H-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile) (Beukers et al., 2004) and BAY60-6583 (2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide) (Kuno et al., 2007; Eckle et al., 2008 a) that are highly selective. LUF5835 (2-amino-4-(4-hydroxyphenyl)-6-(1H-imidazol-2-ylmethylsulfanyl) pyridine-3,5-dicarbonitrile,17) is a full agonist with an EC_{50} of 10 nM for human A_{2B} receptor expressed in CHO cells.

Several potent and relatively selective antagonists have been found among anilide derivatives of xanthines with Ki values in the low nanomolar range, such as MRS1754 (N-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide) (Ji et al., 2001), that is over 200-fold selective for A_{2B} versus all other adenosine receptors (Kim et al., 2000) and the selective

A_{2B} receptor antagonist 8-[4-[4-(4-Chlopehenzyl) piperazide-1-sulfonyl) phenyl]]-1-propylxanthine (PSB603) (Fusco et al., 2018).

Further antagonists xanthine-derived are MRE 2029-F20 (N-(2H-1,3-benzodioxol-5-yl)-2-{[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3yl] oxy}acetamide) and OSIP339391 (N-[2-[[2-phenyl-6-[4-(3-phenylpropyl) piperazine-1-carbonyl]-7H-pyrrolo[3,2-e]pyrimidin-4-yl]amino]ethyl]acetamide) (Gessi et al., 2005; Kim et al., 2001; Stewart et al., 2004).

3.4.4 A_3 receptor: molecular structure, distribution and function

A_3 receptor is a protein of approximately 39 kDa, coupled to G_i protein. It evokes the same effects mentioned for A_1 receptor (Englert et al., 2002), induces Ca^{2+} mobilization (Englert et al., 2002; Shneyvays et al., 2005; Shneyvays et al., 2004) and interacts with MAPKs (Schulte & Fredholm, 2003) (Figure 16).

The expression of A_3 adenosine receptor in the brain is generally lower than that of the other subtypes (Ji et al., 1994) and is highly species-dependent (Fredholm et al., 2000, 2001). By the sensitive technique of real time PCR, A_3 receptors are found in both neuronal and non-neuronal elements, i.e. astrocytes, microglia, and vasculature of the cerebral tissue (Zhao et al., 1997) with widespread distribution. In the rat, a significant expression of A_3 receptors is found in cerebellum and hippocampus (Dixon et al., 1996) where they are mainly expressed at the presynaptic level (Lopes et al., 2003).

Discrepancies are present in literature about the role of adenosine A_3 receptors in the CNS. An excitatory role of A_3 receptor has been supported by evidence indicating that, in the rat hippocampus, its activation attenuates long-term depression (LTD) and allows induction of LTP elicited by a subliminal weak-burst protocol (Costenla et al., 2001). Additional evidence for an excitatory role of adenosine A_3 receptors came from studies carried out in hippocampal slices (Pugliese et al., 2007). In the same brain area, A_3 receptor activation through a selective agonist has been shown to antagonize the adenosine A_1 receptor-mediated inhibition of excitatory neurotransmission (Dunwiddie & Masino, 2001). However, further electrophysiological studies refused this hypothesis, since several authors demonstrated that no significant interaction between A_1 and A_3 receptors occurs in the rat cortex and hippocampus (Brand et al., 2001; Lopes et al., 2003). Conversely, an inhibitory action has been attributed to A_3 receptors by Brand and colleagues, who demonstrated that, in rat cortical neurons, the selective activation of A_3 receptor is involved in inhibition of excitatory neurotransmission, suggesting a synergic action with the inhibitory effect mediated by A_1 receptor activation.

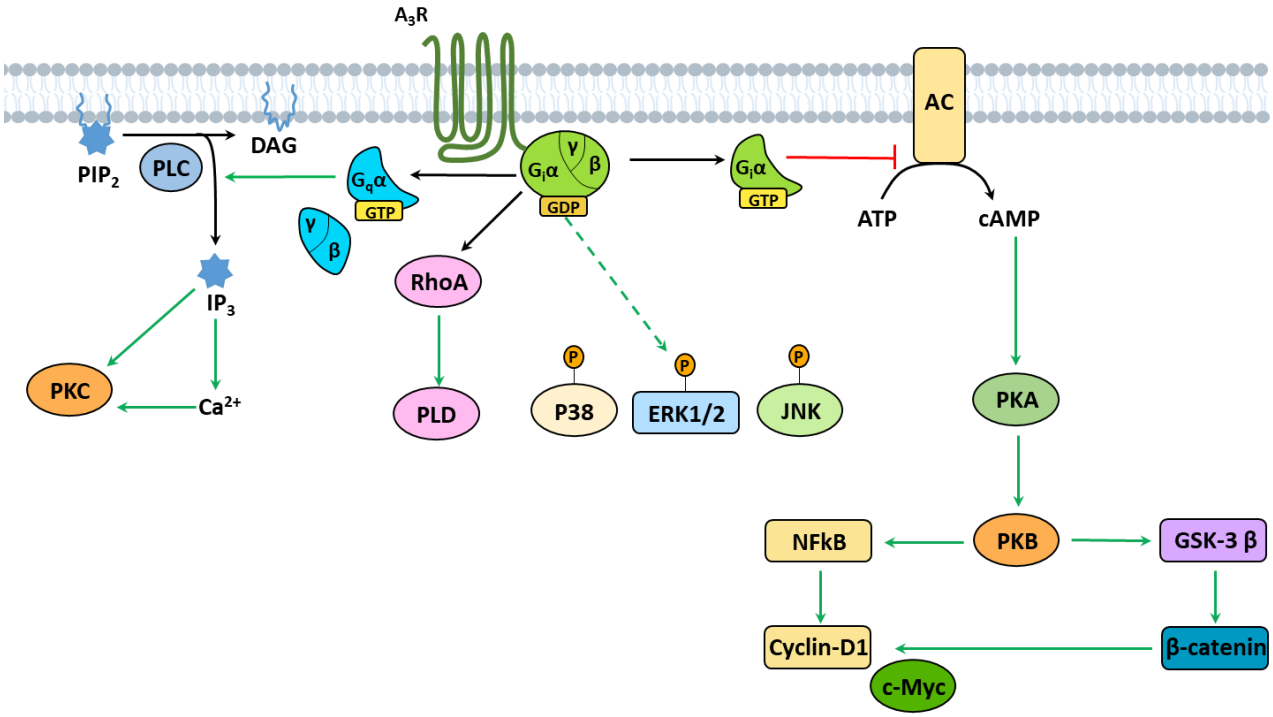

Figure 16. Overview of A_3 receptor (A_3R) intracellular signaling pathways. A_3R stimulation triggers decrease of adenylate cyclase (AC) activity and cAMP production, activation of glycogen synthase kinase-3β (GSK-3β), and consequent decrease of β-catenin, cyclin D1, and c-Myc. Increase induced by A_3R activation of phospholipase C (PLC) and Ca^{2+}, as well as of RhoA and phospholipase D (PLD) is shown. Mitogen-activated protein kinases p38, ERK1/2, and JNK1/2 phosphorylation are induced by A_3R activation.

Despite results obtained by A_3 receptor stimulation, evidence that selective block of A_3 receptors does not affect neurotransmission in the CA1 region of the hippocampus under normoxic conditions, indicates that endogenous adenosine at physiological concentration does not exert tonic activation of A_3 receptors (Dunwiddie & Masino, 2001; Pugliese et al., 2003).

In the periphery, A_3 subtype is mainly found in rat testis (Meyerhof et al., 1991) and mast cells, in accordance with the fact that for a long time the unique role assigned to this receptor have been mast cell degranulation and histamine release. Intermediate levels are found in the lung, spleen, thyroid and liver. Interestingly, A_3 receptor is overexpressed in several cancer cells and tissues and is therefore likely to have an important antitumoral role (Pier Andrea Borea et al., 2015). Moreover, a recent paper from our research group proved that A_3 receptor activation has pain-relieving effects mediated through N-type Ca channel block in isolated rat DRG neurons (Coppi et al., 2019).

Pharmacology

The most widely used A_3 receptor agonist is Cl-IB-MECA (2-Chloro-N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide) for its high affinity (low nanomolar range) and selectivity (more than 100-fold

versus A_1 and A_{2A} receptors) towards A_3 receptors. For these features Cl-IB-MECA is the most used pharmacological tool to investigate A_3-mediated effects. Cl-IB-MECA affinity does not vary beyond an order of magnitude among mammal species. The new, highly selective, A_3 receptor agonist (1S,2R,3S,4R,5S)-4-(2-((5-chlorothiophen-2-yl)ethynyl)-6-(methylamino)-9H-purin-9-yl)-2,3 dihydroxy-N-methylbicyclo [3.1.0]hexane-1-carboxamide (MRS5980) was later synthesized (Fang et al., 2015; Tosh et al., 2014).

Particularly potent agonists can be obtained through the 4'-thio modification of adenosine derivatives, such as in LJ 568 (Jeong et al., 2003).

An emblematic feature of the adenosine A_3 receptor is its insensitivity to the antagonistic actions of methylxanthines, such as caffeine and theophylline, the traditional blockers of adenosine receptors. Hence, A_3 receptor antagonists are dihydropyridines, pyridines and flavonoids. Another class of highly selective compounds are isoquinoline and quinazoline derivatives, such as VUF5574 (N-(2-methoxyphenyl)-N'-[2-(3-pyrindinyl)-4-quinazolinyl]-urea) that presents a Ki value of 4 nM versus human A_3 receptors but not versus the rat isoform. In this regard, it is worth noticing that significant species differences in the affinity of adenosine A_3 receptor antagonists have been noted, as expected from the high structural inter-species variability already mentioned. The affinity values of several A_3 blockers are typically more than 100-fold greater for human than rat receptors, as described for MRS1220 (N-[9-Chloro-2-(2-furanyl) [1,2,4]-triazolo[1,5-c] quinazolin-5yl] benzene acetamide).

Among the antagonists are also CCPA and DPMA (N(6) -[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine), whose structures have been used to get the drug MRS1292 which is a selective antagonist of A_3 receptors, both in rat and in humans (Gao et al., 2002; Yang et al., 2005). Finally a selective adenosine antagonist is MRS1523 (3-propyl-6-ethyl-5-[(ethylthio) carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate) (Li et al., 1998).

Chapter IV: Role of adenosine A_{2B} receptor in cerebral ischemia

4.1 Adenosinergic signaling in brain ischemia

During *in vivo* ischemia, extracellular adenosine concentration increases (Dux et al., 1990; Hagberg et al., 1987; Matsumoto et al., 1992; Melani et al., 1999; Phillis et al., 1994; Sciotti et al., 1992). Soon after ischemia (in the first 20 minutes after ischemia onset), this increased adenosine concentration is mainly due to extracellular release of ATP (Melani et al., 2012), which is hydrolyzed by ecto-nucleotidases (NTPDases 1, 2 and 3 that convert ATP to ADP and AMP) and by ecto-5'-nucleotidase, that convert AMP to adenosine (Fausther et al., 2012; Zimmermann, 2000). Subsequently, in the hours after ischemia, adenosine *per se* is released from cells, likely by the ENT2 (Melani et al., 2012).

Inhibition of adenosine-uptake processes due to down-regulation of CNT 2 and 3 and of the ENT1 also contributes to the extracellular adenosine increase after stroke (Medina-Pulido et al., 2013). During ischemia the extracellular adenosine concentrations are able to stimulate all of four adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3). The wide distribution of ATP receptors on neurons, glial cells and peripheral inflammatory cells (Brodie et al., 1998; Fiebich et al., 1996; Hettinger et al., 2001; Svenningsson et al., 1999; Yu et al., 2004) suggests that their role in ischemia is the consequence of an interplay among different receptor activation in neuronal, glial and inflammatory cells, which may vary depending on the time-related development of the pathological condition (Pedata et al., 2016).

During ischemia, adenosine has long been identified as a neuroprotectant endogenous agent (Cunha, 2001; Pedata et al., 2008; Fredholm et al., 2005; Ongini et al., 1997; Ribeiro et al., 2002; Schwarzschild et al., 2002). Indeed, it was demonstrated that adenosine infusion into the ischemic striatum significantly ameliorated neurological outcome and reduced infarct volume after transient focal cerebral ischemia (Kitagawa et al., 2002). This protective effect has been attributed to A_1 receptor, whose activation leads to reduced Ca^{2+} influx and thus to lowering presynaptic release of excitatory neurotransmitters, in particular glutamate, which during ischemia exerts an early excitotoxic effect mainly by overstimulation of NMDA receptors (Andiné, 1993; Choi, 1990; Corradetti et al., 1984; Dunwiddie, 1984; Kitagawa et al., 2002; Pedata et al., 1993; Zetterström & Fillenz, 1990). In addition, by increasing the K^+ and Cl^- ion conductances, adenosine stabilizes the neuronal membrane potentials, thus reducing neuronal excitability (Choi, 1990). However, A_1 receptor-selective agonists are not used clinically due to important side effects such as bradycardia, sedation and hypotension (Sollevi, 1986; Von Lubitz et al., 1994) White et al., 1996; Fredholm et al., 2005). For this reason, in the last years research focused on A_{2A} and A_{2B} receptors subtypes.

Indeed, it was reported that both antagonists and agonists of adenosine A_{2A} receptor exert protective effects after ischemia.

In the *in vitro* OGD model in acute rat hippocampal slices, the selective A_{2A} receptor antagonists, ZM241385 and SCH58261, delay the appearance of AD, an unequivocal sign of neuronal injury (Somjen, 2001), protect from the synaptic activity depression brought about by a severe (7 min) OGD period, and protect CA1 neuron and astrocyte from injury (Pugliese et al., 2009). The A_{2A}R antagonists also delay AD onset invariably induced during a long period, 30 min, OGD in the CA1 region of the hippocampus (Pugliese et al., 2009).

In vivo, it has been demonstrated that the A_{2A} receptor-selective antagonist, ZM241385, injected peripherally or directly intra hippocampus, is protective against excitotoxicity induced by kainate and by the combinations of quinolinic acid and IL-1β (Jones et al., 1998). Moreover, A_{2A} receptor

antagonists protect from ischemia–induced activation of MAPKs expression (Stone & Behan, 2007). In addition, it has been demonstrated that the selective A_{2A} receptor antagonist, SCH58261, acutely (5 min) or sub-chronically (5 min, 6 h, and 20 h) administered, was protective against neurological deficit and brain damage 24 hours after ischemia (Melani et al., 2006). The neuroprotective properties of adenosine A_{2A} receptor antagonists largely reside in direct central effects mediated by A_{2A} receptors located on brain cells, where the A_{2A} receptor antagonists exert early neuroprotection by controlling the excessive glutamatergic transmission and thus the ensuing acute excitotoxicity after ischemia. Several mechanisms contribute to such protective effect of the A_{2A} receptor in brain ischemia. A_{2A} receptor stimulation might regulate extracellular glutamate by modulation of glutamate uptake transporter. In the brain, adenosine A_{2A} receptors are expressed on both neurons and glia (Fiebich et al., 1996; Fink et al., 1992). In particular, A_{2A} receptors located on astrocytes mediate inhibition of glutamate uptake by glutamate transporter-1 (Nishizaki et al., 2002; Pinto-Duarte et al., 2005; Pintor et al., 2004). Moreover, the protective effect can be related to the reduction of activation of deleterious pathways of MAPK expressed in microglia, such as p38 (Melani et al., 2006). This is consistent with the finding that, 24 hours after focal ischemia, a definite overexpression of A_{2A} receptors (Trincavelli et al., 2008) and an activation of p38 and ERK1/2 MAPKs was found in neurons and in microglia of the ischemic tissue (Irving et al., 2000; Melani et al., 2006; Piao et al., 2003; Takagi et al., 2000). However, 7 days after ischemia onset, the A_{2A} receptor antagonist SCH58261, chronically administered, failed to be protective from ischemic brain damage (Melani et al., 2015).

In an apparent paradoxical manner, also A_{2A} receptor agonists exert protective effect in cerebral ischemia and such protection is exerted 7 days after ischemia (Melani et al. 2014). Activation of central A_{2A} receptors is known to increase expression and release of neurotrophic factors (A. M. Sebastião & Ribeiro, 1996) such as nerve growth factor in microglia (Heese et al., 1997), BDNF in mice hippocampus (Tebano et al., 2008), in rat cortical neurons (Jeon et al., 2011) and in primary cultures of microglia (Gomes et al., 2013), and glial cell line-derived neurotrophic factor in striatal neurons (Gomes et al., 2006). The increase in neurotrophic factor expression by adenosine A_{2A} receptor stimulation may contribute to restore neurological functions and cerebral damage after brain ischemia. Besides protective effect attributable to A_{2A} expressed at central level, a bulk of evidences indicate that peripheral effects on A_{2A} receptors located on blood cells greatly account for protective effects of adenosine A_{2A} receptor agonists after ischemia. Adenosine A_{2A} receptors are present, in fact, on peripheral blood marrow derived cells including platelets, monocytes, mast cells, neutrophils and T cells, where selective activation of A_{2A} receptors reduces production of adhesion cell factors, reduces

platelet aggregation and neutrophil activation exerting therefore an antiplatelet, antioxidant and anti-inflammatory effect (Haskó et al., 2000; Lappas et al., 2006; Sitkovsky et al., 2004; Varani et al., 1998).
In particular, it has been demonstrated that A_{2A} receptor activation reduced ischemia-induced rolling, adhesion and transmigration of various peripheral inflammatory cells (such as lymphocytes and neutrophils) (Haskó et al., 2008). In agreement, A_{2A} receptor agonists, systemically administered, protected from inflammation in various models of autoimmune disease such as rheumatoid arthritis (Mazzon et al., 2011; Szabó et al., 1998), colitis (Di Paola et al., 2010; Odashima et al., 2005), and hepatitis (Choukèr et al., 2008) and in models of spinal cord trauma (Genovese et al., 2009; Li et al., 2006; Paterniti et al., 2011) and of traumatic brain injury (Dai et al., 2010). The protracted protection provided by the A_{2A} receptor agonists is thus attributable to reduced secondary damage, i.e. neuroinflammation, which in the hours and days after ischemia overwhelms early glutamate-mediated excitotoxicity (Coppi et al., 2020).
Regarding adenosine A_3 receptor, the few studies present in the literature concerning their role in cerebral ischemia are rather contradictory. The effects of adenosine A_3 receptor stimulation appears to depend on drug administration (acute vs chronic), dosage and timing of treatment with respect to the onset of the ischemic insult (Borea et al., 2009; Pedata et al., 2010).

4.1.1 Adenosine A_{2B} receptor in cerebral ischemia

Adenosine A_{2B} receptor is expressed at low levels uniformly throughout the CNS (Dixon et al., 1996; Fredholm et al., 2000; Puffinbarger et al., 1995) and has a low affinity for adenosine. The A_{2B} receptor mRNA and protein expression levels increase on endothelial cells, neurons, and astrocytes to a greater extent than do those of the other three adenosine receptors (A_1, A_{2A}, and A_3) 24 hours after tMCAo in the rat (Q. Li et al., 2017). Thus, during conditions of hypoxia or ischemia, when the extracellular adenosine levels rise, A_{2B} receptors might be well activated (J. Xu et al., 2013). Because of paucity of A_{2B} selective agonists and antagonists (Müller & Jacobson, 2011) few studies are till now present on the role of A_{2B} receptors in brain ischemia.
Recently, the role of A_{2B} receptor selective antagonists was investigated under OGD. Fusco et al., (2018) demonstrated that, in the CA1 area of the rat hippocampus, the selective A_{2B} receptor antagonists, MRS1754 and PSB603 prevented the appearance of AD, a phenomenon strictly related to cell damage and death (Pugliese et al., 2006) and protected from the synaptic activity depression, bringing to a significant recovery of an otherwise disrupted neurotransmission induced by 7 min OGD (Fusco et al., 2018). Adenosine A_{2B} receptor antagonism also counteracted the reduction of neuronal density in CA1 stratum pyramidale, decreased apoptosis at least up to 3 hours after the end of OGD and prevented astrocyte modification (Fusco et al., 2018).

The selective A_{2B} receptor antagonist, N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) henoxy] acetamide (MRS1706), completely prevented in human astroglial cells elongation of astrocytic processes (a morphological hallmark of *in vivo* reactive astrogliosis) induced by selective stimulation of A_{2B} receptors (Trincavelli et al., 2004). Moreover, a short-term TNF-α treatment induced A_{2B} adenosine receptor desensitization in human astroglial cells (Trincavelli et al., 2004). These results suggest that in the acute phase of brain ischemia characterized by both cytokine and adenosine high release, A_{2B} receptor desensitization on astroglia might represent a cell defense mechanism (Trincavelli et al., 2004).

Few studies have investigated the role of A_{2B} receptors in brain ischemia *in vivo*. It was reported that the selective A_{2B} receptor antagonist, MRS1754, administered befor ischemia induction, reduced the ceramide production in astrocytes and attenuated inflammatory responses and neuronal damage after 30 minutes and 6 hours in a model of global cerebral ischemia induced by four-vessel occlusion in the rat (Gu et al., 2013). This effect was related to an early reduction of p38 MAPK activation. In fact, A_{2B} receptor plays a key role in the rapid activation of p38 and in the subsequent inflammatory process (Koscsó et al., 2012; Wei et al., 2013). Altogether experiments indicated that a precocious antagonism of A_{2B} receptor located on brain cells might be protective from ischemic brain damage.

Besides brain cells, A_{2B} receptors are present on blood immune cells i.e neutrophils, lymphocytes (Eckle et al., 2008; Gessi et al., 2005) where in most cases they are co-expressed with A_{2A} receptors. They are also expressed at low levels on platelets, where they are upregulated following injury and systemic inflammation *in vivo* inducing an inhibition of platelet aggregation (Yang et al., 2010). Moreover, A_{2B} receptors are expressed on the surface of endothelial cells (Feoktistov et al., 2004) where they are up regulated by the HIF-1α (Eltzschig et al., 2004). In agreement, vascular permeability was significantly increased in organs of A_{2B} receptor KO mice subjected to ambient hypoxia. By contrast, hypoxia-induced vascular leak was not accentuated in A_1, A_{2A} or A_3 receptor KO mice, suggesting a specific role of A_{2B} receptor in endothelial cells (Eckle et al., 2007). Moreover, A_{2B} receptor KO mice exposed to hypoxia exhibit increased neutrophil infiltration into hypoxic tissues revealing an inhibitory role for A_{2B} receptors in neutrophil transmigration *in vivo* (Eckle et al., 2008, 2007). Attenuation of hypoxia-associated increases in tissue neutrophil numbers appeared to depend largely on hematopoietic cell A_{2B} receptor signaling (Eckle et al., 2007; Yang et al., 2006).

Recent introduction of new pharmacological tools (Hinz et al., 2014) led to understand the role of A_{2B} receptor in ischemia. Intravenous treatment with the selective A_{2B} receptor agonist BAY60-6583 (1 mg/kg), at the start of reperfusion after brain ischemia induced by tMCAo, reduced lesion volume and attenuated brain swelling and BBB disruption. In the presence of the trombolitic treatment t-PA,

BAY60-6583 also mitigated sensorimotor deficits and reduced t-PA induced hemorrhages at 24 hours after ischemia (Li et al., 2017). The neurovascular protection afforded by BAY60-6583 appears to derive from stimulation of the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) production, inhibition of t-PA-induced MMP activation, and prevention of tight junction protein degradation. In fact, overactivation of MMP leads to increased cerebrovascular permeability after ischemia-reperfusion injury (Mishiro et al., 2012). Thus, this study proposed that A_{2B} receptor agonists might be adjuvant to t-PA and could be a promising strategy for decreasing the risk of hemorrhages during treatment for ischemic stroke (Li et al., 2017).

All together these studies point toward a role of central A_{2B} receptors, in synergy with A_{2A} receptors in promoting brain excitotoxicity, while A_{2B} receptors located on vascular endothelial cells would play a pivotal role in attenuating hypoxia-induced increases in vascular leak. Blood immune cells would be implicated in dampening vascular adhesion signals and hypoxia-induced inflammation (Koeppen et al., 2011). A_{2B} receptor agonists might therephore exert a protective effect at more distant times from ischemia, when a defined neuroinflammation develops.

4.2 Therapeutic potential of multi-target drugs

Despite the successes of traditional single-target drugs, monotherapies seem to fail against complex diseases, showing limited efficacy and increasing incidences of drug resistance (Makhoba et al., 2020). To overcome the therapeutic limitations observed for the single-target directed drugs, in the last decade multi-target drugs (or polypharmacology) have emerged as a promising approach against diseases with complex pathophysiological mechanisms.

Multi-target drugs, first proposed by Morphy et al. and Roth et al. in 2004, refer to drugs that simultaneously modulate the activity of multiple targets at clinically relevant concentrations, and that in the last few years have gained the interest of scientific community. This shifting from single- to multi-target approach is exemplified by the fact that multi-target drugs represent 21% of the new molecular entities approved by FDA from 2015 to 2017 (Ramsay er al., 2018). Research has also focused in understanding polypharmacology and its origins in order to improve its exploitation for therapeutic purposes. In a recent review by Jalencas and Mestres, polypharmacology was analyzed from an evolutionary point of view, suggesting that the ability of early biological organisms to adapt to environmental changes might have favored the selection of biomolecular systems intrinsically prone to chemical promiscuity (Jalencas & Mestres, 2013).

To fight complex diseases, multi target drugs may be an efficient and cost-effective alternative to single-target drugs combination therapies, offering advantages like lower risk of toxicities (Anighoro et

al., 2014), lower drug resistence and development of tolerance (Liu et al., 2013), and lower likelihood of drug-drug interactions in comparison to combination therapies of single-target drugs (Amelio et al., 2015).

Multi-target drugs are promising candidates for several diseases such as cancer and diabetes, and for neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Kondej et al., 2018; Wang et al., 2019). Therephore, dual- or multi-target approach could be useful for the treatment of a complex pathology like brain ischemia (Geldenhuys & Van der Schyf, 2013). For example JM-20, a novel multi-target ligand, 1,5-benzodiazepine fused to a dihydropyridine moiety, showed neuroprotective activity both *in vitro* and *in vivo*. When administered 8 hours after reperfusion, after a 90 min-MCAo, JM-20 protected brain mitochondria, inhibited brain excitatory amino acid release and reduced ischemic brain injury 24 h post-ischemia. (Nuñez-Figueredo et al., 2014).

4.2.1 Dual drugs targeting adenosine receptors

Thanks to the availability of crystal structures, several dual drugs targeting adenosine receptors have been investigated (Burggraaff et al., 2020; Van Westen et al., 2012). A dual adenosine A_{2B}/A_3 receptor antagonist was designed as an anti-asthmatic agent (Press et al., 2005), while dual adenosine A_{2A} receptor agonists/A_3 receptor antagonists have been developed for the treatment of asthma and other inflammatory diseases (Bevan et al., 2007).

Regarding brain diseases, dual adenosine A_1/A_{2A} receptor antagonists were synthesized with the goal to treat neurodegenerative diseases including Parkinson's (Atack et al., 2014; Shook et al., 2010).

Moreover, potent and selective dual adenosine A_1/A_3 receptor antagonists were developed as potential therapeutics for treating kidney failure, pulmonary diseases, and Alzheimer's disease (Abdelrahman et al., 2020).

To date, no data are present in the literature regarding dual adenosine receptors ligands for the treatment of cerebral ischemia. However, since cerebral ischemia is a complex disease, targeting multiple receptors could have a synergic effect for its treatment.

Recently, a potent mixed agonist A_{2A}/A_{2B} (C2-substituted compound, MRS 3997) has been developed by Professor Kennet Jacobson's group, from the Institute of Nationl Health of Washington. MRS3997 has a high potency for both A_{2A} and A_{2B} adenosine receptors, with a EC_{50} of 39.7 (A_{2A}) and 109 (A_{2B}) nM for the stimulation of adenyl cyclase in CHO cells transfected with human receptors (Gao et al., 2014). Moreover, MRS3997 has a low affinity for A_1 and A_3 receptor subtypes (Adachi et al., 2007). The *in vivo* administration of a dual A_{2A}/A_{2B} receptor agonist, could offer several advantages respect to the simultaneous administration of the two A_{2A} and A_{2B} receptor agonists, CGS21680 and BAY60-6583,

respectively. A mixed agonist may reach a uniformly distributed concentration in the plasma and in the tissues, respect to the two compound administered separately. This would guarantee the activation of both receptor subtypes likely offering a greater protective effect . Furthermore, unlike BAY60-6583, which is a partial A_{2B} receptor agonist (Hinz et al., 2014), MRS3997 acts as a full agonist on A_{2B} receptors, thus offering the possibility of a correct interpretation of results. The compound MRS3997 does not present labile groups, and it has an increased hal-life in comparison to CGS21680 and BAY60-6583.

All these characteristics render the mixed A_{2A}/A_{2B} receptor agonist MRS3997 an interesting drug for the treatment of cerebral ischemia.

Aims of the research.

Aim of Section I:

Role of carbonic anhydrase inhibitors in a rat model of cerebral ischemia

The evidence that hypoxic microenvironments elicit the expression of specific isoform of CA (in particular CA IX and CA XII) through the HIF allows to hypothesize a possible relevant role of CA in ischemia.

Recently it has been demonstrated that CAIs, sulfonamide and coumarin, were able to improve neurological functionalities after cerebral ischemic insult in the rats.

On these bases, the first aim of my thesis was to investigate if acetazolamide (ACTZ) and new generation sulphonamide CAIs such as AN11-740 and AN6-277 provided protection in two models of brain ischemia. In an *in vitro* model of acute rat hippocampal slices that underwent to severe, 30 min long, oxygen-glucose deprivation (OGD) episodes and in an *in vivo* model of focal cerebral ischemia induced by permanent occlusion of the middle cerebral artery (pMCAo) in the rat.

Aim of Section II:

Role of A_{2B} receptor agonist, BAY60-6583, in a rat model of transient cerebral ischemia

Adenosine A_{2B} receptors are expressed at low levels uniformly throughout the CNS and have a low affinity for adenosine. An overexpression of A_{2B} receptors occurs on endothelial cells, neurons, and astrocytes 24 hours after tMCAo in the rat. Besides brain cells, A_{2B} receptors are present on blood immune cells that take part in the inflammatory phenomena that developing hours and days after ischemia, contribute to enlarge the ischemic damage. Thus, A_{2B} receptors might play a crucial role in the inflammatory phenomena. No evidences were in literature on the effects of A_{2B} receptor ligands after ischemia, when a defined neuroinflammation develops. On these basis, second purpose of the present thesis was to check the putative neuroprotective effects of the adenosine A_{2B} receptor agonist, BAY60-6583 chronically administered for 7 days after transient (1 hour) focal cerebral ischemia induced by MCAo in the rat.

Aim of Section III:

Role of dual A_{2A}/A_{2B} receptor agonist, MRS3997, in a rat model of transient brain ischemia

Adenosine receptor ligands reported neuroprotective properties after cerebral ischemia. Previous data obtained in our laboratory demonstrated that the A_{2A} receptor agonist CGS21680, chronically

administered, is protective in a rat model of transient brain ischemia by reducing secondary damage. As well in the same ischemia model, studies reported in this thesis have demonstrated that the A_{2B} selective agonist BAY60-6583 is protective against ischemic damage. It was therefore envisaged that A_{2A} and A_{2B} receptor agonists could act in synergy exerting protective effect attenuating neuroinflammation and immune responses that develops after ischemia. Multi-target drugs in the last few years have gained the interest of scientific community representing the 21% of the new molecular entities approved by FDA from 2015 to 2017. Recently the new A_{2A}/A_{2B} receptor mixed agonist, MRS3997, was synthetized. Pharmacodynamic and pharmacokynetic characteristics render this compound favorable to investigate its putative protective effect in brain ischemia models.

The third aim of this thesis was therefore to investigate the putative protective effect of MRS3997, chronically administered for 7 days after transient (1 hour) focal cerebral ischemia induced by MCAo in the rat.

Materials and Methods.

1. Animals

Male Wistar rats (Envigo, Italy) weighting 270–290 g were used. Animals were housed in groups of three with free access to food and water and kept under standardized temperature, humidity and light conditions (12 h light/dark cycle) within the animal house facility of the University of Florence. The experimental procedures described were approved by the local Animal Welfare Body (AWB) of the University of Florence. The ethical policy of the University of Florence complies with to the Directive 2010/63/EU of the European Parliament and to the Italian Regulation DL 26/2014 on the protection of animals used for scientific purposes. According to the law, all efforts were made to fulfill to the principle of 3Rs.

2. In vitro experiments

2.1 Preparation of slices

Animals were killed with a guillotine under anesthesia with isoflurane (Baxter, Rome, Italy) and hippocampi were rapidly removed and placed in ice-cold oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 125, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, $NaHCO_3$ 25, and D-glucose 10. Slices (400 lm nominal thickness) were cut using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, United Kingdom) and kept in oxygenated aCSF for at least 1 h at room temperature. A single slice was then placed on a nylon mesh, completely submerged in a small chamber (0.8 ml) and superfused with oxygenated aCSF (31–32°C) at a constant flow rate of 1.5 ml/min. The treated solutions reached the preparation in 60 s and this delay was considered in our calculations.

2.2 Extracellular recordings

Test pulses (80 ls, 0.066 Hz) were delivered through a bipolar nichrome electrode positioned in the stratum radiatum of the CA1 region of the hippocampus to stimulate the Schaffer collateral-commissural pathway. Evoked potentials were extracellularly recorded with glass microelectrodes (2–10 MX, Crisel Instruments for Harvard Apparatus LTD, United Kingdom) filled with 150mM NaCl. The recording electrode was placed at the dendritic level of the CA1 region to record field excitatory postsynaptic potentials (fEPSPs). Responses were amplified (200X, BM 622, Mangoni, Pisa, Italy), digitized (sample rate, 33.33 kHz), and stored for later analysis with LTP (version 2.30 D) program (W. W. Anderson & Collingridge, 2007). The amplitude of fEPSP was always measured as the difference between the negative peak following the afferent fibre volley and the baseline value preceding the stimulus artefact. When a stable baseline of evoked responses was reached, fEPSP amplitudes were

routinely measured and expressed as the percentage of the mean value recorded 5 min before the application of any treatment. Stimulus-response curves were obtained by gradual increase in stimulus strength at the beginning of each experiment. The test stimulus strength was then adjusted to produce a response whose amplitude was 40% of the maximum and was kept constant throughout the experiment. Simultaneously, with fEPSP amplitude, anoxic depolarization (AD) was recorded as negative extracellular direct current (d.c.) shifts induced by OGD. The d.c. potential is an extracellular recording considered to provide an index of the polarization of cells surrounding the tip of the glass electrode (Farkas et al., 2008). AD latency, expressed in min, was calculated from the beginning of OGD; AD amplitude, expressed in mV, was calculated at the maximal negativity peak. In the text and bar graphs, AD amplitude values were expressed as positive values.

2.3 Application of OGD and drugs

OGD was obtained by superfusing the slice with aCSF without glucose and gassed with nitrogen (95% N_2–5% CO_2) (Pedata et al., 1993). This caused a drop in pO2 in the recording chamber from ~500mm Hg (normoxia) to a range of 35–75mm Hg (after 7-min OGD) (Pugliese et al., 2003). ACTZ and the CAIs, AN11-740 and AN6-277 (Figure 17,18), were applied 20 min before and during OGD application. All drugs were dissolved in dimethyl sulphoxide (DMSO). Stock solutions, of 1000–10,000 times the desired final concentration, were stored at –20°C. The final concentration of DMSO (0.05 and 0.1% in aCSF) did not affect either fEPSP amplitude or the depression of synaptic potentials induced by OGD (data not shown).

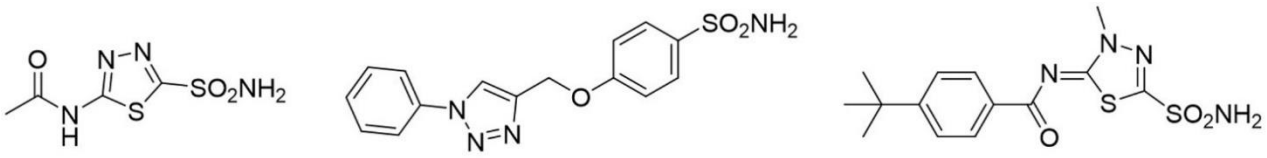

Figure 17. Chemical structure of the investigated CAIs (Nocentini et al., 2016; Rogato et al., 2019).

	Ki (nM)[a]			
Compound	**CA I**	**CA II**	**CA IX**	**CA XII**
ACTZ	250 ± 15	12 ± 0.8	25 ± 1.4	5.7 ± 0.5
AN11-740	1580 ± 110[b]	5.9 ± 0.4[b]	1.3 ± 0.1	3.0 ± 0.2
AN6-277[c]	565.6 ± 34.2	1.2 ± 0.1	2.6 ± 0.2	1.1 ± 0.1

Figure 18. Inhibition data of CA isoforms I, II, IX, and XII by inhibitors ACTZ and AN11-740 by a stopped flow CO_2 hydrase assay (Khalifah, 1971). [a] Inhibition data are expressed as means ± SEM of 3 different assays; [b] data from (Rogato et al., 2019); [c] data from (Nocentini et al., 2016). Adapted from (Dettori et al., 2021).

2.4 Statistical analysis

Statistical significance was evaluated by Student's paired or unpaired t-tests. Analysis of variance (one-way ANOVA), followed by Bonferroni multiple comparison *post-hoc* test was used, as appropriate. p Values from both Student's paired and unpaired t-tests are two-tailed. Data were analyzed using software package GraphPad Prism (version 7.0; GraphPad Software, San Diego, CA, United States). All numerical data are expressed as the mean ± standard error of the mean (SEM). A value of $p<0.05$ was considered significant.

3. In vivo experiments

3.1 Surgery

Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAo) in the right hemisphere. The animals were anesthetized with 5.0% isoflurane (Baxter International) and spontaneously inhaled 1.0 to 2.0% isoflurane in air by the use of a mask. Body core temperature was maintained at 37°C with a recirculating pad and K module and was monitored via an intrarectal type T thermocouple (Harvard, Kent, UK). The surgical procedure to occlude the MCA consisted in insertion of a 4-0 nylon monofilament (Doccol corporation, USA), via the external carotid artery into the internal carotid artery in order to block the origin of the MCA according to the procedure originally described by (Longa et al., 1989) and modified by Melani and co-workers (Melani et al., 1999) (Figure 19).

In permanent middle cerebral artery occlusion (pMCAo), the nylon filament was not removed while in the transient (1 hour) model of focal cerebral ischemia (tMCAo), animals were re-anesthetized with isoflurane and reperfused by withdrawing the filament.

Carprofen (5 mg/kg) was administered intraperitoneally (i.p.) to reduce post-operative pain. The sham operation was conducted by inserting the filament into the internal carotid artery and immediately withdrawing it.

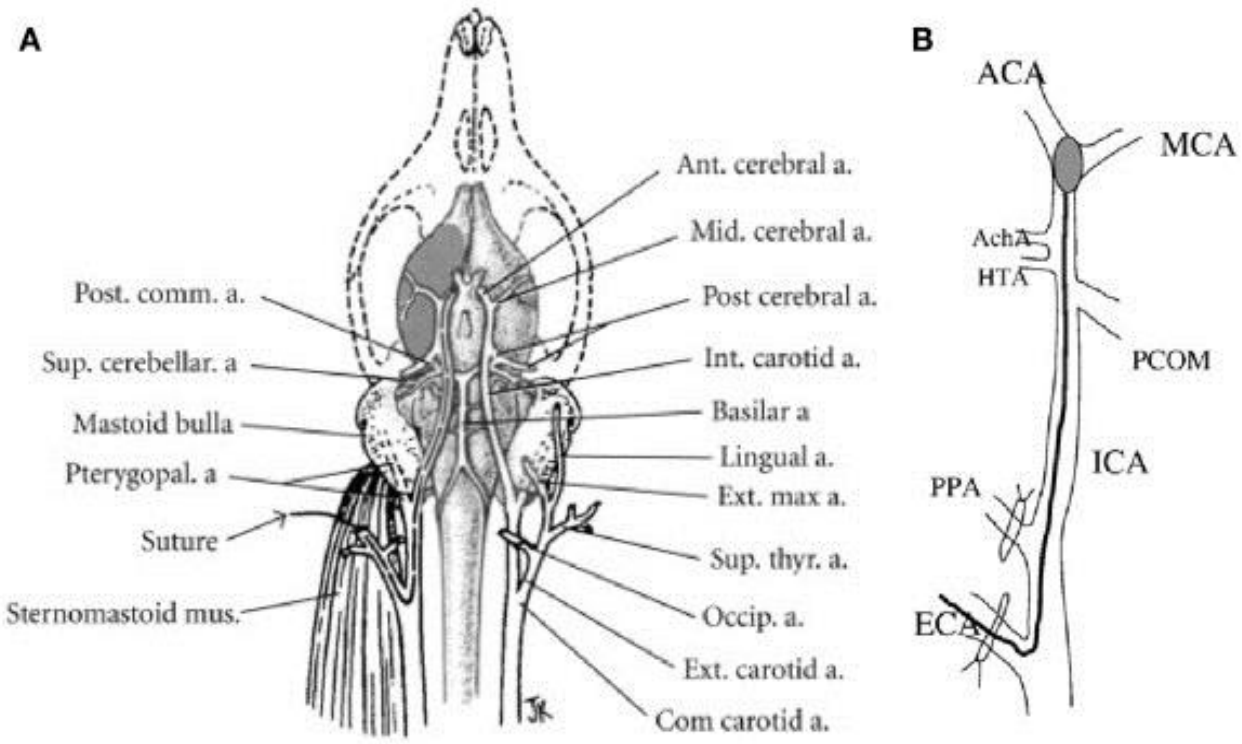

Figure 19. (A) Vascular anatomy of the rat. **(B)** Intraluminal middle cerebral artery occlusion. Abbreviations: ACA: anterior cerebral artery; ECA: external carotid artery; ICA: internal carotid artery; MCA: middle cerebral artery; PCOM: posterior communicating artery; PPA: pterygopalatine artery. Modified from (Canazza et al., 2014).

3.2 Treatments and experimental groups

3.2.1 Sub-chronic treatment with Carbonic Anhydrase Inhibitors

CAIs 5-acetamido-1,3,4-thiadiazole-2-sulfonamide (ACTZ) and the more lipid soluble compound, AN11-740 (Rogato et al., 2019) (Figure 17, 18), were dissolved in saline with 0.05% and 0.01% DMSO, respectively. ACTZ and AN11-740 were sub-chronically administered intraperitoneally (i.p.) at the dose of 4.4 mg/kg and 1.0 mg/ kg, respectively, 5 min, 6 and 20 hours after starting MCAo. Doses of CAIs were calculated on the bases of the concentration that revealed protective in OGD experiments (20 µM ACTZ; 3 µM AN11-740). We choose to test *in vivo* only AN11-740 to be sure, being highly lipophilic, that it would have pass the BBB.

Animals subjected to pMCAo were sacrificed 24 hours after ischemia. Rats were randomly allocated in the following groups: (1) sham-operated rats (n=7): did not receive any treatment; (2) MCAo+vehicle group (n=4): saline with DMSO administered i.p. 5 min, 6 and 20 hours after starting pMCAo; (3) MCAo+ACTZ group (n=4): ACTZ administered i.p. 5 min, 6 and 20 hours after starting pMCAo; (4) MCAo+AN11-740 group (n=5): AN11-740 administered i.p. 5 min, 6 and 20 hours after starting pMCAo.

3.2.2 Chronic treatment with adenosine A_{2B} receptor agonist, BAY60-6583

Adenosine A_{2B} receptor agonist 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide (BAY60-6583) (Tocris, Bristol, United Kingdom, UK) (Figure 20), was dissolved in DMSO 0.5%. BAY60-6583 was chronically administered at the dose of 0.1 mg/kg, i.p. The dose of BAY60-6583 (0.1 mg/kg) was chosen by comparison with the protective dose of CGS21680 (Melani et al., 2014), taking into account the affinity of BAY60-6583 and CGS21680 for the rat A_{2B} and A_{2A} receptors (Alnouri et al., 2015). A further criterion was to refer to the dose of BAY60-6583 (0.1 mg/kg) which in previous studies had shown protection from peripheral arterial lesions (Bot et al., 2012) and from lung injury induced by mechanical ventilation in mice (Eckle et al., 2008).

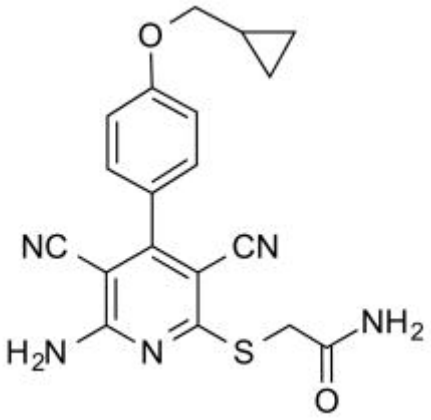

Figure 20. Chemical structure of the A_{2B} receptor agonist BAY60-6583.

Animals subjected to tMCAo were sacrificed 7 days after ischemia. Rats were randomly allocated in the following groups: (1) sham-operated rats (n=7): did not receive any treatment; (2) tMCAo+vehicle group (n=6): DMSO (0.1%) administered (i.p.) twice/day for 7 days, starting 4 hours after tMCAo; (3) tMCAo+ BAY60-6583 group (n=6): BAY60-6583 administered (i.p.) twice/day for 7 days, starting 4 hours after tMCAo.

A second group of animals was operated as described above and sacrificed 2 days after tMCAo. Rats were randomly allocated to the following groups treated as described above: (1) sham rats (n=3); (2) tMCAo+vehicle-treated rats (n=3); (3) tMCAo+BAY60-6583-treated rats (n=4).

3.2.3 Chronic treatment with dual adenosine A_{2A}/A_{2B} receptor agonist, MRS3997

Adenosine A_{2A}/A_{2B} receptor agonist 2-(2-(6-bromo-indol-3-yl)ethyloxy)adenosine (MRS3997) (Figure 21), was dissolved in DMSO 0.5%. MRS3997 was chronically administered at the dose of 0.1 mg/kg, i.p.

The dose of MRS3997 (0.1 mg/kg) was chosen based on the protective effects resulting from our previous studies (Dettori et al., 2020; Melani et al., 2014), considering the molecular weight and the affinity of MRS3997 for A_{2A} and A_{2B} receptors.

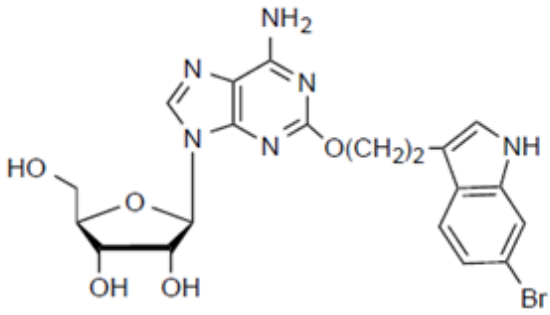

Figure 21. Chemical structure of the mixed A_{2A}/A_{2B} receptor agonist MRS3997.

Animals subjected to tMCAo were sacrificed 7 days after ischemia. Rats were randomly allocated in the following groups: (1) sham-operated rats (n=7): did not receive any treatment; (2) tMCAo+ vehicle group (n=3): DMSO (0.1%) administered (i.p.) twice/day for 7 days, starting 4 hours after tMCAo; (3) tMCAo+ MRS3997 group (n=3): MRS3997 administered (i.p.) twice/day for 7 days, starting 4 hours after tMCAo.

3.3 Neurological deficit

The neurological deficit was evaluated by modified Neurological Severity Score (mNSS) test described by Chen et al., (2001). All tests were carried out before pMCAo or tMCAo and 1, 5 and 7 days after MCAo. The mNSS test evaluates the sensorimotor deficit: it is composed of motor, sensory, reflex and beam balance tests (Figure 22). The score assigned to each rat at completion of the evaluation equals the sum of all test scores. The test is graded on a scale from 0 (normal score) to 18 (maximal deficit score). In the beam balance test, a score between 0 (normal score) and 6 (maximal deficit score) was assigned to each animal in function of the ability to stay and walk on the beam. Beam balance test score affects 1/3 of the total mNSS score.

Modified Neurological Severity Score Points	
Motor tests	
Raising rat by tail	**3**
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30 s	1
Placing rat on floor (normal = 0; maximum = 3)	**3**
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Falls down to paretic side	3
Sensory tests	**2**
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing pew against table edge to stimulate limb muscles)	1
Beam balance test (normal = 0; maximum = 6)	**6**
Balances with stady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs falls down from beam, or spins on beam (>60 s)	3
Attempts to balance on beam but falls off (>40 s)	4
Attempts to balance on beam but falls off (>20 s)	5
Falls off; no attempt to balance or hang on to beam (<20 s)	6
Reflex absence and abnormal movements	**4**
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when comes is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystony	1
Maximum points	**18**

One point is awarded for inability to perform the tasks or for lack of a tested reflex: 13-18, severe injury; 7-12, moderate injury; 1-6, mild injury.

Figure 22. Example of modified Neurological Severity Severity (mNSS) score points scale for sensorimotor deficit evaluation.

3.4 Body weight evaluation

Rats used to induce cerebral ischemia were in the body weight ranging from 270 to 290 g. The body weight was evaluated before tMCAo and after 1, 5, 7 days from occlusion. The weight variation after ischemia of each animal was evaluated with respect to its own pre-ischemia weight.

3.5 Ischemic brain damage

Rats were anesthetized with Zoletil 50/50 (100 mg/kg i.p., Virbac, Carros, France) and were perfused transcardially with an ice-cold 4% paraformaldehyde solution (in phosphate buffer, pH 7.4). Brains were post-fixed overnight and cryoprotected in a 18% sucrose solution (in phosphate buffer) for at least 48 hours. Brains were cut with a cryostat and coronal sections (30 µm) were collected at 210 µm intervals at 12 different levels through the striatum (König et al., 1964). One and seven days after pMCAo and tMCAo respectively, brain slices were stained by acetate cresyl violet (1%) or by hematoxylin and eosin (H&E). Histological analysis by cresyl violet staining allows to clearly define the infarct area and volume up to 1 week after ischemia (Rousselet et al., 2012). To evaluate area and volume of ischemic damage, 12 cresyl violet-stained brain sections per animal were placed directly on the scanning screen of a color flatbed scanner (CanoScan LiDE 90; Canon). Following image acquisition, the images were analyzed using ImageJ software. The measurements of infarct area in striatum and cortex were obtained by manually outlining the margins of infarcted area. Ischemic cortical and striatal volumes were calculated by multiplying the infarcted area by the slice thickness and summing the volume of the 12 slices.

After H&E staining, heterochromatic nuclei were counted at Bregma level within an optical field at 40X in ischemic cortex and striatum. Data were then averaged and expressed as mean±SEM of number cells per optical field of “n” animals.

3.6 Neuronal damage, gliosis and blood cell infiltration

Immunohistochemistry was performed with the free-floating method (Giovannini et al., 2002; Lana et al., 2014) on rat brain coronal sections in striatum and cortex.

The following primary antibodies were used: for neurons a mouse antineuronal nuclei (NeuN) antibody, dilution 1:400 (Product Code #MAB377, Millipore, Billerica, MA, United States); for astrocytes a mouse monoclonal antibody, anti-GFAP, diluition 1:300 (Product code: #610565; BD Transduction Laboratories), or a mouse anti-GFAP antibody conjugated with the fluorochrome AlexaFluor 488, dilution 1:500 (Product Code #MAB3402X, Millipore, Billerica, MA, United States); for microglia a rabbit anti-ionized calcium binding adaptor molecule 1 (IBA1) antibody, dilution 1:400

(Product Code #016-20001, WAKO, Osaka, Japan); for granulocytes a mouse monoclonal antibody anti-HIS-48, dilution 1:50 (Santa Cruz Biotechnology). The following fluorescent secondary antibodies were used: AlexaFluor 555 donkey anti-mouse, dilution 1:400 (Product Code #A31570, Thermo Fisher Scientific); AlexaFluor 635 goat anti-rabbit, dilution 1:400 (Product Code #A31577, Thermo Fisher Scientific). Nuclei were stained using DAPI, contained in the mounting medium for glass slides, Vectashield (Product Code #H1200, Vector Laboratories, Burlingame, CA, United States). The sections were mounted onto gelatin-coated slides using Vectashield with DAPI (Vector Laboratories).

3.6.1 Microscopy techniques, qualitative and quantitative analysis

Microscopy images were obtained with epifluorescent microscope or confocal microscope.

Slices observed with an epifluorescent Olympus BX63 microscope (Olympus, Hamburg, Germany) were photographed using a digital camera (Olympus DP50). Images were assembled into montages using Adobe Photoshop Cs 6.1 (Adobe Systems, Mountain view, CA, USA). To quantify neurons, astrocytes and microglia, cells were counted within an optical field at 20X in ischemic and perischemic areas of cortex and striatum at Bregma level. All quantitative analyses were performed blind to the treatment by two independent experimenters and data were averaged and expressed as mean± SEM of number cells per optical field of “n” animals.

The confocal microscopy images were obtained with a LEICA TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany). The parameters of acquisition were maintained constant: frame dimension 1024 x 1024 points, frequency of acquisition 200 Hz.

Two experimenters performed all quantitative analyses blind, and data were averaged. All evaluations of cell density were made on z projections of 10 consecutive confocal scans (total thickness 12 µm). Cells were counted, and the area of analysis was measured. Density of cells (neurons, astrocytes, microglial cells) was calculated as cells/mm^2 in striatum and cortex, on confocal z projections of ten scans (total 12 µm inside the section).

3.7 Determination of TNF-α and IL-10 plasma levels

The levels of TNF-α, a pro-inflammatory cytokine, and IL-10, a regulatory cytokine, were measured on aliquots (100µl) of plasma using commercial ELISA kits (Rat TNF-α Platinum ELISA, Catalogue no: BMS622, Affymetrix eBioscience, Vienna, Austria; Rat IL-10 Platinum ELISA, Catalogue no: BMS629, Affymetrix eBioscience, Vienna, Austria), following the protocol provided by the manufacturer. Results are expressed as pg of protein/ml of plasma.

3.8 Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test, Repeated Measures two-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test and by unpaired Student's t-test as specified in text and in figure legends. A value of $p<0.05$ was considered statistically significant. The statistical analysis was performed utilizing GraphPadPrism.

Results.

Section I: Carbonic anhydrase inhibitors (CAIs) for the management of cerebral ischemia

1.1 The application of selective CAIs delayed the appearance of AD induced by OGD

The experiments were performed on 49 slices taken from 20 rats to test the role of CAIs during severe, 30 min long OGD, a time duration that is invariably harmful for the tissue (Pearson et al., 2006; Pugliese et al., 2006). The effects of two new selective CAIs, AN6-277 and AN11-740, on the time of the AD appearance and amplitude, were evaluated (Figure 23) and compared to those obtained in the presence of the prototypical CAI, ACTZ. In accord with previous data (Fusco et al., 2018; Pugliese et al., 2006) no recovery of fEPSP was recorded after interruption of 30 min OGD in all experimental conditions (not shown). As illustrated in Figure 23 A, 30-min OGD elicited the appearance of AD in untreated OGD slices, which presented a mean latency of 6.2 ± 0.3 min (calculated from the beginning of OGD; n=20, Figure 23 A,E) and a mean peak amplitude of –6.3 ± 0.5mV (n=20, Figure 23 A,F). When OGD was applied in the presence of CAIs, the d.c. shifts were always delayed (Figure 23 B–E). Indeed, the latency of AD was postponed to 8.65 ± 0.5 min in the presence of 20 mM ACTZ, (n=5, $p<0.05$, Figure 23 B,E), to 8.8 ± 0.6 min in the presence of 3 mM AN11-740 (n=8, $p<0.01$, Figure 23 C,E) and to 9.04 ± 0.7 min in the presence of 5 mM AN6-277 (n=7, $p<0.001$, Figure 23 D,E). AN6-277 affected AD latency also when tested at lower concentrations (Figure 23 E). All compounds tested did not significantly change AD amplitude in comparison to that found in OGD untreated slices (Figure 23 F).

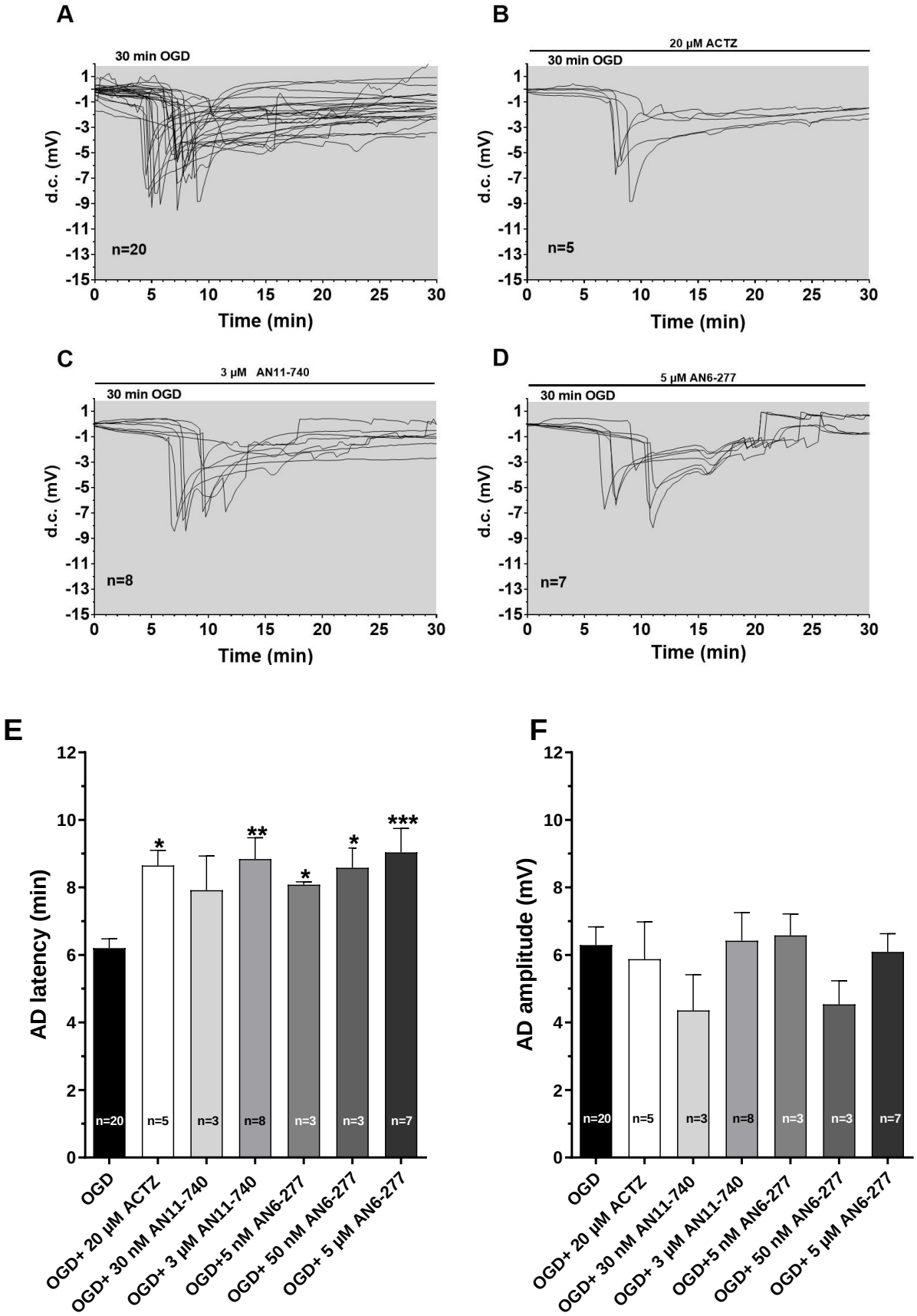

Figure 23. Effects of different CA inhibitors on AD development during 30 min OGD in the CA1 region. **(A–D)** The graphs show the d.c. shift traces during 30 min OGD in untreated OGD slices (A, n=20), in the presence of 20 mM ACTZ

(B, n=5), 3 mM AN11-740 (C, n=8) and 5 mM AN6-277 (D, n=7). Each inhibitor was applied at least 20 min before OGD and maintained for all the insult. **(E)** Each column represents the mean ± SEM of AD latency recorded in hippocampal slices during 30 min OGD in different experimental groups. AD was measured from the beginning of OGD insult. *p<0.05, **p<0.01, ***p<0.001 vs. OGD, One-way ANOVA followed by Bonferroni post hoc test. **(F)** Each column represents the mean ± SEM of AD amplitude recorded in the CA1 region during 30 min OGD. The number of slices is reported in the columns.

1.2 Effect of treatment with CAIs on neurological deficit after pMCAo

The mNSS test was performed according to Chen and cooworkers before and 24 hours after pMCAo. Figure 24 shows that sham-operated rats had a neurological score of 0.6±0.3, 24 hours after pMCAo. Twenty-four hours after pMCAo, vehicle-treated rats showed a clear neurological deficit with a neurological score of 14.0±0.7 (mean±SEM) that defines a severe injury. Sub-chronic treatment with both carbonic anhydrase inhibitors, ACTZ (4.4 mg/kg i.p.) and AN11-740 (1.0 mg/kg i.p.), significantly reduced the neurological score by 28.6 and by 42.9%, respectively, 24 h after pMCAo (One-way ANOVA: F3,16=67.24, p<0.0001; Newman-Keuls *post-hoc* test: **at least p<0.01). Sham-operated rats had a neurological score significantly different from vehicle-, ACTZ- and AN11-740-treated rats (One-way ANOVA: F3,16=67.24, p<0.0001; Newman-Keuls *post-hoc* test: **at least p<0.01; ***p<0.001). Sub-chronic treatment with both CAIs, ACTZ (4.4 mg/kg i.p.) and AN11-740 (1.0 mg/kg i.p.) significantly reduced by 28.6% and by 41.4% respectively the neurological deficit 24 hours after pMCAo (One-way ANOVA: p<0.001-0.0001).

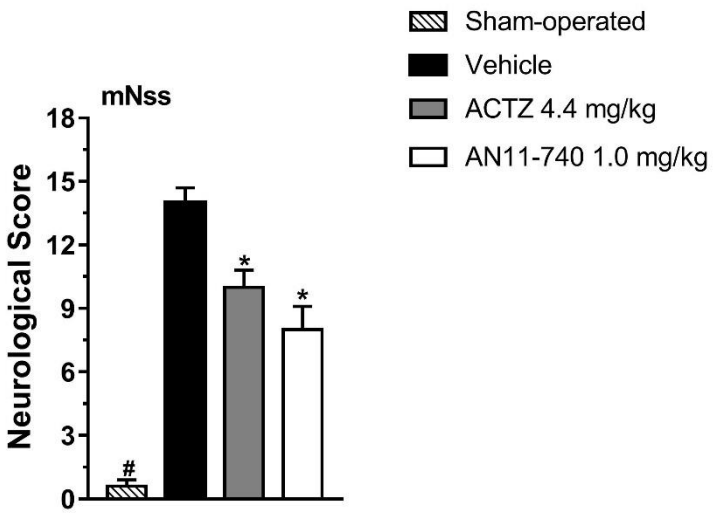

Figure 24. Effect of sub-chronic treatment with ACTZ and AN11-740 on neurological deficit, 24 h after pMCAo. mNSS test: the score is evaluated 24 h after pMCAo in sham-operated (n = 7), vehicle-treated (n = 4), ACTZ-treated (n = 4) and AN11-740-treated (n = 5) rats. Data are expressed as mean ± SEM, one-way ANOVA followed by Newman-Keuls *post-hoc* test: $^{\#}p < 0.001$ sham-operated rats vs. vehicle- ACTZ- AN11-740-treated rats; *at least p < 0.01 ACTZ- and AN11-740-treated rats vs. vehicle-treated rats.

1.3 Effect of treatment with CAIs on brain ischemic damage after pMCAo

Figure 25 shows the extent of ischemic damage evaluated as infarct area (Figure 25 A,C) and infarct volume (Figure 25 B,D) in ischemic striatum and cortex of vehicle-, ACTZ and AN11-740-treated rats, 24 h after pMCAo. Sub-chronic treatment with both ACTZ and AN11-740 significantly reduced the infarct volume in ischemic cortex by 55.2 and 66.2%, respectively (One-way ANOVA: $F_{2,10}=5.9$, $p<0.02$; Newman-Keuls *post-hoc* test: *$p<0.05$) and in ischemic striatum by 53.4 and 45.6%, respectively (One-way ANOVA: $F_{2,9}=9.5$, $p<0.006$; Newman-Keuls *post-hoc* test: *at least $p<0.05$). Sham-operated rats did not show any damage. To characterise the cytoarchitecture of the ischemic cortex and ischemic striatum 24 h after pMCAo, ischemic tissue was stained by H&E (Figure 26 A–H). Twenty-four hours after ischemia, the typical cytoarchitecture of these two regions (for a description see Danner & Pfister (1981) was lost. In sham-operated rats, the typical caudate-putamen cytoarchitecture of the dorsal striatum was appreciable (Figure 26 A), with numerous transversally sectioned white matter *fascicula* (f) surrounded by grey matter containing diverse types of neurons, distinct on the bases of their size and shape. In the fronto-parietal cortex (Figure 26 B), the typical columnar organisation was recognisable (Danner & Pfister, 1981). In vehicle-treated rats, the striatal tissue was clearly damaged, the cytoarchitecture was lost, the *fascicula* were much less recognisable, the distinction between white and grey matter no more appreciable, the interstitial spaces increased, and numerous heterochromatic small nuclei were present (Figure 26 C). In the fronto-parietal cortex, the columnar organisation was hardly visible, the interstitial spaces were enlarged and dilated, and numerous heterochromatic small nuclei were present (Figure 26 D). Qualitative analysis (Figure 26 E–H) shows that administration of both ACTZ and AN11-740 was associated with a recovery of tissue cytoarchitecture, and with a reduction of heterochromatic small nuclei in both brain regions. The white matter *fascicula* were recognisable in the dorsal corpus striatum (Figure 26 E,G), and the columnar organisation was appreciable in the fronto-parietal cortex (Figures 26 F,H). Quantitative analysis showed that treatment with both ACTZ and AN11-740 significantly reduced the number of heterochromatic nuclei in the ischemic striatum (One-way ANOVA: $F_{2,6}=10.1$, $p<0.001$; Newman-Keuls *post-hoc* test: *at least $p<0.05$, Figure 26 I) and in the ischemic cortex (One-way ANOVA: $F_{2,6}=115.6$, $p<0.0001$; Newman-Keuls *post-hoc* test: ***at least $p<0.001$, Figure 26 J).

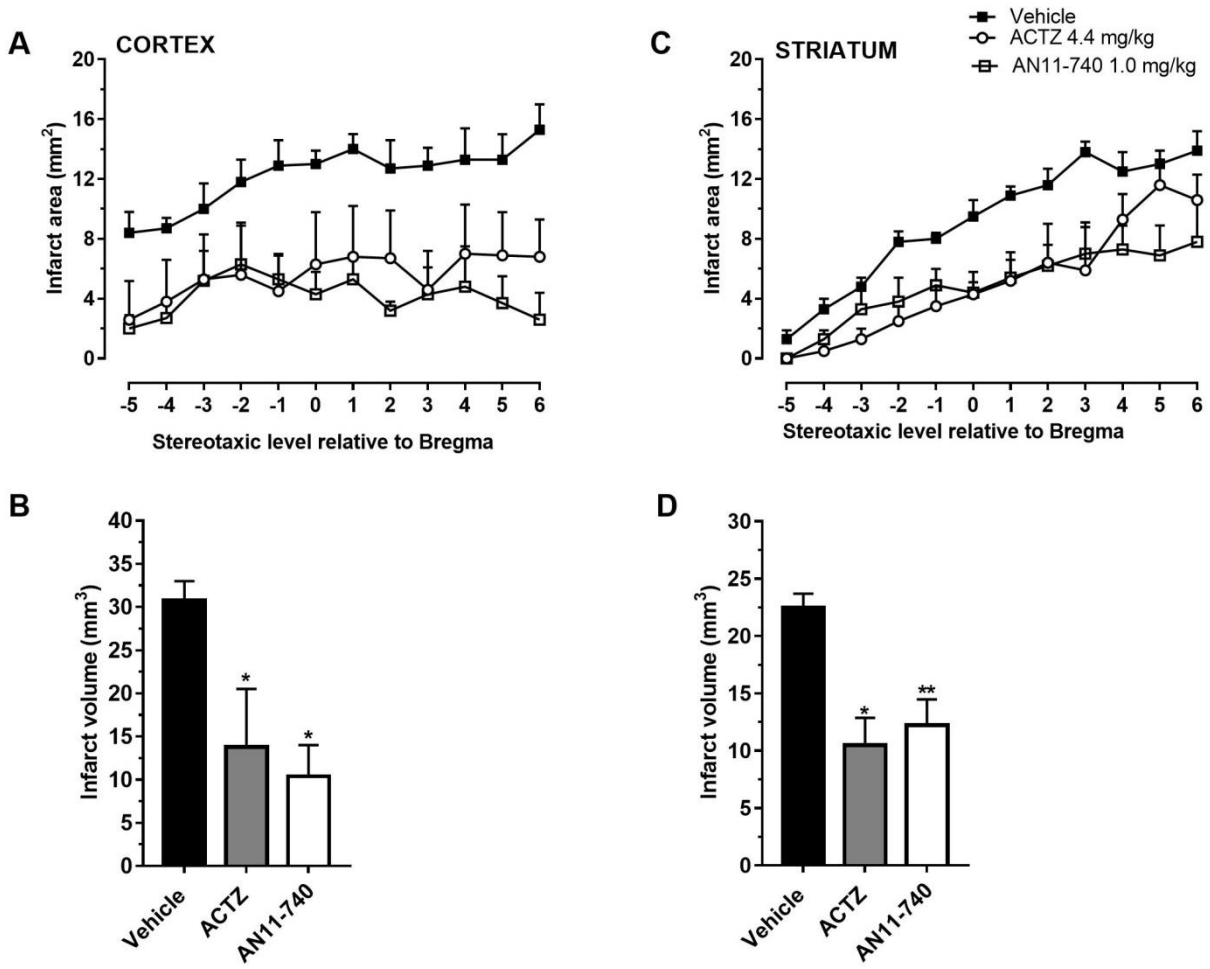

Figure 25. Effect of sub-chronic treatment with ACTZ and AN11-740 on infarct area **(A, C)** and infarct volume **(B, D)** in the cortex and striatum 24 hours after pMCAo. Data are mean±SEM of infarct area measured at 12 predetermined coronal levels through the brain of vehicle-treated (n = 4), ACTZ-treated (n = 4) and AN11-740-treated (n = 5) rats. Bregma = 0 (Rousselet et al., 2012). **(B,D)** Bar graphs indicate the infarct volume calculated as mean ± SEM in the striatum and cortex. One-way ANOVA followed by Newman–Keuls *post-hoc* test: *p < 0.05 and **p < 0.01 vs. vehicle-treated rats.

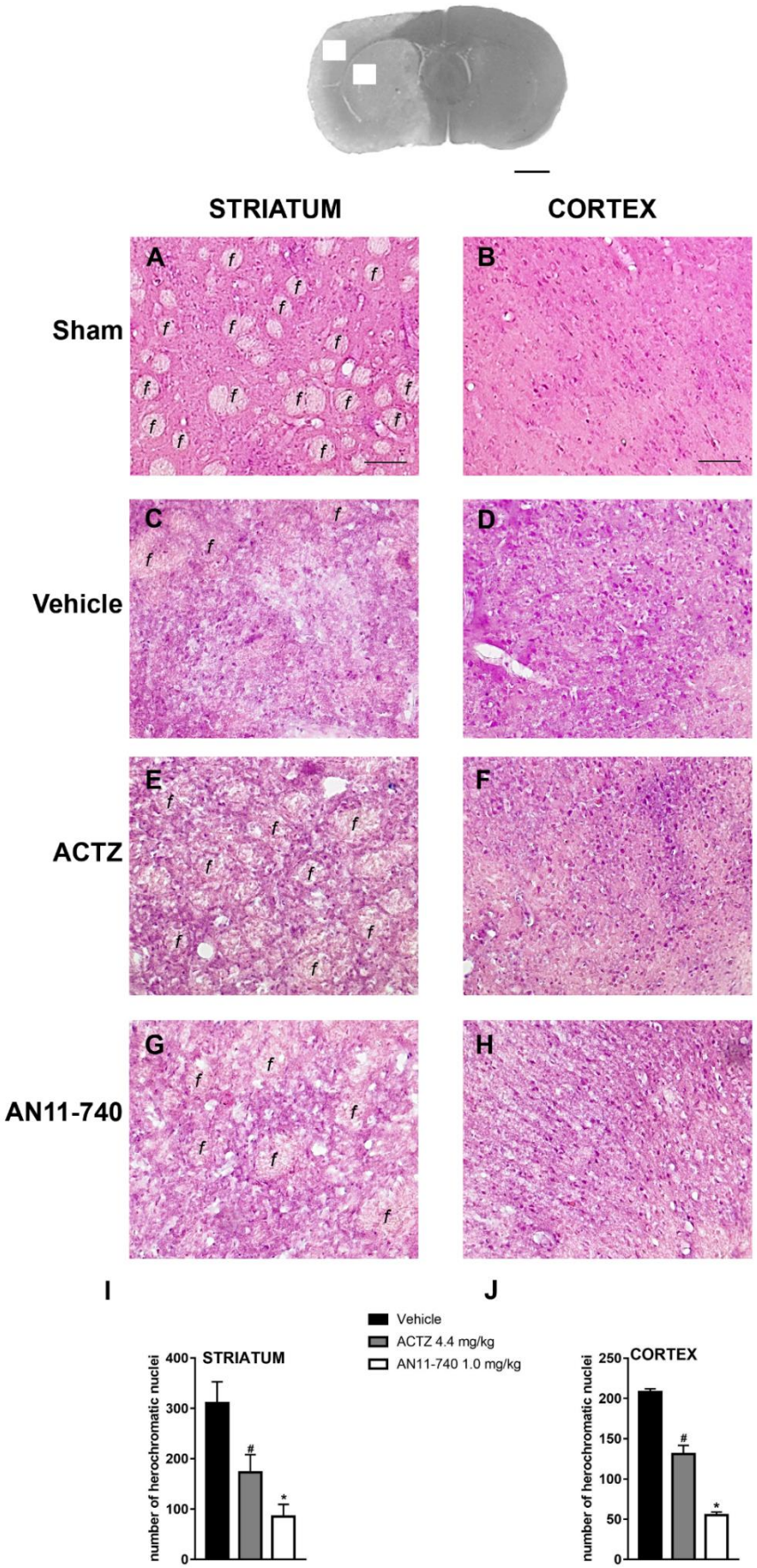

Figure 26. Effect of sub-chronic treatment with ACTZ and AN11-740 on the cytoarchitecture of the striatum and cortex 24 h after pMCAo. Upper part: Representative photomicrograph of a coronal section (at Bregma = 0, (König et al.,

1964)) showing the ischemic area in a vehicle-treated rat. The two white squares indicate regions within the ischemic area where photomicrographs were captured. Scale bar: 2 mm. **(A–H)** Representative microphotographs of H&E staining from dorsal striatum and fronto-parietal cortex of a sham-operated **(A,B)**, a vehicle- **(C,D)**, an ACTZ- **(E,F)** and an AN11-740-treated rat **(G, H)**. The white matter *fascicula* (f) are evidenced. Scale bar: 100 mm. **(I,J)** Quantitative analyses of heterochromatic nuclei per striatal **(I)** and cortical areas **(F)** at coronal level AP = 0 from Bregma. Data represent the mean ± SEM of 3 rats/group. One-way ANOVA followed by Newman-Keuls *post-hoc* test: $^{\#}$at least $p < 0.05$ ACTZ- vs. vehicle-treated rats; *at least $p < 0.001$ AN11-740- vs. vehicle-treated rats.

1.4 Effect of treatment with CAIs on neuronal damage after pMCAo

Twenty-four hours after pMCAo, the extent of neuronal damage in ischemic striatum and cortex was assessed by immunohistochemistry using anti-NeuN antibody. Representative images of NeuN immunostaining of each group were shown in figure 27 A-H. In the ischemic cortex and striatum of vehicle-treated rats, we observed the presence of many damaged neurons characterised by shrunken cell bodies (Garcia et al., 1995) (Figure 27 C,D,K,L) or by damaged nuclei that had lost NeuN immufluorescence, while the immunofluorescence persisted in the cytoplasm (shown by the arrows in Figures 27 C,K). Sub-chronic treatment with CAIs antagonised neuronal damage in both ischemic area (Figure 27 E,G; M,O) and perischemic area (Figure 27 F,H; N,P). Figures 27 Q,R show the quantitative analysis of $NeuN^+$ cells in the ischemic and in the perischemic areas of both cortex and striatum of sham-operated, vehicle- ACTZ- and AN11-70-treated rats. Statistical analysis performed by One-way ANOVA evidenced that the number of $NeuN^+$ cells significantly decreased both in the cortical ischemic and perischemic areas (One-way ANOVA: $F_{3,11}=28.9$, $p<0.0001$; Newman-Keuls *post-hoc* test: ***$p<0.001$; One-way ANOVA: $F_{3,12}=9.3$, $p<0.001$; Newman-Keuls *post-hoc* test: **$p<0.01$; Figures 27 Q,R) and in the striatal ischemic and perischemic areas (One-way ANOVA: $F_{3,11}=15.4$, $p<0.0003$; Newman–Keuls *post-hoc* test: ***$p<0.001$; One-way ANOVA: $F_{3,12}=22.96$, $p<0.0001$; Newman–Keuls *post-hoc* test: ***$p<0.001$; Figures 27 Q,R) of vehicle-treated rats in comparison to sham-operated rats. Sub-chronic treatment with both ACTZ and AN11-740 significantly counteracted the decrease in neuron number in ischemic areas (One-way ANOVA followed by Newman-Keuls *post-hoc* test: *$p<0.05$; Figures 27 Q,R) and in perischemic areas (One-way ANOVA followed by Newman-Keuls *post-hoc* test: *at least $p<0.05$; Figures 27 Q,R) of both cortex and striatum.

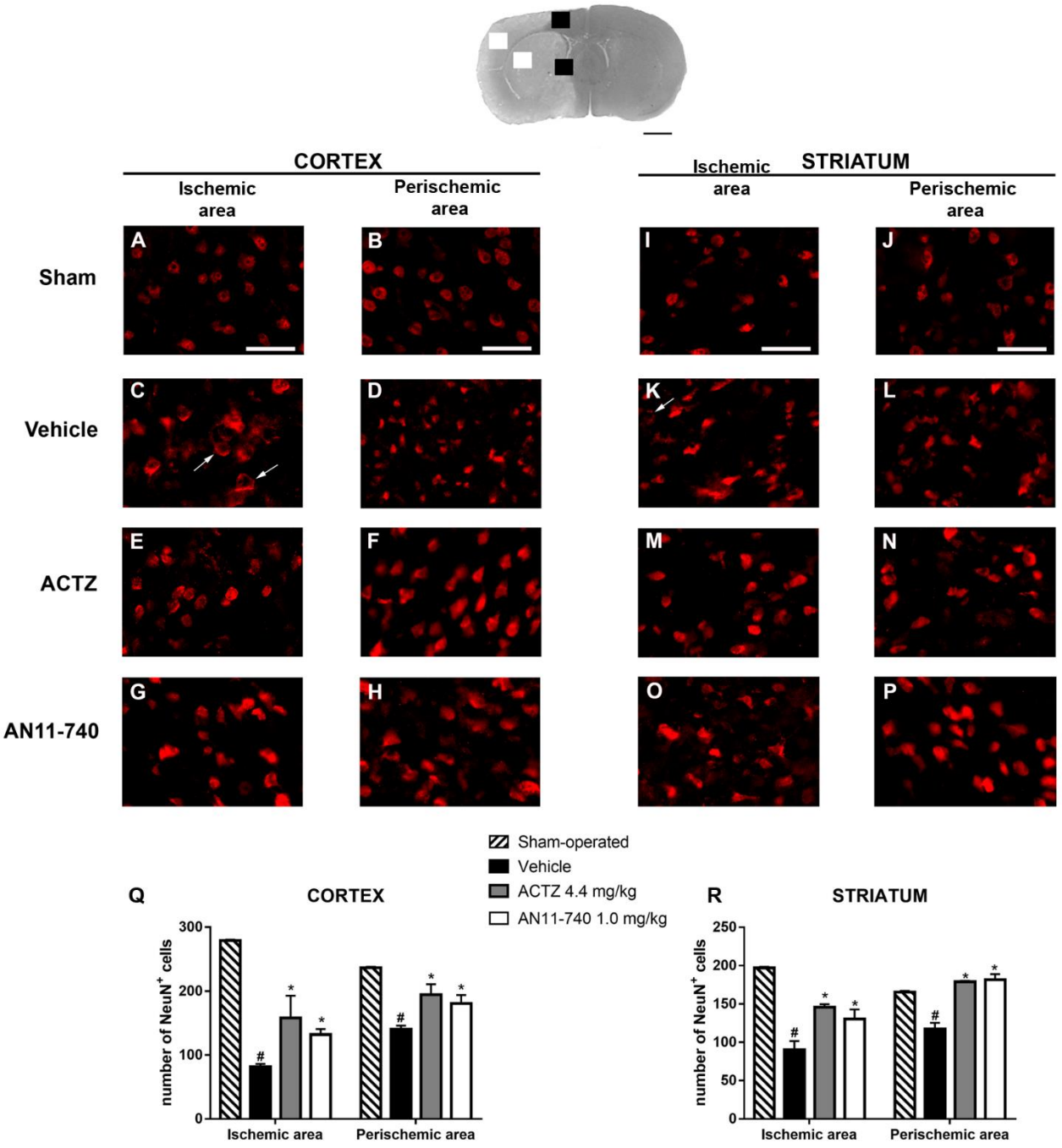

Figure 27. Effect of sub-chronic treatment with ACTZ and AN11-740 on neuronal damage in cortex and striatum 24 h after pMCAo. Upper part: Representative photomicrograph of a coronal section (at Bregma = 0, (König et al., 1964)) showing the ischemic area in a vehicle-treated rat. The two white and black squares indicate regions within the ischemic area and within the perischemic area, respectively, where photomicrographs were captured. Scale bar: 2 mm. **(A–P)** Representative microphotographs of neurons (red) in cortical ischemic and perischemic areas and in striatal ischemic and perischemic areas of sham-operated (**A, B; I, J**; n = 3), vehicle- (**C, D; K, L**; n = 4) ACTZ- (**E, F; M, N;** n = 4) and AN11-740- (**G, H; O, P;** n = 5) treated rats. Scale bar: 50 mm. **(Q, R)** Quantitative analysis of $NeuN^+$ cells in cortical and striatal ischemic and perischemic areas, bar graphs represent mean ± SEM of the number of neurons per optical field (20x). One-way ANOVA followed by Newman–Keuls *post-hoc* test: $^{\#}$at least $p<0.01$ vehicle-treated vs. sham-operated rats; *at least $p<0.05$ ACTZ- and AN11-740- vs. vehicle-treated rats.

1.5 Effect of treatment with CAIs on microglia activation after pMCAo

Twenty-four hours after ischemia, microglia cells were characterised using immunofluorescent staining with anti-IBA1 antibody, as shown by the representative images of Figure 28 A–P. In sham-operated rats, resting microglia was diffusely distributed throughout the cortex and the striatum and was characterised by small cell body and thin and ramified branches (Figure 28 A,B; I,J). Twenty-four hours after pMCAo, we found a strong pattern of activation of microglia cells, both in the ischemic cortex and ischemic striatum of vehicle-treated rats. In the cortical and striatal ischemic areas, microglia cells appeared round-shaped and amoeboid, morphology typical of activated cells (shown by the arrows in Figure 28 C,K). In the cortical and striatal perischemic areas, microglia showed a hyperthrophic cell body with thick and short processes, a morphology defined reactive microglia (Cerbai et al., 2012; Melani et al., 2006) (Figure 28 D,L). Sub-chronic treatment with both ACTZ and AN11-740 appeared to revert the morphological alterations caused by ischemia in both ischemic (Figure 28 E,G; M,O) and perischemic

areas (Figure 28 F,H; N,P). Quantitative analysis perfomed by One-way ANOVA showed that $IBA1^+$ cells increased in vehicle-treated rats both in cortical ischemic and perischemic areas (One-way ANOVA: $F_{3,8}=7.9$, $p<0.008$; Newman–Keuls *post-hoc* test: $^{**}p<0.01$; One-way ANOVA: $F_{3,8}=7.4$, $p<0.01$; Newman-Keuls *post-hoc* test: $^{*}p<0.05$; Figure 28 Q) and both in striatal ischemic and perischemic areas (One-way ANOVA: $F_{3,9}=8.0$, $p<0.006$; Newman-Keuls *post-hoc* test: $^{**}p<0.01$; One-way ANOVA: $F_{3,9}=5.4$, $p<0.02$; Newman–Keuls *post-hoc* test: $^{*}p<0.05$; Figure 28 R) in comparison to sham-operated rats. Sub-chronic treatment with both CAIs significantly reduced the number of $IBA1^+$ cells in ischemic (One-way ANOVA followed by Newman–Keuls *post-hoc* test: *at least $p<0.05$; Figure 28 Q,R) and perischemic areas (One-way ANOVA followed by Newman–Keuls *post-hoc* test: *at least $p<0.05$; Figure 28 Q,R) of both ischemic cortex and striatum.

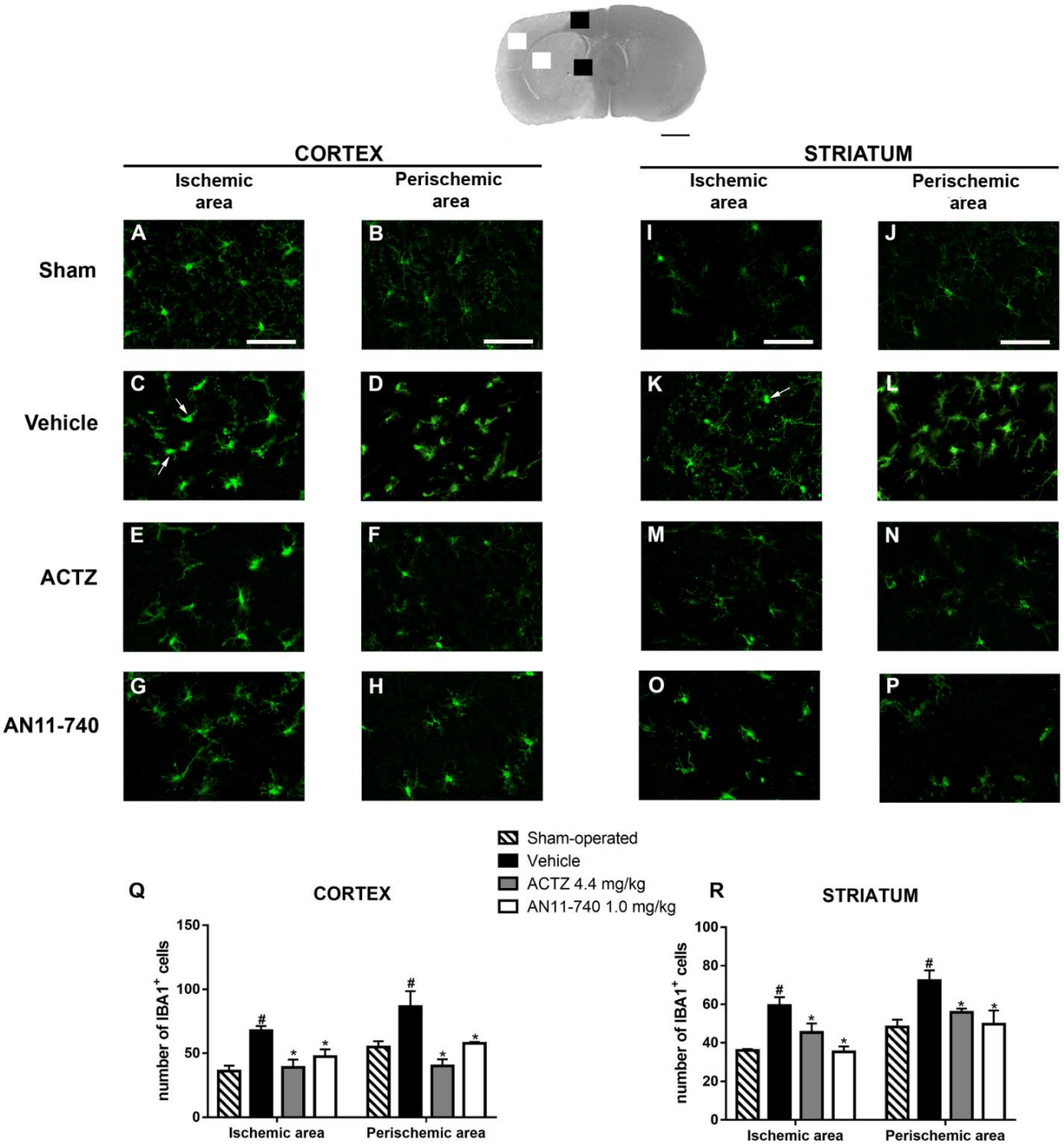

Figure 28. Effect of sub-chronic treatment with ACTZ and AN11-740 on microglia activation in cortex and striatum 24 h after pMCAo. Upper part: Representative photomicrograph of a coronal section (at Bregma = 0, (König et al., 1964)) showing the ischemic area in a vehicle-treated rat. The two white and black squares indicate regions within the ischemic area and within the perischemic area, respectively, where photomicrographs were captured. Scale bar: 2 mm. **(A–P)** Representative microphotographs of microglia (green) in cortical ischemic and perischemic areas and in striatal ischemic and perischemic areas of sham-operated (**A, B; I, J;** n = 3), vehicle- (**C, D; K, L;** n = 3) ACTZ- (**E, F; M, N;** n = 4) and AN11-740- (**G, H; O, P;** n = 3) treated rats. Scale bar: 50 mm. **(Q, R)** Quantitative analysis of IBA1$^+$ cells in cortical and striatal ischemic and perischemic areas, bar graphs represent mean ± SEM of the number of microglia cells per optical field (20x). One-way ANOVA followed by Newman–Keuls *post-hoc* test: $^{\#}$at least $p < 0.05$ vehicle-treated vs. sham-operated rats; *at least $p < 0.05$ ACTZ- and AN11-740- vs. vehicle-treated rats.

1.6 Effect of treatment with CAIs on astrocytes alteration after pMCAo

Immunofluorescence analysis using anti-GFAP antibody specific for astrocytes, are shown by the representative images of Figure 29 A–P. In sham-operated rats, resting astrocytes were diffusely distributed throughout the cortex and the striatum, and presented small cell body and faintly stained thin processes (Figure 29 A,B; I, J). Twenty-four hours after ischemia, in cortical and striatal ischemic areas of vehicle-treated rats, $GFAP^+$ cells showed small cell bodies and fragmented branches (Figure 29 C,K), morphological features typical of suffering cells. In cortical and striatal perischemic areas of vehicle-treated rats, astrocytes have hyperthrophic cell bodies and long and thick processes (Figure 29 D,L), characteristic of reactive cells (Qin et al., 2010). Sub-chronic treatment with ACTZ and AN11-740 amiliorates astrocytes morphology both in ischemic cortex and ischemic striatum (Figure 29 E–H; M–-P): astrocytes appeared less hyperthrophic and less activated. Quantitative analysis evidenced that the number of $GFAP^+$ cells decreased in vehicle-treated rats both in cortical ischemic and perischemic areas (One-way ANOVA: $F_{3,8}=8.7$, $p<0.006$; Newman–Keuls *post-hoc* test: *$p<0.05$; One-way ANOVA: $F_{3,8}=16.4$, $p<0.0009$; Newman-Keuls *post-hoc* test: ***$p<0.001$; Figure 29 Q) and both in striatal ischemic and perischemic areas (One-way ANOVA: $F_{3,8}=6.6$, $p<0.01$; Newman–Keuls *post-hoc* test: *$p<0.05$; One-way ANOVA: $F_{3,9}=28.3$, $p<0.0001$; Newman–Keuls *post-hoc* test: ***$p<0.001$; Figure 29 R) in comparison to sham-operated rats. Sub-chronic treatment with CAIs did not modify the number of $GFAP^+$ cells neither in the cortical nor in the striatal ischemic areas (Figure 29 Q,R), but partially conteracted the decrease in number of astrocytes in the cortical and striatal perischemic areas (One-way ANOVA followed by Newman–Keuls *post-hoc* test: **$p<0.01$; Figure 29 Q,R; One-way ANOVA followed by Newman–Keuls *post-hoc* test: ***at least $p<0.001$; Figure 29 Q,R).

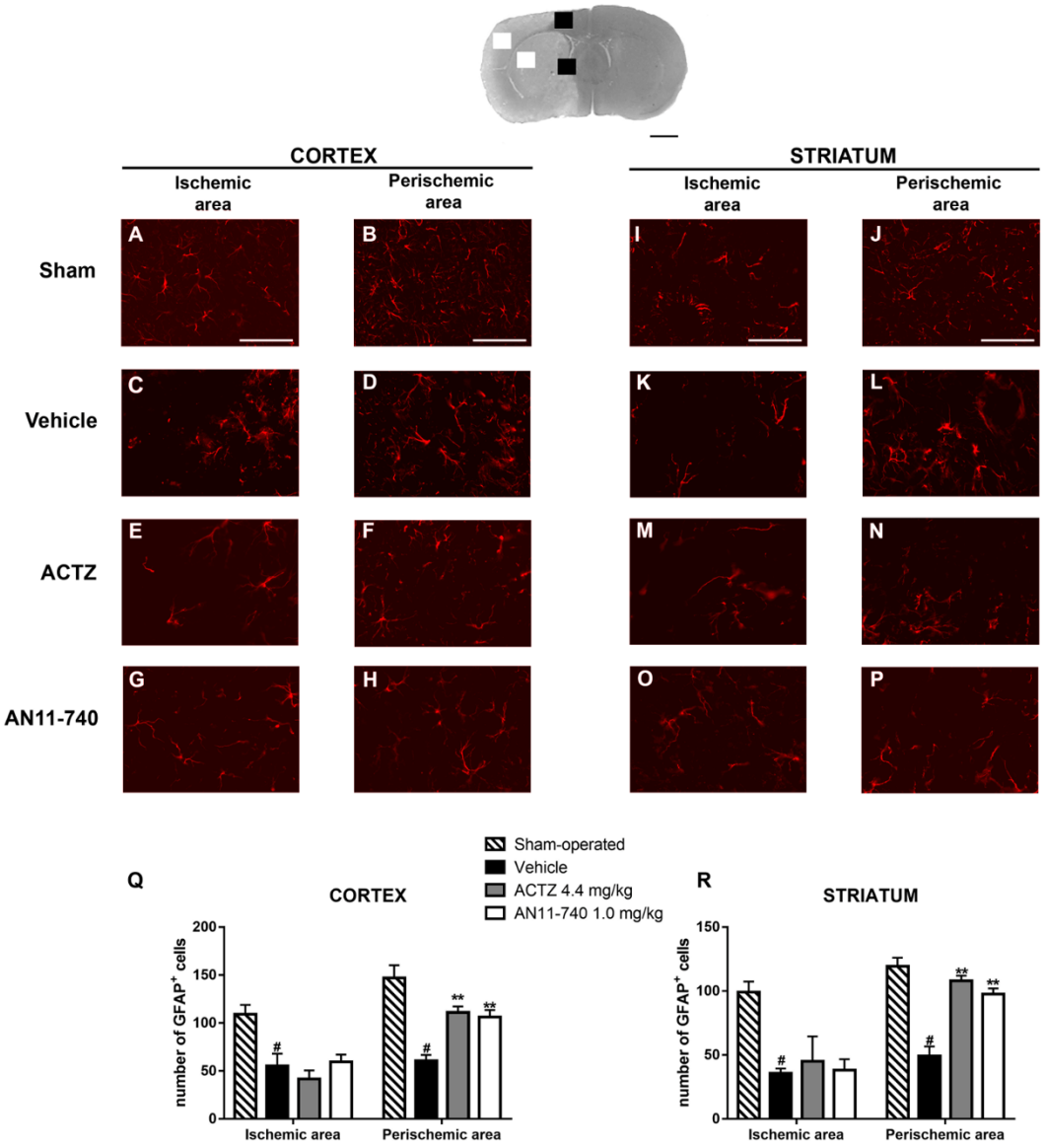

Figure 29. Effect of sub-chronic treatment with ACTZ and AN11-740 on astrocytes alteration in cortex and striatum 24 h after pMCAo. Upper part: Representative photomicrograph of a coronal section (at Bregma = 0, (König et al., 1964)) showing the ischemic area in a vehicle-treated rat. The two white and black squares indicate regions within the ischemic area and within the perischemic area, respectively, where photomicrographs were captured. Scale bar: 2 mm. **(A–P)** representative microphotographs of astrocytes (red) in cortical ischemic and perischemic areas and in striatal ischemic and perischemic areas of sham-operated (**A, B; I, J;** n = 3), vehicle- (**C, D; K, L;** n = 3) ACTZ- (**E, F; M, N;** n = 4) and AN11-740- (**G, H; O, P;** n = 3) treated rats. Scale bar: 50 mm. **(Q, R)** Quantitative analysis of GFAP$^+$ cells in cortical and striatal ischemic and perischemic areas, bar graphs represent mean ± SEM of the number of astrocytes per optical field (20x). One-way ANOVA followed by Newman–Keuls *post-hoc* test: $^{\#}$at least $p < 0.05$ vehicle-treated vs. sham-operated rats; **at least $p < 0.01$ ACTZ- and AN11-740- vs. vehicle-treated rats.

1.7 Effect of treatment with CAIs on cytokines plasma levels after pMCAo

Twenty-four hours after pMCAo, we evaluated plasma levels of the pro-inflammatory cytokine TNF-α, and of IL-10, a regulatory cytokine with an anti-inflammatory action. In vehicle-treated rats TNF-α plasma levels significantly increased as compared to sham-operated rats (One-way ANOVA: $F_{3,8}=10.7$, $p<0.003$; Newman–Keuls *post-hoc* test:**$p<0.01$; Figure 30 A) while the plasma level of IL-10 was significantly reduced in vehicle-treated rats as compared to sham-operated rats (One-way ANOVA: $F_{3,7}=13.7$, $p<0.002$; Newman–Keuls *post-hoc* test: **$p<0.01$; Figure 30 B). Sub-chronic treatment with both CAIs did not modify neither TNF-α nor IL-10 plasma levels 24 h after pMCAo (Figure 30 A,B).

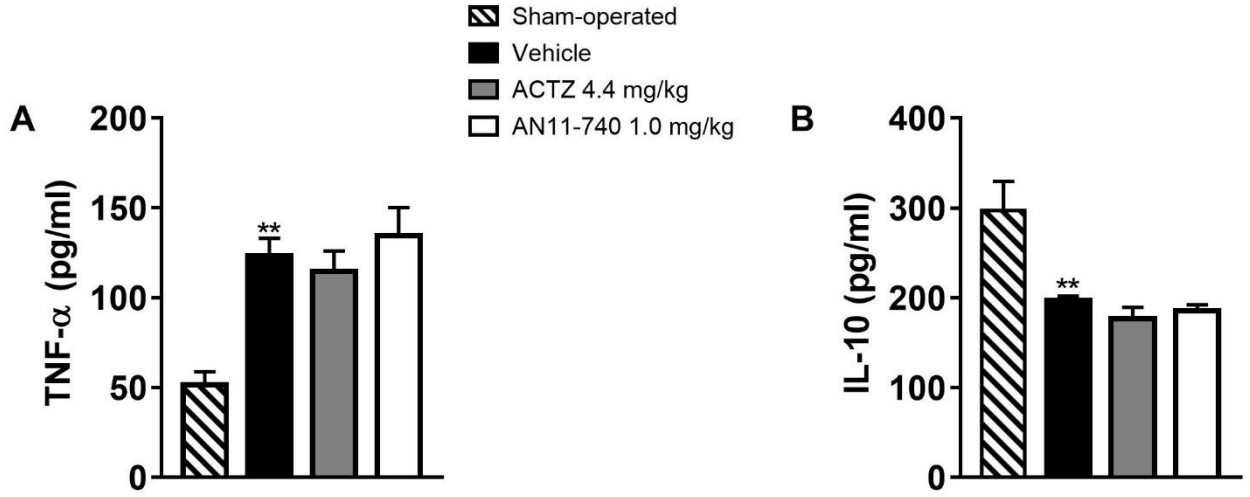

Figure 30. Effect of sub-chronic treatment with ACTZ and AN11-740 on TNF-α **(A)** and IL-10 **(B)** plasma levels. Results are expressed as pg of protein/ml of plasma and values are mean ± SEM. One-way ANOVA followed by Newman–Keuls *post-hoc* test: **$p < 0.01$ vs. sham-operated rats.

Section II: The adenosine A_{2B} receptor agonist, BAY60-6583, is protective in a rat model of transient cerebral ischemia

2.1 Effect of treatment with BAY60-6583 on neurological deficit and on body weight loss after tMCAo

Figure 31 A shows that in the mNSS test, sham-operated rats had a neurological score of 0.1-0.6 in the period from 1 to 7 days after tMCAo showing any neurological deficit. Twenty-four hours after tMCAo, vehicle-treated rats showed a clear neurological deficit with a neurological score of 10.5±1.3 (mean±SEM) that defines a moderate injury. The neurological impairment spontaneously recovered up to 7 days after tMCAo. Five days after tMCAo the neurological score was reduced to 7.3±0.5 and seven days after tMCAo, the neurological score was further reduced to 6.8±0.9. Chronic treatment with the A_{2B} receptor agonist, BAY60-6583, at the dose of 0.1 mg/kg, significantly reduced the neurological

deficit at each time point up to 7 days after tMCAo. Repeated Measures two-way ANOVA calculated for the two factors: treatment and time after tMCAo, showed that treatment factor ($F_{2,14}=35.2$; $p<0.0001$), time factor ($F_{2,28}=13.2$; $p<0.0001$) and interaction between treatment and time ($F_{4,28}=2.9$; $p<0.04$) were statistically significant. The Tukey *post-hoc* test indicated that sham-operated rats had a neurological score significantly different from vehicle-treated and BAY60-6583-treated rats at each time point (1, 5, 7 days after tMCAo, $p<0.0001$-0.03). The chronic treatment with BAY60-6583 significantly reduced the neurological deficit with respect to vehicle-treated rats at 1, 5 and 7 days ($p<0.001$-0.02) after tMCAo.

Twenty-four hours after the surgery, sham-operated rats were not affected in weight, then they increased in body weight as evaluated up to 7 days after tMCAo. Vehicle-treated rats lost 16.8±5.7 g 1 day after tMCAo, 15.0±4.4 g 5 days after tMCAo and 0.8±5.3 g 7 days after tMCAo (Figure 31 B). Treatment with the A_{2B} receptor agonist, BAY60-6583, did not modify the body weight loss at any time point after tMCAo respect to vehicle-treated rats. Repeated Measures two-way ANOVA, calculated for the two factors: treatment and time after tMCAo, showed that treatment factor ($F_{2,14}=27.5$; $p<0.0001$), time factor ($F_{2,28}=59.7$; $p<0.0001$) and the interaction between treatment and time ($F_{4,28}=13.9$; $p<0.0001$) were statistically significant. The Tukey *post-hoc* test indicated that in sham-operated rats, body weight was different with respect to vehicle-treated rats 5 and 7 days after tMCAo ($p<0.0001$) and to BAY60-6583-treated rats at each time point ($p<0.0001$-0.003) after tMCAo.

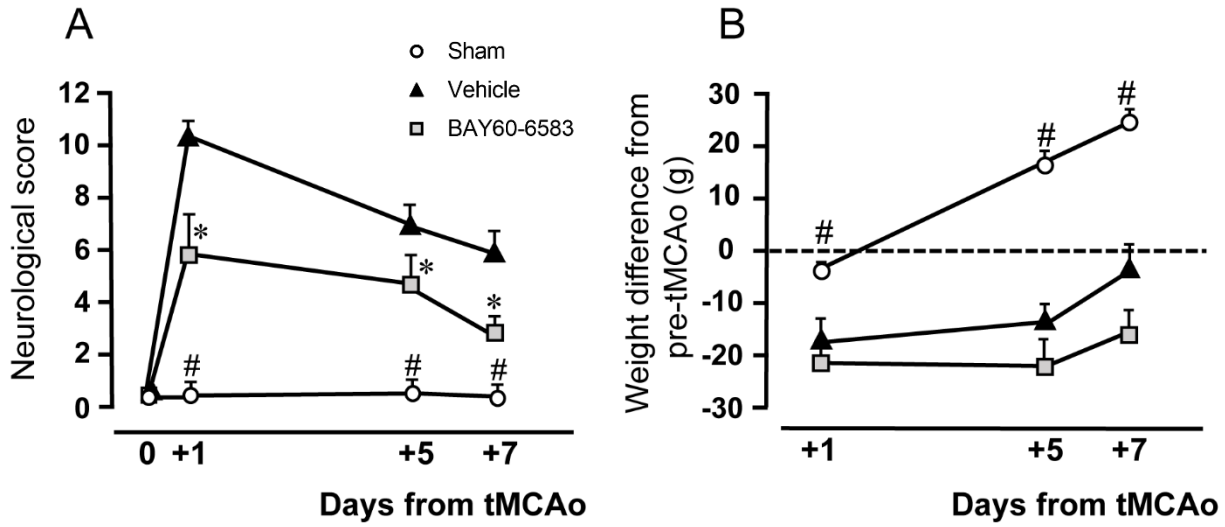

Figure 31. Effect of BAY60-6583 (0.1 mg/kg, i.p.) on neurological deficit and body weight. **(A)** The score of mNSS test was evaluated before and 1, 5 and 7 days after tMCAo. Statistical significance was evaluated by Repeated Measures two-way ANOVA followed by Tukey *post-hoc* test. Significativity: #at least $p < 0.03$, sham vs. BAY60-6583- and vehicle-treated rats; *at least $p < 0.05$, BAY60-6583- vs. vehicle-treated rats. **(B)** The body weight was calculated as the difference in g of the rat's body weight at each time point minus its own pre-operation body weight. Significativity: #at least $p < 0.03$, sham vs. BAY60-6583- and vehicle-treated rats, Repeated Measures two-way ANOVA followed by Tukey *post-hoc* test.

2.2 Effect of treatment with BAY60-6583 on brain ischemic damage after tMCAo

Figure 32 shows the extent of the ischemic damage evaluated as infarct area (Figures 32 A,B) and infarct volume (Figures 32 C,D) in the striatum and cortex of vehicle- and BAY60-6583-treated rats 7 days after tMCAo. Chronic treatment with BAY60-6583, at the dose of 0.1 mg/kg, significantly reduced the infarct area and volume in both areas. In BAY60-6583-treated rats the striatal and cortical infarct volumes were reduced by 44.7% and 46.3%, respectively (*$p<0.05$, **$p<0.001$ vs. vehicle-treated rats, unpaired Student's t-test: Figures 32 C,D). In BAY60-6583-treated rats the striatal and cortical infarct volumes were reduced by 44.7% and 46.3%, respectively (*$p<0.05$, **$p<0.001$ vs. vehicle-treated rats, unpaired Student's t-test: Figures 32 C,D). Sham rats did not show any damage both in the striatum and in the cortex (not shown).

To characterize the cytoarchitecture of the ischemic cortex and striatum at day 7 after tMCAo, striatal and cortical sections from the three experimental groups were stained with H&E (Figures 33 A–F). Seven days after transient ischemia, H&E staining showed a decrease of staining intensity in vehicle-treated rats (Figures 33 B,E) compared to the sham rats (Figures 33 A,D). The decrease of staining intensity is attributable to an increase in extracellular fluid consequent to edema. The typical cytoarchitecture of cortex and striatum was lost. In the dorsal striatum of sham rats (A), the typical caudate-putamen cytoarchitecture was appreciable, with numerous transversally sectioned white matter *fascicula* (f) surrounded by gray matter containing diverse types of neurons, distinct on the bases of their size and shape (Danner & Pfister, 1981). In vehicle-treated rats, the striatal tissue was clearly damaged, the cytoarchitecture was lost, the *fascicula* were much less recognizable, the distinction between white and gray matter no more appreciable, the interstitial spaces increased, and numerous heterochromatic small nuclei were present (shown by the arrows in the insets, Figure 33 B1). In the fronto-parietal cortex, the columnar organization was no longer appreciable, the interstitial spaces were enlarged and dilated, and numerous heterochromatic small nuclei were present (shown by the arrows in the insets, Figure 33 E1). No heterochromatic nuclei were ever found in the striatum and cortex of sham rats. Qualitative analysis (Figures 33 G,H) shows that administration of BAY60-6583 at the dose of 0.1 mg/kg, was associated with a recovery of staining intensity, and with a reduction of heterochromatic small nuclei in both brain regions. In BAY60-6583-treated rats, the cytoarchitecture of the white matter *fascicula* was maintained in the dorsal corpus striatum (Figure 33 C) and the columnar organization was still appreciable in the fronto-parietal cortex (Figure 33 F). Quantitative analysis showed that treatment with BAY60-6583 significantly reduced the number of heterochromatic nuclei in the ischemic striatum (−72.4%, *$p<0.02$ vs. vehicle-treated rats, unpaired Student's t-test;

Figure 33 G) and in the ischemic cortex (−84.4%, **p<0.005 vs. vehicle-treated rats, unpaired Student's t-test; Figure 33 H).

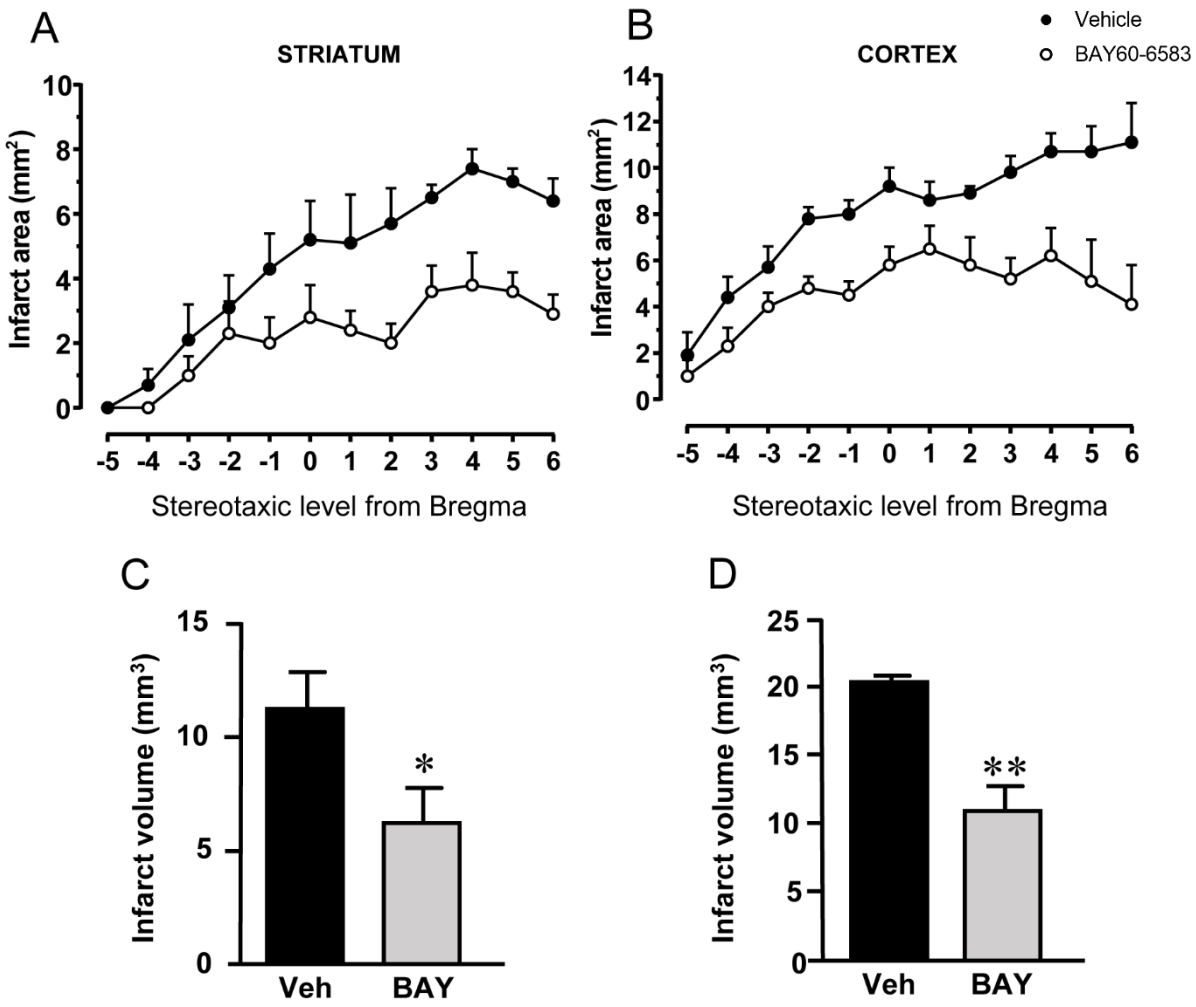

Figure 32. Effect of chronic treatment with BAY60-6583 (0.1 mg/kg i.p.) on infarct area and infarct volume in the striatum and cortex 7 days after tMCAo. **(A,B)** Data represent infarct area measured at 12 coronal levels between AP: +2.2 mm to −2.0 mm from Bregma of vehicle- (n = 6) and BAY60-6583-treated rats (n = 6) (König et al., 1964). **(C,D)** Graphs show the infarct volume calculated in the striatum and cortex as mean ± SEM of 6 rats per group. Significativity: *p < 0.05; **p < 0.001 vs. vehicle-treated rats, unpaired Student's t-test.

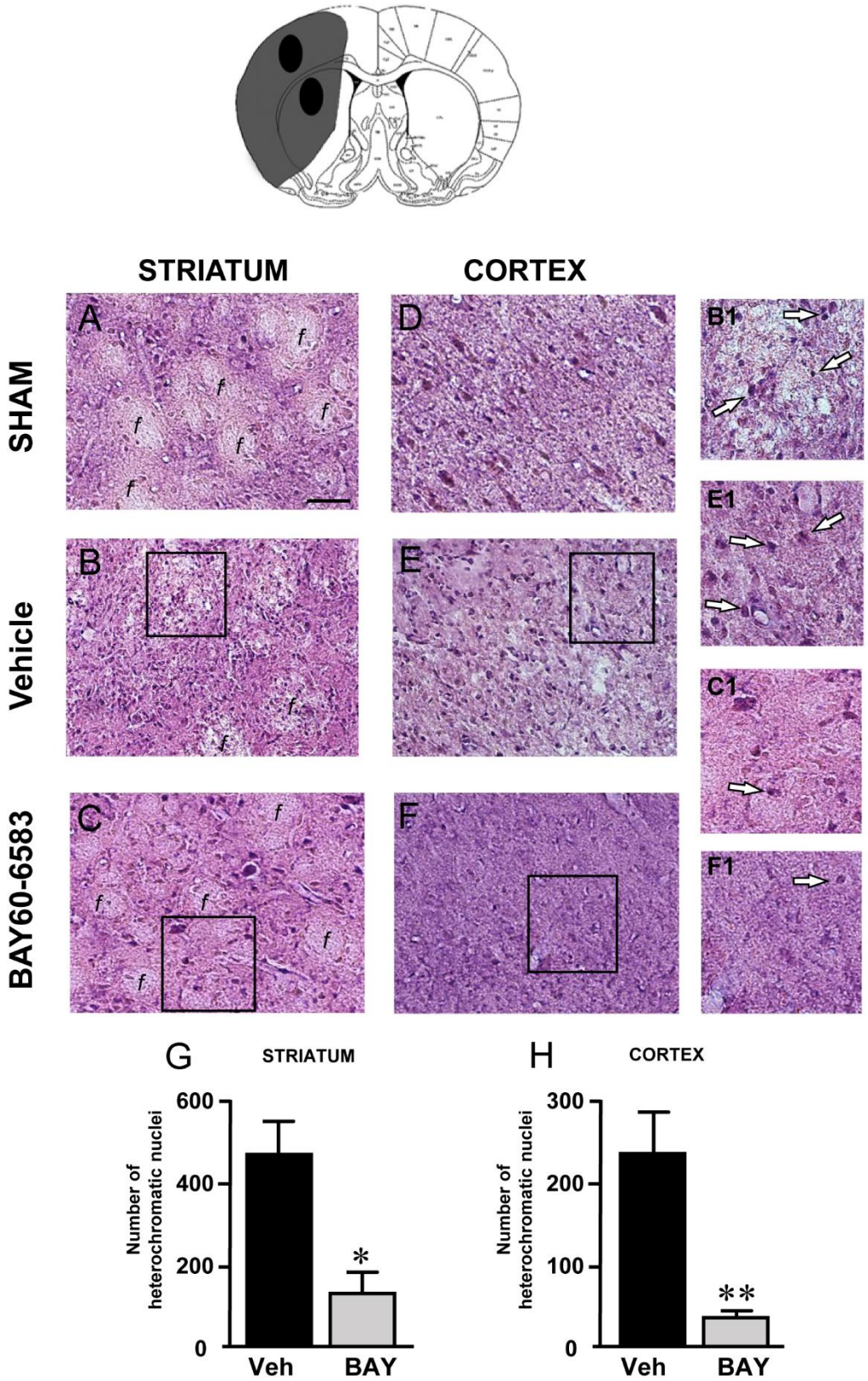

Figure 33. Effect of BAY60-6583 (0.1 mg/kg, i.p.) on the cytoarchitecture of the striatum and cortex 7 days after tMCAo. Upper part: Schematic brain picture of a coronal section (at Bregma = 0, König et al., 1964) showing the infarct area in a vehicle-treated rat. The two black circles indicate regions within the infarct area where photomicrographs were captured. **(A–F)** Representative microphotographs of H&E staining from dorsal striatum and fronto-parietal cortex of a sham **(A,D)**, a vehicle- **(B,E)** and a BAY60-6583-treated rat **(C,F)**. The white matter *fascicula* (f) are

evidenced. Scale bar: 100 µm. Insets show magnifications of cells present in the square areas of panels **(C–F)**. **(G,H)** Quantitative analyses of heterochromatic nuclei per striatal **(G)** and cortical areas **(H)** at coronal level AP = 0 from Bregma. Data represent the mean ± SEM of three rats/group (*p < 0.02; **p < 0.005 BAY60-6583- vs. vehicle-treated rats, unpaired Student's t-test.

2.3 Effect of treatment with BAY60-6583 on neuronal damage 7 days after tMCAo

To estimate the neuronal damage caused by tMCAo in the striatal boundary zone, fluorescent confocal immunohistochemical analysis were performed using neuron specific anti-NeuN antibody in control slices taken from sham-operated rats, in slices taken from vehicle-treated rats and in slices taken from BAY60-6583-treated rats.

Representative images (each obtained stacking 10 consecutive confocal scans, z step of 1.2 µm, total thickness 12 µm) of NeuN immunostaining in the striatal boundary zone are shown in Figure 34 A-C. Figure 34 D shows the quantitative analysis of NeuN-positive cells in the striatum of sham, vehicle- and BAY60-6583 rats. Statistical analysis performed with One-way ANOVA showed that the density of NeuN positive neurons decreased significantly in vehicletreated rats in comparison to sham rats ($F_{2,12} = 4.788$, $p<0.05$; −34%, *$p<0.05$, vehicle-treated vs. sham rats, Newman-Keuls *post-hoc* test). The treatment with BAY60-6583 significantly antagonized this effect ($^{\#}p<0.05$, BAY60-6583- vs. vehicle-treated rats, Newman-Keuls *post-hoc* test). Indeed, the density of neurons in BAY60-6583-treated rats was not different from that of sham rats (+0.01%, n.s., BAY60-6583-treated vs. sham rats). In the ROI of the contralateral striatum we found no difference in the density of neurons among the three experimental groups (data not shown).

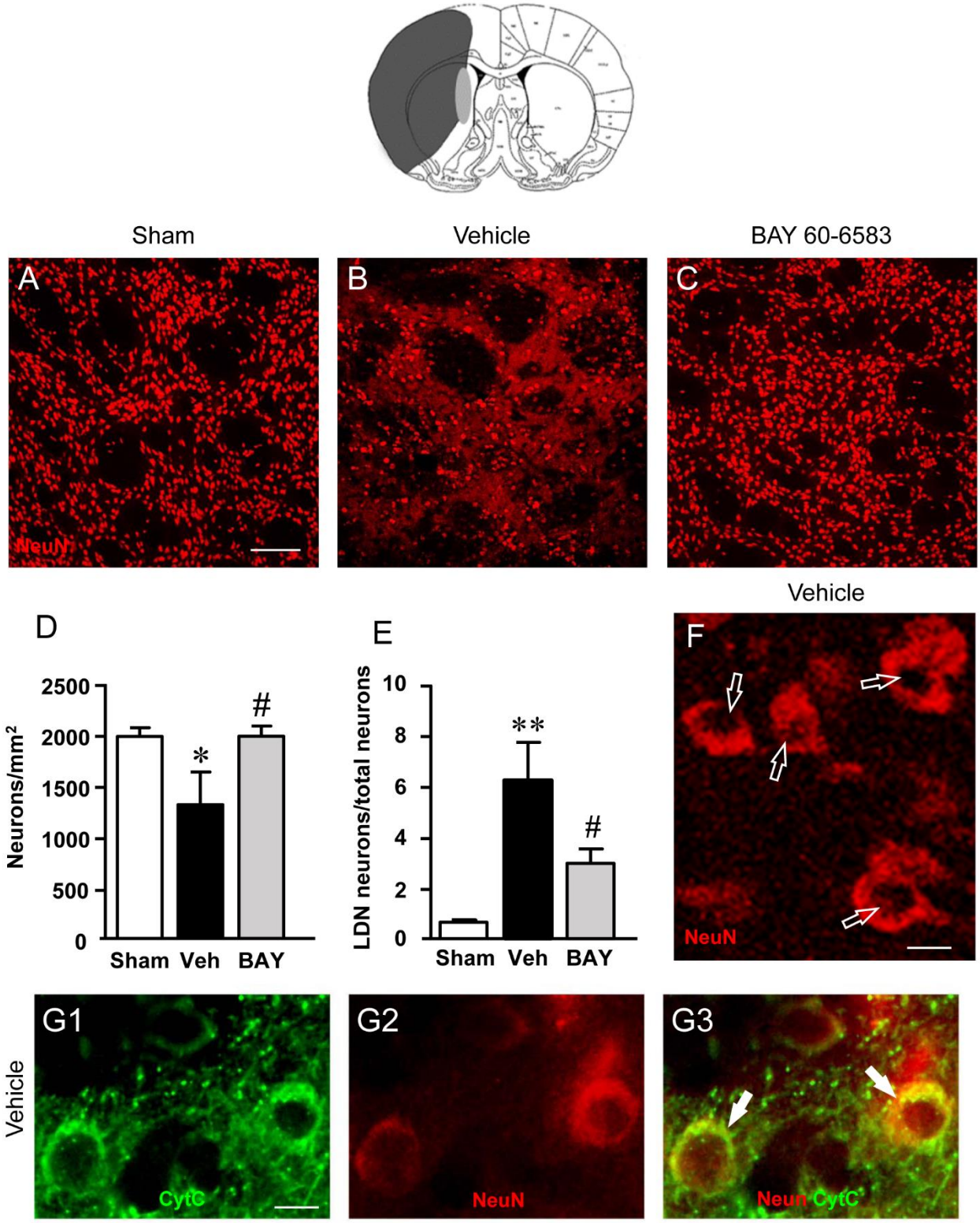

Figure 34. Analysis of neurons of sham, vehicle- and BAY60-6583-treated rats 7 days after tMCAo. Upper part: Schematic brain picture of a coronal section (at Bregma = 0, König et al., 1964) showing the infarct area in a vehicle-treated rat. The gray circle indicates region within the boundary zone where photomicrographs were captured. **(A–C)** Representative confocal microphotographs of NeuN immunostaining of neurons (red) in the ipsilateral striatum of a sham **(A)**, a vehicle- **(B)** and a BAY60-6583-treated **(C)** rat. Scale bar: 100 µm. **(D)** Quantitative analysis of the density

of neurons (neurons/mm^2) in the ROI of sham (n = 6), vehicle- (n = 4) and BAY60-6583-treated (n = 5) rats. Neuronal density was significantly lower in the striatum of tMCAo rats treated with vehicle in comparison to sham rats. BAY60-6583 reverted significantly this effect. Significativity: *p < 0.05 vehicle-treated vs. sham rats; $^{\#}$p < 0.05 BAY60-6583- vs. vehicle-treated rats, One-way ANOVA followed by Newman-Keuls *post-hoc* test. **(E)** Quantitative analysis of NeuN-positive LDN neurons in the striatum of sham (n = 6), vehicle- (n = 5) and BAY60-6583-treated (n = 5) rats, expressed as percent of total neurons in the ROI. The percent of LDN neurons was significantly higher in striatum of vehicle-treated vs. sham rats. BAY60-6583 reverted significantly this increase. Significativity: **p < 0.01 vehicle-treated vs. sham rats; $^{\#}$p < 0.05 BAY60-6583- vs. vehicle-treated rats, One-way ANOVA followed by Newman-Keuls *post-hoc* test. Data are reported as mean ± SEM. **(F)** Note the presence of many LDN neurons (open arrows) in the striatum of vehicle-treated rats. Scale bar: 7.5 µm. **(G1-G3)** Representative microphotographs of CytC (**G1**, green), NeuN (**G2**, red) immunostaining of LDN neurons, and the merge of the previous images (**G3**, orange-yellow) in the ipsilateral striatum of a vehicle-treated rat. The arrows show the colocalization of CytC and NeuN immunostaining in the neuronal cytoplasm. Scale bar: 7.5 µm.

2.3.1 Characterization of neuronal damage in the striatum after tMCAo

A closer examination of the neurons in the striatal boundary zone with confocal microscopy indicated the presence of many damaged neurons in both vehicle- and BAY60-6583-treated rats seven days after tMCAo. A representative image of a vehicle-treated rat is shown in Figure 34 F. Figure 34 F shows that in vehicle-treated rats many of the surviving neurons were damaged, as shown by signs of karyorrhexis (Figure 34 F). Indeed, we observed many neurons that had lost their nuclear NeuN positive immunofluorescence (Figure 34 F, open arrows), which is a clear index of damaged nuclei, while NeuN positive immunofluorescence persisted in the cytoplasm. We had previously found and characterized neurons showing karyorrhexis in different models of ischemia *in vitro* (Fusco et al., 2018) and *in vivo* (Lana et al., 2017), and we had named them Low Density Nucleus neurons, “LDN neurons”.

In order to better characterize this phenomenon, we performed the quantitative analysis of LDN neurons in sham-operated, vehicle- and BAY60-6583-treated rats (Figure 34 E). Statistical analysis performed with One-way ANOVA showed that the percent of LDN neurons over total (surviving) neurons was significantly higher in vehicle-treated rats compared with sham rats ($F_{2,13} = 10.85$, $p<0.002$; +780%, **$p<0.01$ vehicle treated vs. sham rats, Newman-Keuls *post-hoc* test). Interestingly, treatment with BAY60-6583 decreased significantly, although not completely, the percent increase of LDN neurons/total neurons in the ipsilateral striatum (−52%, $^{\#}p<0.05$, BAY60-6583- vs. vehicle-treated rats, Newman-Keuls *post-hoc* test). Indeed, the percent of LDN neurons in BAY60-6583-treated rats was not significantly different from that in sham rats (n.s., sham vs. BAY60-6583-treated rats).

To determine whether these effects might be caused by apoptosis, we used double immunohistochemistry for NeuN and CytC, a protein which, in the most advanced stages of apoptosis,

is intensely and diffusely released from mitochondria to the cytoplasm, where it activates caspases (Jiang & Wang, 2004; Kluck et al., 1997; Suen et al., 2008; Yang et al., 1997). CytC can be used as a marker of apoptosis by immunohistochemistry (Martínez-Fábregas et al., 2014). The results are shown in Figures 34 G1–G3 where it is possible to visualize that CytC (Figure 34 G1, green) is colocalized in the cytoplasm of two LDN neurons (Figure 34 G2, red), as evidenced in the merge of the previous pictures (Figure 34 G3), indicating that LDN neurons are apoptotic.

2.4 Effect of treatment with BAY60-6583 on microglia morphology in the striatum after tMCAo

Total microglia cells were identified in the striatal boundary zone using the fluorescent immunostaining for IBA1, as shown by the representative images of Figure 35 A-C, each obtained stacking 10 consecutive confocal z scans (1.2 µm each, total thickness 12 µm) through the thickness of striatal boundary zone. Figure 35 shows the magnifications of the framed areas in panels A1-C1. Quantitative analysis of IBA1-positive cells in Figure 35 D revealed that microglia cells increase in vehicle-treated rats in comparison with sham-operated rats (+130% vehicle vs sham) 7 days after tMCAo, although not in a statistically significant manner. The qualitative analysis shows that in vehicle-treated rats there is a strong pattern of microglia activation (magnification B1): microglial cells appeared with hypertrophic branches, round-shaped and ameboid morphology in comparison with those of sham-operated rats, that are in a resting ramified state (Figure 35 A1). BAY60-6583 reverted these morphological changes reducing the strong pattern of activation. As shown in figure 35 C1, microglia cells belonging to BAY60-6583-treated rats have similar morphology to that of sham-operated rats (Figure 35 A1). Analysis of IBA1 positive pixel/cell (Figure 35 E) with One-way ANOVA ($F_{2,14} = 7.675$, $p<0.01$) indicated that in vehicle-treated rats the number of pixel/cell was significantly decreased (−55%, **$p<0.01$ vehicle-treated vs. sham rats, Newman-Keuls *post-hoc* test). Since the volume of a microglial cell in ameboid state is reduced in comparison to a resting cell, the number of pixel/cell indirectly confirms the reactive state of the cell. Therefore, from the data presented in Figures 5 D,E, it is possible to conclude that in vehicle-treated rats the number of microglia cells increased (although not significantly), and the cells were in an activated state. The density of microglia cells in BAY60-6583-treated rats was not different from that of sham rats (Figure 5D). Treatment with BAY60-6583 caused a significant increase in pixel/cell ($^{\#}p<0.05$, BAY60-6583-treated vs. vehicle-treated rats, One-way ANOVA followed by Newman-Keuls *post-hoc* test) (Figure 35 E) indicating that it has significantly protected microglia from reactivity (Figure 35 C1).

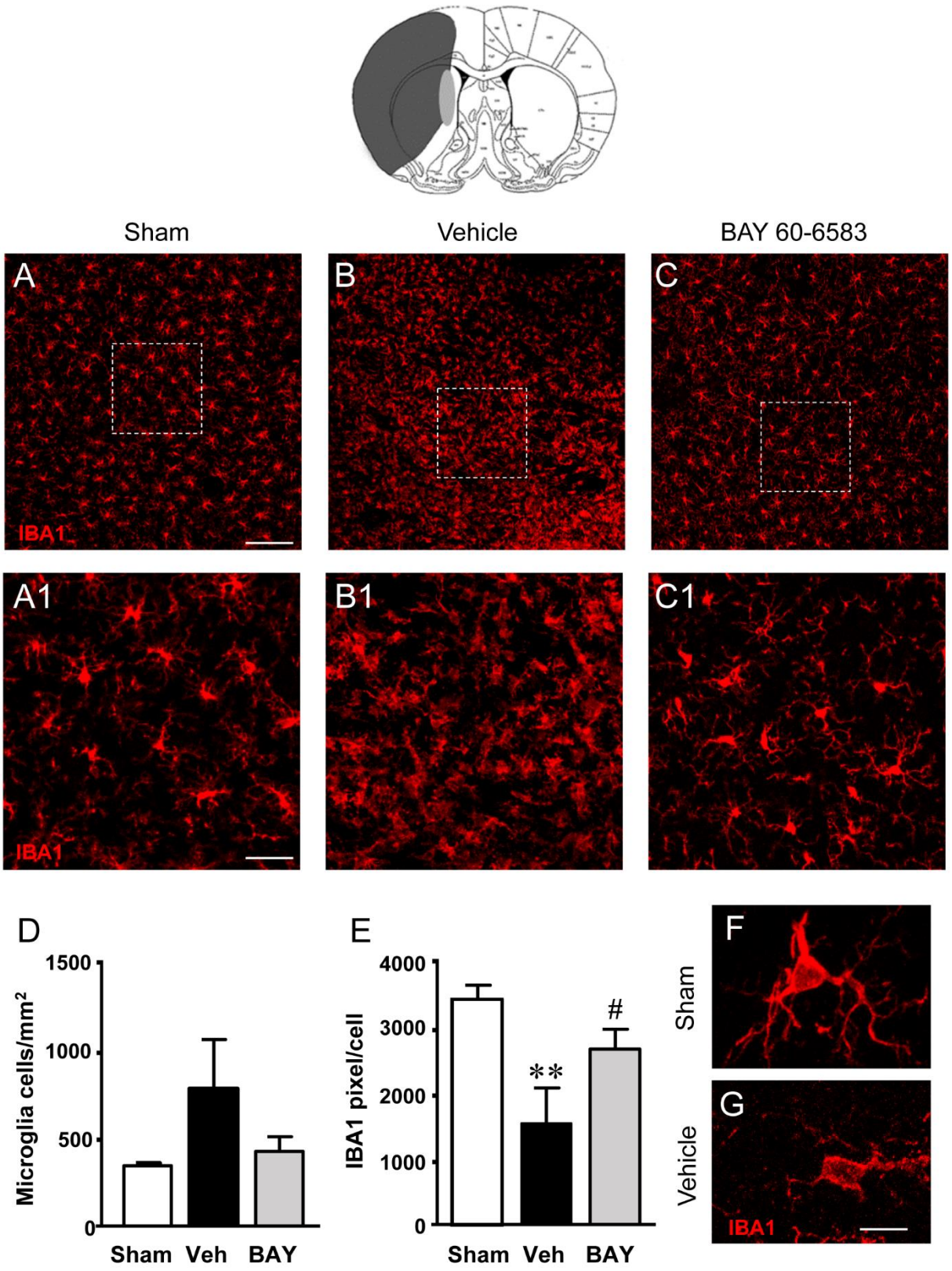

Figure 35. Analysis of microglia of sham, vehicle- and BAY60-6583-treated rats 7 days after tMCAo. Upper part: Schematic brain picture of a coronal section (at Bregma = 0) (König et al., 1964) showing the infarct area in a vehicle-treated rat. The gray circle indicates region within the boundary zone where photomicrographs were captured. **(A–C1)** Representative confocal photomicrographs of IBA1 immunostaining of microglia (red) in the striatum of a sham **(A)**, a vehicle- **(B)**, and a BAY60-6583-treated **(C)** rat. Scale bar = 100 µm. Panels **(A1–C1)** show the magnification of areas

framed in Panels **(A–C)**. Scale bar = 20 µm. **(D)** Quantitative analysis of IBA1 positive microglia/mm^2 in striatum of sham (n = 5), vehicle- (n = 5) and BAY60-6583-treated (n = 6) rats. **(E)** Quantitative analysis of IBA1 positive pixels/cell in the striatum of sham (n = 5), vehicle- (n = 5), and BAY60-6583-treated (n = 6) rats. Significativity: **p < 0.01 vehicle-treated vs. sham rats, $^{\#}$p < 0.05 BAY60-6583- vs. vehicle-treated rats, One-way ANOVA followed by Newman-Keuls *post-hoc* test. Data represent the mean ± SEM. **(F,G)** Representative confocal z projections (z stacks of 65 consecutive scans, each 0.4 µm, total thickness 26 µm) of a microglia cell from a sham **(F)** and a vehicle-treated **(G)** rat. Scale bar: 5 µm.

2.5 Effect of treatment with BAY60-6583 on astrocytes morphology 7 days after tMCAo

Astrocytes were immunolabelled in the striatal boundary zone with anti-GFAP antibody.

Figure 36 A-C show the representative images, each obtained stacking 10 consecutive confocal z scans (1.2 µm each, total thickness 12 µm) through the thickness of striatal boundary zone.

Panels 36 A1-C1 show the magnifications of the framed areas in figures 36 A-C. Figure 36 B1 shows that 7 days after tMCAo the density and morphology of striatal astrocytes of vehicle-treated rats was significantly different from that of the sham-operated rats (Figure 36 A1). Indeed, in vehicle-treated rats, astrocytes morphology is strongly altered: they are smaller with shorter and highly fragmented branches. Chronic treatment with BAY60-6583, at the dose of 0.1 mg/kg, reverted the alterations caused by ischemia in the morphology of astrocytes (Figure 36 C1): astrocytes in BAY60-6583-treated rats show a morphology mostly similar to that of sham-operated rats.

Quantitative analysis of GFAP$^+$ astrocytes reported in Figure 36 E, shows that the density of GFAP$^+$ astrocytes decreased significantly in vehicle-treated rats in comparison to sham-operated rats (One-way ANOVA: $F_{2,12} = 4.137$, $p<0.05$; -83%, *$p<0.05$ vehicle-treated vs. sham rats, Newman-Keuls *post-hoc* test, Figure 36 A1). Treatment with BAY60-6583 tended to revert this effect (-28% BAY60-6583-treated rats vs sham-operated rats) although the difference respect to vehicle-treated rats was not different in a statistically significant manner. Although astrocytes of vehicle-treated rats were decreased in number, the number of GFAP positive pixel/cell (Figure 36 E) was significantly higher than in sham rats and BAY60-6583 reverted significantly this effect (One-way ANOVA: $F_{2,13} = 4.547$, $p<0.05$; +107%, *$p<0.05$, vehicle-treated vs. sham rats and BAY60-6583-treated rats, Newman-Keuls *post-hoc* test; +19%, n.s., BAY60-6583-treated vs. sham rats). Since the volume of reactive, hypertrophic astrocytes is increased in comparison to resting astrocytes, the increase of the number of pixel/cell in vehicle-treated rats indirectly confirms that astrocytes are more activated than those of the sham rats and that BAY60-6583 has significantly protected astrocytes from reactivity (Figure 36 C1).

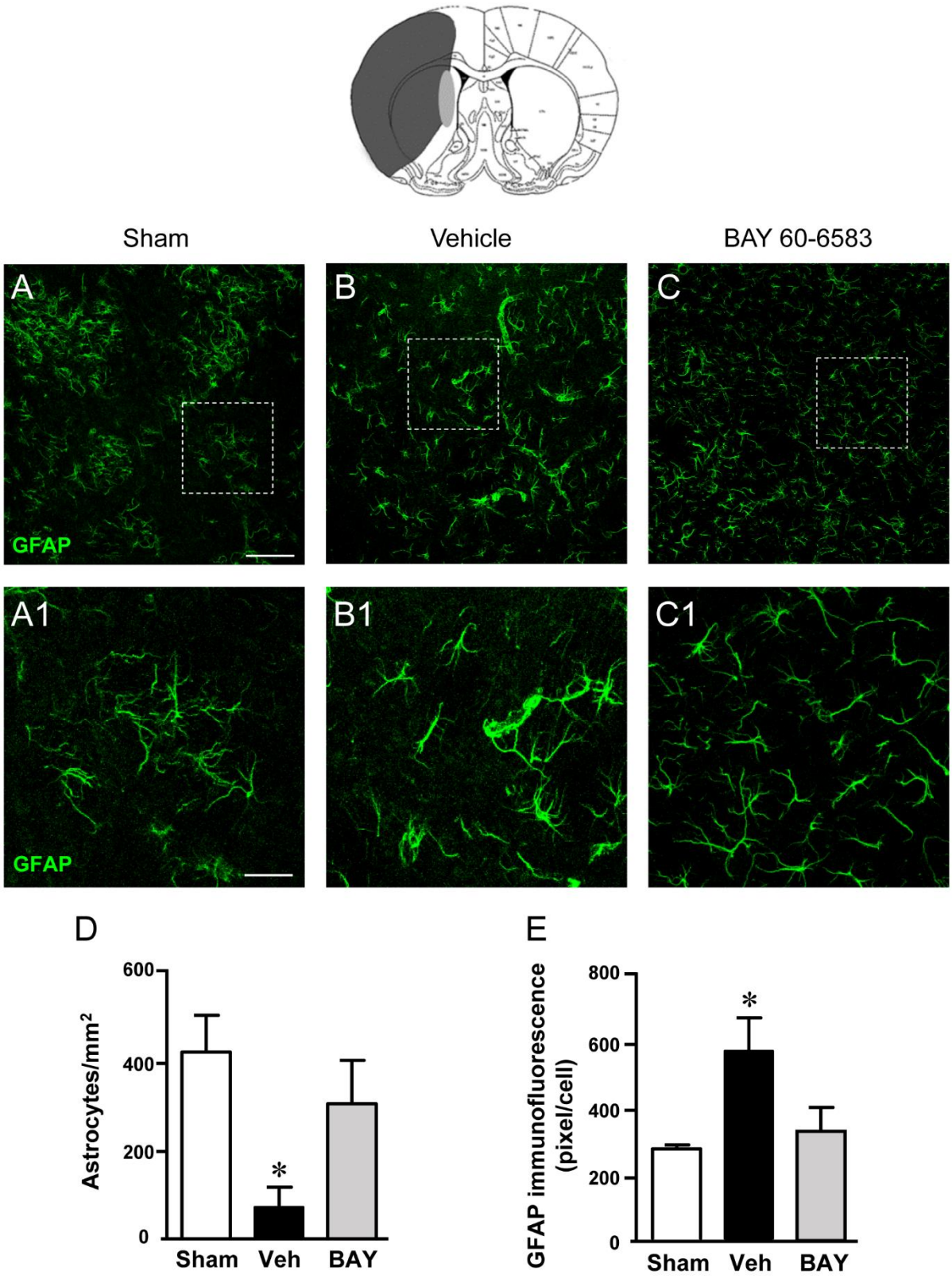

Figure 36. Analysis of astrocytes of sham, vehicle- and BAY60-6583-treated rats 7 days after tMCAo. Upper part: Schematic brain picture of a coronal section (at Bregma = 0) (König et al., 1964) showing the infarct area in a vehicle-treated rat. The gray circle indicates region within the boundary zone where photomicrographs were captured. **(A–C1)** Representative confocal microphotographs of GFAP immunostaining of astrocytes (green) in the striatum of a sham **(A)**, a vehicle- **(B)**, and a BAY60- 6583-treated **(C)** rats. Scale bar: 100 µm. Panels **(A1–C1)** show the magnification of

areas framed in Panels **(A–C)**, respectively. Scale bar: 30 µm. **(D)** Quantitative analysis of GFAP positive astrocytes/mm^2 in the striatum of sham (n = 5), vehicle- (n = 5) and BAY60-6583- (n = 6) rats. Significativity: *p < 0.05 vehicle-treated vs. sham rats, One-way ANOVA followed by Newman-Keuls *post-hoc* test. Astrocytes were significantly less numerous in striatum of vehicle-treated rats in comparison to sham rats. BAY60-6583 reverted this effect. **(E)** Quantitative analysis of GFAP in the striatum of sham (n = 5), vehicle- (n = 5), and BAY60-6583-treated (n = 6) rats. Significativity: *p < 0.05 vehicle-treated vs. sham rats and BAY60-6583-treated rats, One-way ANOVA followed by Newman-Keuls *post-hoc* test. Data are reported as mean ± SEM.

2.6 Effect of treatment with BAY60-6583 on cytokines plasma levels after tMCAo

Seven days after tMCAo, plasma levels of pro-inflammatory cytokine TNF-α were significantly increased in vehicle-treated rats as compared to sham-operated rats (One-way ANOVA: F2,7 = 17.51, p<0.001; *p<0.05, vehicle-treated vs. sham rats, Newman-Keuls *post-hoc* test; Figure 37 A), while the plasma level of IL-10, a regulatory cytokine with anti-inflammatory action was reduced in vehicle-treated rats as compared to sham-operated rats (One-way ANOVA: F2,8 = 7.03, p<0.01; *p<0.05, vehicle-treated vs. sham rats, Newman-Keuls *post-hoc* test; Figure 37 B). Chronic treatment with BAY60-6583, at the dose of 0.1 mg/kg, significantly decrease TNF-α levels (*p<0.05, BAY60-6583 vs. vehicle-treated rats, Newman-Keuls *post-hoc* test; Figure 37 A) and significantly increase the plasma level of IL-10 (*p<0.05, BAY60-6583 vs. vehicle-treated rats, Newman-Keuls *post-hoc* test; Figure 37 B) to those of sham rats.

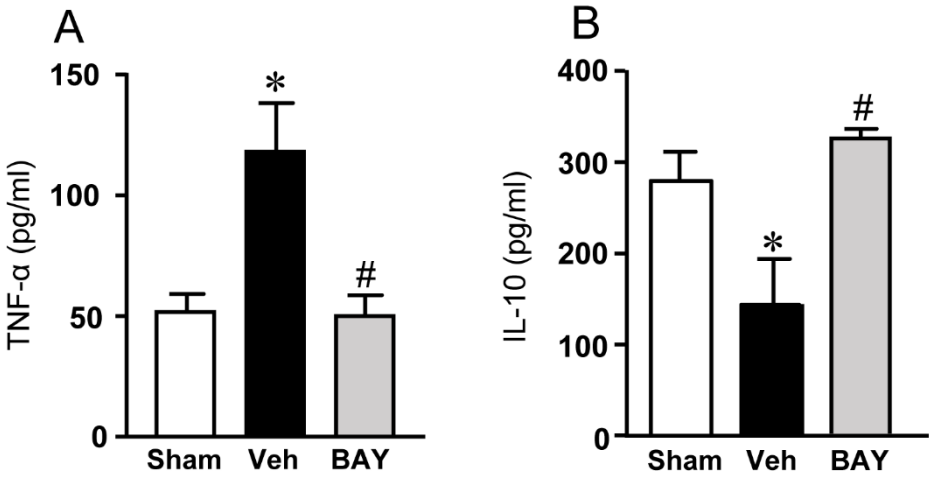

Figure 37. Effect of chronic treatment with BAY60-6583 (0.1 mg/kg i.p.) on TNF-α **(A)** and IL-10 **(B)** plasma levels. Results are expressed as pg of protein/ml of plasma in sham (n = 3), vehicle- (n = 3) and BAY60-6583-treated (n = 4) rats. Values are mean ± SEM: *p < 0.05, vehicle-treated vs. sham rats; $^{\#}$p < 0.05 BAY60-6583- vs. vehicle-treated rats, One-way ANOVA followed by Newman-Keuls *post-hoc* test.

2.7 Effect of treatment with BAY60-6583 on blood cell infiltration after tMCAo

Granulocytes were evaluated using anti-HIS-48 antibody, as shown in Figures 38 A–F. Infiltration of HIS-48 positive granulocytes was never found in any section of the striatum and cortex of sham rats (n = 3). On the contrary, in both cortical and striatal ischemic areas of vehicle-treated rats, HIS-48 positive cells were clearly detectable 2 days after tMCAo (Figures 38 B–F, open arrows). BAY60–6583 reduced significantly the number of HIS-48 positive cells in the cortical and striatal ischemic core 2 days after tMCAo. In the cortex, the effect was significant at Stereotaxic level −1 from Bregma (*$p < 0.05$, two-way ANOVA followed by Bonferroni *post-hoc* test, Figure 38 I). Statistical analysis performed with two-way ANOVA, calculated for the two factors: Treatment and Stereotaxic levels, showed that Treatment ($F_{1,35} = 18.82$; $p < 0.0001$) was statistically significant, while Stereotaxic levels ($F_{6,35} = 0.4687$; $p<0.8$) and the Interaction between Treatment and Stereotaxic levels ($F_{6,35} = 1.057$; $p<0.4$) were not significant. In the striatum, treatment with BAY60-6583 significantly reduced the number of HIS-48 positive cells at Stereotaxic level 0 (*$p<0.05$, two-way ANOVA followed by Bonferroni *post-hoc* test, Figure 38 G).

Two-way ANOVA, calculated for the two factors: Treatment and Stereotaxic levels, showed that Treatment ($F_{1,28} = 6.846$; $p<0.01$) and Stereotaxic levels ($F_{6,28} = 5.322$; $p<0.0009$) were statistically significant, but the Interaction between Treatment and Stereotaxic levels ($F_{6,28} = 1.485$; $p < 0.2$) was not. The mean effect of BAY60-6583 was significant between −3 and +3 stereotaxic levels in the cortex (**$p<0.004$, unpaired Student's t-test, Figure 38 J), but the effect did not reach statistical significance in the ischemic striatum (n.s., unpaired Student's t-test, Figure 38 H). Seven days after tMCAo, the infiltration of granulocytes was no longer detectable in the ischemic tissues (Melani et al., 2014).

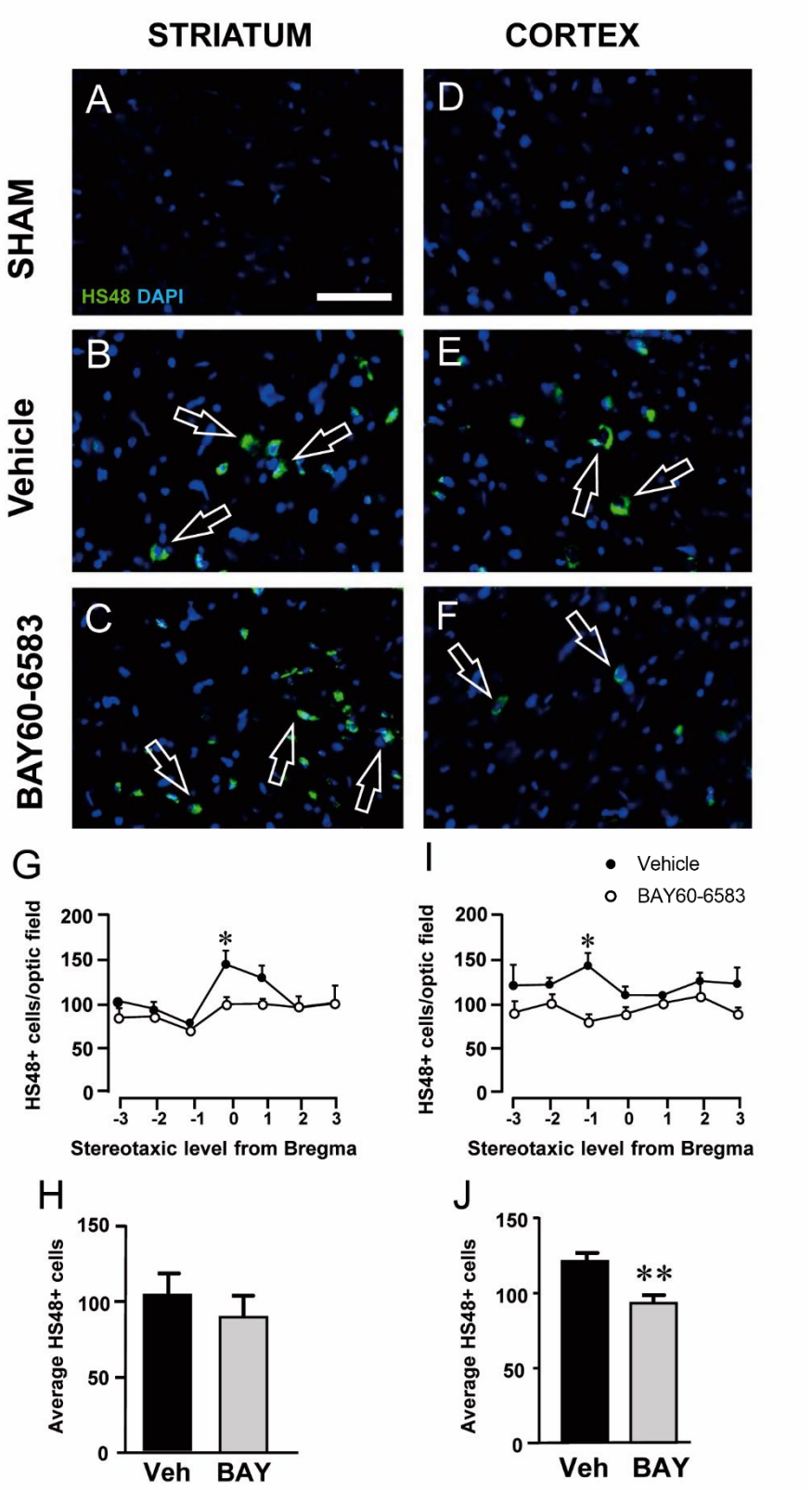

Figure 38. Effect of chronic treatment with BAY60-6583 (0.1 mg/kg i.p.) on blood cell infiltration in the ischemic core 2 days after tMCAo. **(A–F)** Representative microphotographs of HIS-48 positive cells (green label, open arrows) detected in ischemic striatum **(A–C)** and cortex **(D–F)** of sham, vehicle- and BAY60-6583-treated rats taken at AP = 0 from Bregma (König and Klippel, 1967). Cell nuclei were labeled with DAPI (blue). Scale bar: 50 µm. **(G–J)** Quantitative analyses of HIS-48^{+} cells in the striatum **(G, H)** and cortex **(I, J)** of vehicle- and BAY60-65803-treated rats. **(G, I)** Number

of HIS-48 positive cells per optical field counted in seven coronal levels of striatum **(G)** and cortex **(I)** between AP = +2.0 mm to −1.0 mm from Bregma of vehicle- (n = 3) and BAY60-6583-treated rats (n = 4). Significativity was evaluated by two-way ANOVA followed by Bonferroni *post-hoc* test (*p < 0.05 vs. vehicle-treated rats). **(H, J)** Average of HIS-48 positive cells between AP +2.0 mm to −1.0 mm from Bregma of vehicle- (n = 3) and BAY60-6583-treated rats (n = 4). Significativity was evaluated by unpaired Student's t-test (**p < 0.01 vs. vehicle-treated rats). Data in the graphs are mean ± SEM.

Section III. Preliminary results on the protective effect of the dual A_{2A}/A_{2B} receptor agonist, MRS3997, in a rat model of transient cerebral ischemia

3.1. Effect of treatment with MRS3997 on neurological deficit and on body weight loss after tMCAo

Figure 39 A shows that in the mNSS test, sham-operated rats had a neurological score of 0.1-0.6 in the period from 1 to 7 days after tMCAo showing any neurological deficit. Twenty-four hours after tMCAo, vehicle-treated rats showed a clear neurological deficit with a neurological score of 13±1.2 (mean±SEM) that defines a severe injury. The neurological impairment spontaneously recovered up to 7 days after tMCAo. Five days after tMCAo the neurological score was reduced to 10.2±2.5, seven days after tMCAo, the neurological score was further reduced to 7±1.5. Chronic treatment with the A_{2A}/A_{2B} receptor agonist, MRS3997, at the dose of 0.1 mg/kg, significantly reduced the neurological deficit at day 1 and 5 after tMCAo. Repeated Measures two-way ANOVA calculated for the two factors: treatment and time after tMCAo, showed that treatment factor ($F_{2,11}$= 34.75; $p<0.0001$), time factor ($F_{2,22}$=42.58; $p<0.0001$) and interaction between treatment and time ($F_{4,22}$=12.05; $p<0.0001$) were statistically significant. The Tukey *post-hoc* test indicated that sham-operated rats had a neurological score significantly different from vehicle-treated and MRS3997-treated rats at each time point (1, 5, 7 days after tMCAo, $p<0.0001$). The chronic treatment with MRS3997 significantly reduced the neurological deficit with respect to vehicle-treated rats at 1 and 5 days ($p<0.002$-0.02) after tMCAo.

Twenty-four hours after the surgery, sham-operated rats were not affected in weight, then they increased in body weight as evaluated up to 7 days after tMCAo. Vehicle-treated rats lost 26.3±10.1 g 1 day after tMCAo, 42±18 g 5 days after tMCAo and 27±19.3 g 7 days after tMCAo (Figure 39 B). Treatment with the A_{2A}/A_{2B} receptor agonist, MRS3997, did not modify the body weight loss at any time point after tMCAo respect to vehicle-treated rats. Repeated Measures two-way ANOVA, calculated for the two factors: treatment and time after tMCAo, showed that treatment factor ($F_{2,9}$=6; $p<0.02$), time factor ($F_{2,18}$=6.5; $p<0.007$) and the interaction between treatment and time ($F_{4,18}$=8;

p<0.0007) were statistically significant. The Tukey *post-hoc* test indicated that in sham-operated rats, body weight was different with respect to vehicle-treated rats 1, 5 and 7 days after tMCAo (p<0.02).

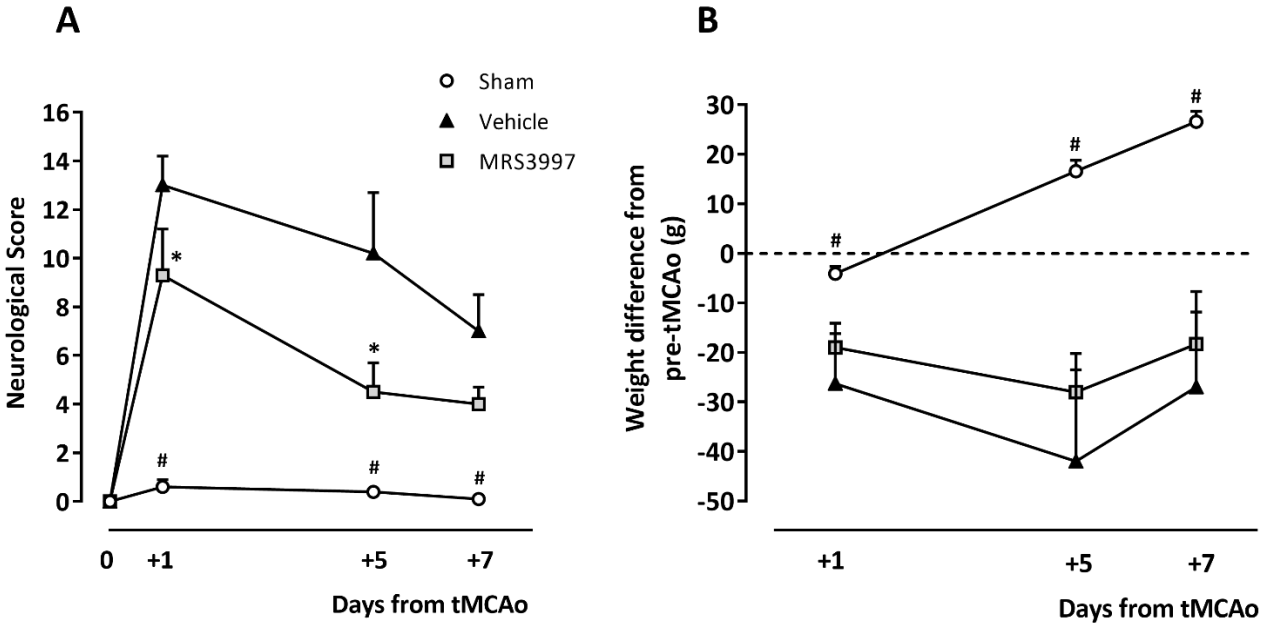

Figure 39. Effect of MRS3997 (0.1 mg/kg, i.p.) on neurological deficit and body weight. **(A)** The score of mNSS test was evaluated before and 1, 5 and 7 days after tMCAo. Statistical significance was evaluated by Repeated Measures two-way ANOVA followed by Tukey *post-hoc* test. Significativity: #at least p < 0.01, sham vs MRS3997- and vehicle-treated rats; *at least p < 0.02, MRS3997- vs. vehicle-treated rats. **(B)** The body weight was calculated as the difference in g of the rat's body weight at each time point minus its own pre-operation body weight. Significativity: #at least p < 0.02, sham vs. vehicle-treated rats, Repeated Measures two-way ANOVA followed by Tukey *post-hoc* test.

3.2. Effect of treatment with MRS3997 on brain ischemic damage after tMCAo

Figure 40 shows the extent of the ischemic damage evaluated as infarct area (Figures 40 A,B) and infarct volume (Figures 40 C,D) in the striatum and cortex of vehicle- and MRS3997-treated rats 7 days after tMCAo. Chronic treatment with MRS3997, at the dose of 0.1 mg/kg, significantly reduced the infarct area and volume in the ischemic cortex and tended to reduce the volume of the ischemic striatum. In MRS3997-treated rats the striatal and cortical infarct volumes were reduced by 25.2% and 14.4%, respectively (*p<0.02 vs. vehicle-treated rats, unpaired Student's t-test: Figures 40 C,D). Sham rats did not show any damage both in the striatum and in the cortex (not shown).

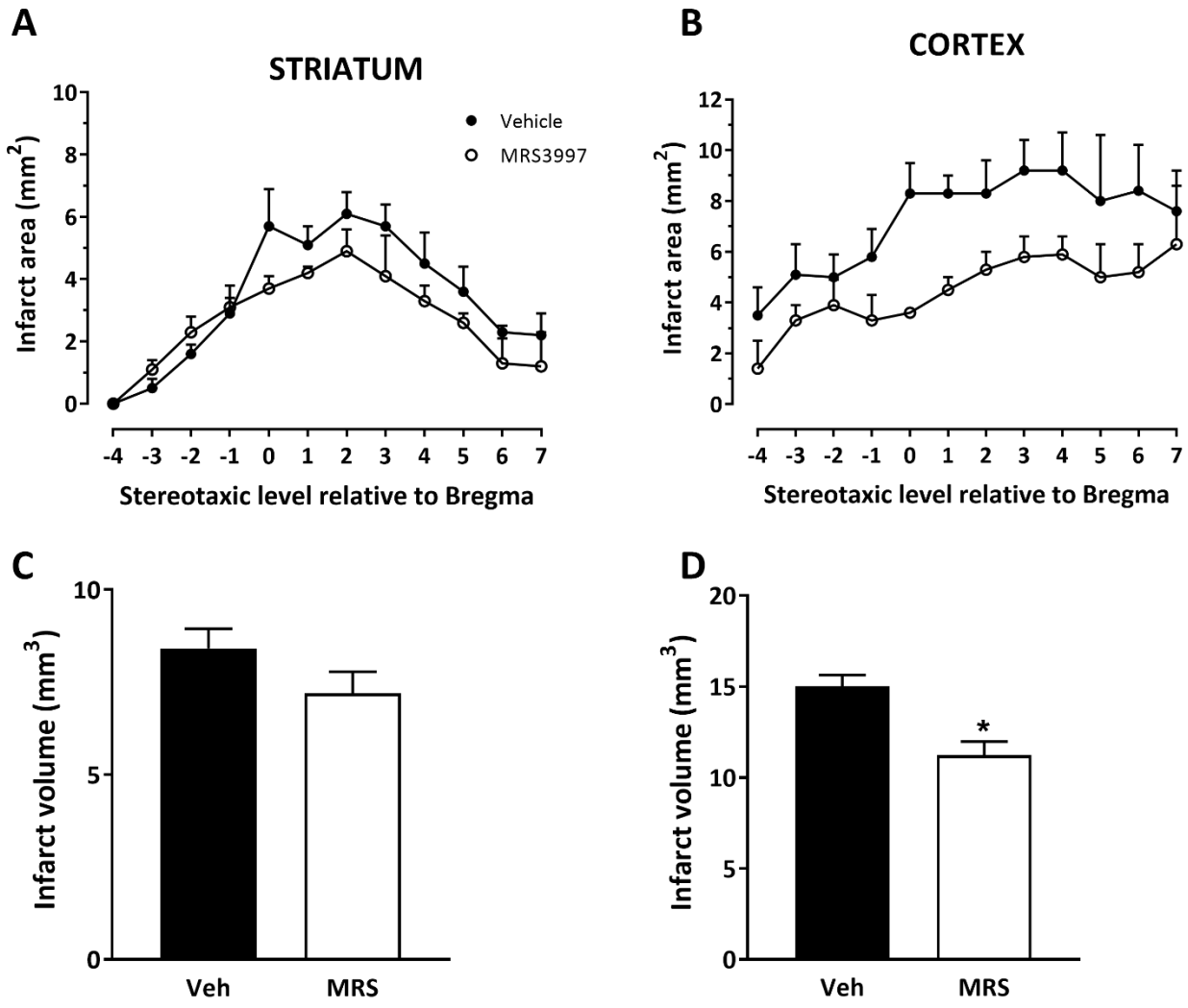

Figure 40. Effect of chronic treatment with MRS3997 (0.1 mg/kg i.p.) on infarct area and infarct volume in the striatum and cortex 7 days after tMCAo. **(A,B)** Data represent infarct area measured at 12 coronal levels between AP: +2.2 mm to −2.0 mm from Bregma of vehicle- (n = 3) and MRS3997-treated rats (n = 3) (König et al., 1964). **(C,D)** Graphs show the infarct volume calculated in the striatum and cortex as mean ± SEM of 3 rats per group. Significativity: *p < 0.02.

Discussion.

Section I: Carbonic anhydrase inhibitors (CAIs) for the management of cerebral ischemia

In this thesis it is reported that ACTZ and two different sulphonamide CAIs, AN11-740 and AN6-277, protect from a strong neuron depolarization induced in a "simil-ischemia" model in hippocampal slices. Furthermore, ACTZ and the highly lipophilic compound AN11-740, systemically and sub-chronically administered in the rat, reveal highly protective also from *in vivo* ischemia i.e. against neurological deficit and against the ischemic damage to all neural cells induced by 24-hour pMCAo.

In agreement with previous results (Frenguelli et al., 2007; Pugliese et al., 2007; Pugliese et al., 2006), a severe OGD, 30 min long, induced the appearance of the electrophysiological phenomenon of AD recorded in all the hippocampal slices. AD is an early and critical event after ischemia, demonstrated both *in vivo* (Somjen, 2001) and *in vitro* (Pugliese et al., 2006; Tanaka et al., 1997) and consists in a robust neuronal depolarization seen as a negative d.c. shift of membrane potential. AD triggers a broad range of molecular events which lead to cell death and represents an unequivocal sign of neuronal injury (Somjen, 2001), as indicated by the fact that after its appearance, the evoked field potential was permanently lost (Pugliese et al., 2007; Pugliese et al., 2006). Recurring peri-infarct depolarization arises at the border of the ischemic core during the first 3–4 h poststroke (Back et al., 1994; Nallet et al., 1999; Nedergaard et al., 1986; Röther et al., 1996; Strong et al., 1996). It is well established that sustained activation of NMDA-type glutamate receptors is essential to AD initiation and propagation leading to excitotoxic neuronal death in stroke (Somjen, 2001). Therefore, it is well accepted that a pharmacological treatment that postpones the onset of a peri-infarct depolarization helps to protect brain tissue after ischemia (Jarvis et al., 2001; Somjen, 2001).

The maintenance of pH homeostasis in the CNS is pivotal for neurotransmission mechanisms, and variations from this homeostasis are crucial for processes underlying a spectrum of pathological conditions including ischemia (Obara et al., 2008). Indeed, under ischemia, the lack of oxygen caused by hypoxia leads to a switch from aerobic to anaerobic glucose metabolism, characterized by increased production of lactic acid and a lowered intracellular pH (Swietach et al., 2007) in neurons and glial cells (Obara et al., 2008).

The CA isoforms IV, IX and XII, all extracellular and membrane-bound enzymes, are highly expressed in glial cells (Svichar et al., 2006; Tong et al., 2000) and contribute to pH homeostasis, both in physiological and pathological conditions. Astrocytes have a key role in pH regulation in the brain (Bélanger & Magistretti, 2009). Glial CA converts neuron derived CO_2 to bicarbonate and protons, which are extruded of the glial cell by a $Na^+/HCO3^-$ cotransporter and monocarboxylate transporters

(Deitmer et al., 2019). Extracellularly, CA is pivotal in buffering extracellular pH by recycling CO_2 in bicarbonate and protons (Tong et al., 2000).

The evidence that hypoxic microenvironments elicit the expression of specific isoforms of CA (in particular CA IX and CA XII) through the hypoxia inducible factor (Wykoff et al., 2000) stresses the relevance of CA in ischemia and supports the importance of CAIs as protective drugs after ischemia. CAIs by reducing H^+ concentration and thus contributing to the pH homeostasis (Pettersen et al., 2015; Supuran, 2008) can protects from damage especially neurons, which are particularly sensitive to pH decrease (Obara et al., 2008). Acidosis is also known to augment the vulnerability of glia to injury induced by OGD (Giffard et al., 1990), and intracellular lactate-induced acidification of astrocytes is reduced in the presence of a non-specific CAI (Tong et al., 2000). Changes in the intracellular pH may affect neurotransmitters release, since lowering of pH results in increased release of dopamine (Cannizzaro et al., 2003; Pittaluga et al., 2005), noradrenaline and serotonin from rat brain synaptosomes (Pittaluga et al., 2005). Glial acidosis has been shown to trigger glial glutamate release and neuronal cell death (Beppu et al., 2014), and it is well established that sustained activation of NMDA-type glutamate receptors is essential in leading to early excitotoxic neuronal death in stroke (Somjen, 2001). Results demonstrating that, in hippocampal OGD slices, CAIs significantly delayed the AD, a phenomenon closely related to cell damage and death (Pugliese et al., 2006) and strictly dependent on NMDA receptor activation, strongly supports the assertion that CAIs, by reestablishing H^+ concentration during ischemia and reducing the ensuing excitatory amino acid efflux, protect from glutamate-induced early excitotoxic damage.

Evidence that CAIs are protective in an *in vitro* model of ischemia from an acute OGD-induced depolarization support the fact that CAIs protect *in vivo* from the functional and tissue damage because of a direct central effect in decreasing excitotoxicity and precocious brain depolarization. CAIs by reestablishing H^+ concentration and thus contributing to the pH homeostasis during ischemia (Mishra et al., 2020; Neri & Supuran, 2011; Supuran, 2008) can protect from ischemic injury by reducing excitatory amino acid outflow and therefore the participation of glutamate in triggering the AD. Moreover, since multiple CA isoforms are expressed in cerebral arteries, CAIs, by decreasing intracellular acidosis, may protect the ischemia-induced BBB breakdown in the cerebrovascular wall during MCAo. Indeed, it has been found that ACTZ (100 µM) reduces the rate of intracellular acidification in the cerebrovascular wall of isolated rat middle cerebral arteries (Rasmussen & Boedtkjer, 2018) and that CAIs decrease hypoxic-mediated brain vascular leakage in a rat model of high-altitude sickness (Gao et al., 2007; Lisk et al., 2013).

Although one of the most likely mechanisms by which CAIs can be protective in brain ischemia is the reduction of hydrogen ions and thus the maintenance of pH homeostasis, protection by CAIs may also be related to different effects. CAIs could mitigate the brain damages induced by cerebral ischemia by reducing brain edema. The presence of edema is a key aggravating factor in ischemic stroke, since brain volume is limited by the rigidity of the skull, so that even little volume increase lead to high intracranial pressure and to compression of the nervous tissue and vasculature (Klatzo, 1985). Rodent brains that have undergone MCAo develop brain edema and therefore swelling during the acute and subacute phases of ischemic injury (Katzman et al., 1977). Gao et al. (2007) demonstrated that subdural infusion of CA in rats increases cerebral vascular permeability, suggesting that it might have relevance in brain edema. In agreement, intracaudate injection of CA increases brain water content and neuronal death, whereas intracerebral injection of ACTZ (5 µL, 1 mM) reduces brain edema, neuronal death and neurological deficit 24 hours after intracerebral hemorrhage in Sprague–Dawley rats (Guo et al., 2012). ACTZ is also known to reduce the permeability of the predominant water channel in the brain, aquaporin-4, known to be involved in cerebral edema (Hladky & Barrand, 2016; Kamegawa et al., 2016; Papadopoulos & Verkman, 2007). The reduction of brain edema after ischemia could also be due to the diuretic action of CAIs (Supuran, 2018b). Currently, CAIs are clinically used to reduce body fluid volume in pathologies like glaucoma (Supuran et al., 2019), idiopathic intracranial hypertension (Uldall et al., 2017), congestive heart failure-induced or drug-induced edema (Bua et al., 2019), and to prevent high-altitude cerebral edema (Davis & Hackett, 2017). CAIs, by regulating the volume of body fluids and thus brain water content, may therefore alleviate cerebral edema, which contributes to poor outcomes in ischemic strokes (Dostovic et al., 2016; Klatzo, 1985).

CAIs may be protective in ischemic stroke also by regulating the vascular tone, leading to vasodilatation of cerebral arterioles and thus to increased cerebral blood flow and oxygen supply (Domoki et al., 2008; Vorstrup et al., 1984). Indeed, CAIs have been related to increased production of NO, a vasodilator molecule (Tuettenberg et al., 2001). Even if, in cerebral circulation, the vasodilating effect of ACTZ appears independent of NO (Kiss et al., 1999), its vasodilator effect might be mediated by vascular calcium-activated potassium channel activation (Pickkers et al., 2001). Moreover, ACTZ inhibits vasoconstriction during intracellular acidification, as occurs during cerebral ischemia (Rasmussen & Boedtkjer, 2018). This is in agreement with a recent paper in which ACTZ, used as a collateral therapeutic to promote cerebral arteriolar vasodilation, decreased the infarct size 24 hours after tMCAo in the rat (Beretta et al., 2017). Since emerging data suggest that vasodilatation of arterioles and the microvasculature may contribute to recruitment of collaterals (Chan et al., 2016; Lin et al., 2020) and it is known that patients with good collateral status have better outcome compared to

those who have poor collateral status (Kim et al., 2018), recruitment of collateral circulation to perfuse the ischemic region could be clinically useful.

Interestingly, 24 hours after pMCAo, the pro-inflammatory cytokine TNF-α is increased while levels of IL-10, a regulatory cytokine with an anti-inflammatory action, is reduced in the plasma of vehicle-treated rats as compared to sham-operated rats. This is in agreement with previous results that have reported an increase of TNF-α in the peripheral plasma 6 and 24 hours after pMCAo in mice (Clausen et al., 2017) and 7 days after tMCAo in the rat (Dettori et al., 2018). Significant increases of TNF-α serum levels were also found in patients with acute stroke, reaching the peak values on day 7 (Intiso et al., 2004) in comparison with controls. Modifications of inflammatory markers in the blood likely reflect cytokines that are produced in the brain and that reach the systemic circulation. On their turn, peripheral inflammatory cytokines might contribute to control damage after stroke. Precise cellular-molecular mechanisms underlying are unclear, but likely reflect a vicious circle responsible of endothelial cell and BBB permeability changes bringing to blood cell infiltration and further brain damage (what is called the secondary damage after stroke). Thus, a TNF-α increase and an IL-10 decrease in the systemic circulation represent valuable markers over time of an ischemic "secondary damage" due to the production of inflammation mediators following an ischemic event (Jickling & Sharp, 2011). Treatment with ACTZ and AN11-740 did neither modify TNF-α or IL-10 levels in the plasma. Likely, a 24 hours after stroke time lapse is too early to appreciate a protective effect of the CAIs from a later ischemic "secondary damage".

In conclusion, data indicate that CAIs could represent an innovative pharmacological tool for the treatment of cerebral ischemia, and may complement t-PA-based therapy in its therapeutic time-window. Although ACTZ is able to reach the nervous tissue (Hanson et al., 1981), more lipophilic CAIs such as AN11-740 could be particularly relevant for clinical translatability, because crossing the BBB soon after ischemia may induce early neuroprotection (Dettori et al., 2021). Drug design studies of isoform-selective CAIs able to easily cross the BBB are recommended in order to develop more effective pharmacological agents. However, the main limitation at present is poor understanding of the differential role/s of various brain CA isoforms in this pathology, as there are at least 9 CAs present in the brain. Further and more detailed pharmacological studies are needed to assess if CAIs can be protective at a later time after ischemia induction.

Section II: The adenosine A_{2B} receptor agonist, BAY60-6583, is protective in a rat model of transient cerebral ischemia

Results demonstrate that the selective adenosine A_{2B} receptor agonist, BAY60-6583, chronically and systemically administered improves the neurological deficit, reduces the ischemic brain damage, counteracts the neurodegeneration and microglia activation and tends to protect from astrocyte death 7 days after tMCAo. Moreover, BAY60-6583 reduces blood cell infiltration in ischemic areas two days after tMCAo.

The chronic treatment with BAY60-6583 at the dose of 0.1 mg/kg protects from the neurological deficit as evaluated up 7 days after tMCAo, but does not protect ischemic rats from the body weight loss. On this regard it is worth mentioning that adenosine released from fat cells in adipose tissue (Capogrossi et al., 1986; Schwabe et al., 1975) participates in the regulation of adipocyte function (Eisenstein et al., 2014). In particular, stimulation of A_{2B} receptor inhibits adipogenesis (Gharibi et al., 2012; Gnad et al., 2020). This effect might account for the lack of the chronic treatment with BAY60-6583 in protecting from the body weight loss.

BAY60-6583 reduces significantly the volume of the ischemic infarct both in the cortex and striatum. Immunohistochemical analysis of neurons, in the striatum of ischemic rats, 7 days after tMCAo induction revealed remarkable alterations in comparison with sham-operated rats. We found a significant decrease of total neuron density and a significative increment of the low-density nuclei (LDN) neurons. Fusco et al. (2018) characterized LDN neurons as damaged and apoptotic neurons, a clear sign of an ischemic insult. BAY60-6583 significantly prevented the loss of neurons and reduced the percent of LDN neurons on total neurons, a clear sign of its protective role against neurodegeneration. Indeed, seven days after ischemia, H&E staining shows that chronic treatment with BAY60-6583 has reconstituted the cortex and striatum cytoarchitecture and has decreased heterochromatic small nuclei in the ischemic area. Melani et al. (2014) reported that 7 days after ischemia the heterochromatic small nuclei belong to activated astrocytes and microglia. It is today well known that after an ischemic event, overactivation of microglia is detrimental and these resident immune cells are involved in the neuroinflammation process (Block et al., 2007; Kriz, 2006; Schwartz, 2003). Seven days after ischemia, the chronic treatment with BAY60-6583 definitely reduced the morphological features of microglia activation (i.e. the amoeboid state). Adenosine A_{2B} receptor is expressed on microglia and their stimulation reduces TNF-α levels in primary microglia cultures (Merighi et al., 2015) and augments the release of IL-10 by murine microglial cells (Koscsó et al., 2012). Moreover, adenosine A_{2B} receptor is also expressed on astrocytes, where its levels increase 24 hours after transient ischemia (Li et al., 2017). The chronic treatment with BAY60-6583 tended to decrease

the loss of astrocytic cells. In a model *in vitro* of hypoxia/reoxygenation, A_{2B} receptor stimulates the increase of IL-6 from astrocytes (Maeda et al., 1994) and it was demonstrated that IL-6 exerts neuroprotective actions in a model of permanent MCAo (Loddick et al., 1998). Interestingly, analysis of astrocytes gave an unexpected result: after ischemia, astrocytes density decreased and their morphology was altered with shorter and thinner branches, suggesting a clasmatodendrotic process. Astrocyte clasmatodendrosis is induced *in vitro* by mild acidosis, a microenvironmental condition commonly associated with aging (Mercatelli et al., 2016) and ischemia (Hulse et al., 2001). BAY60-6583 shows a tendency to revert this effect. Further analyses are necessary to better investigate this phenomenon. On the all, the results support that the protective effect of chronic BAY60-6583 treatment along the days after ischemia can be attributed to direct agonism of A_{2B} receptor located on rat microglial cells and on astrocytes cells.

Besides microglia and astrocytes, A_{2B} receptor is present also on endothelial cells, where their expression increases after transient ischemia (Li et al., 2017). It has been reported that stimulation of A_{2B} receptor reduces vascular leak in a model of hypoxia in mice (Eckle et al., 2008). After ischemia, it is known that damage evolves in time and space (Dirnagl et al., 1999) and it is today accepted that after a first excitotoxic damage, a secondary damage characterized by neuroinflammation further damages the brain tissue. In the days after ischemia, activated blood cells infiltrate brain tissue and by secreting cytotoxic factors contribute to further enlarge the ischemic damage. A_{2B} receptors localized on blood immune cells, i.e. neutrophils and lymphocytes (Eckle et al., 2008; Gessi et al., 2005) exert anti-inflammatory actions, inhibiting vascular adhesion (Yang et al., 2006) and inflammatory cells migration (Konrad et al., 2012; Wakai et al., 2001).

The observation that 2 days after tMCAo the A_{2B} receptor agonist, BAY60-6583, has significantly reduced granulocyte infiltration in cortex, supports that A_{2B} receptor activation on endothelial and blood cells is involved in counteracting neutrophils infiltration and then inflammation of brain parenchyma. In agreement A_{2B} receptor KO mice, exposed to hypoxia, exhibit increased neutrophils infiltration in the brain (Eckle et al., 2008, 2007).

Interestingly, seven days after ischemia an inflammatory parameter such as TNF-α is increased in the peripheral plasma and the chronic administration of the adenosine A_{2B} receptor agonist, BAY60-6583, clearly reduced TNF-α in the plasma. Such reduction might reflect a central effect of BAY60-6583 that reduces pro-inflammatory cytokine produced in the brain and reaching peripheral blood. It is also possible that TNF-α reduction in the plasma reflect a peripheral mechanism of action of BAY60-6583 that reduces neutrophils activation. On the other hand, stroke and inflammation are strictly interrelated. Brain ischemia induces profound inflammatory changes in the periphery (Szigeti et al.,

2015). Inflammatory changes in peripheral organs (especially the lungs and the gut) as early as 2 hours after tMCAo in mice, has been detected by whole-body-SPECT-based imaging protocol (Szigeti et al., 2015). Such peripheral inflammatory changes on their turn might contribute to a worse recovery after stroke. These results stress the key research questions of the predictive value of blood biomarkers in stroke.

A previous study reported that intravenous treatment with the selective A_{2B} receptor agonist BAY60-6583 (1 mg/kg), at the start of reperfusion after brain ischemia induced by tMCAo, 24 hours later reduced the lesion volume, attenuated brain swelling and BBB disruption. In the presence of t-PA (administered after ischemic stroke to dissolve intravascular clots), BAY60-6583 also reduced t-PA induced hemorrhages after ischemia (Li et al., 2017). The neurovascular protection afforded by BAY60-6583 was related to stimulation of the inhibitor of matrix metalloproteinase-1 (TIMP-1), to inhibition of t-PA-induced MMP activation, and prevention of tight junction protein degradation. Since MMPs by degradating extracellular matrix complex and tight junction proteins on endothelial cells (Tsuji et al., 2005; Yepes et al., 2009) can increase BBB permeability, protection afforded by A_{2B} receptor agonist after MCAo was largely attributed to protection of the endothelium.

In conclusion, the results presented points toward the possibility that stimulation of adenosine A_{2B} receptors located on central neural cells, on vascular cells and on blood cells attenuate neuroinflammation that develops days after ischemia, and suggest that BAY60-6583, by controlling a secondary inflammatory damage, represents a new interesting target after brain ischemia. Importantly, A_{2B} receptor agonists might be adjuvant to the today accepted pharmacological t-PA administration, a promising strategy for decreasing the risk of hemorrhages during treatment for ischemic stroke.

Section III: Preliminary results on the protective effect of the dual A_{2A}/A_{2B} receptor agonist, MRS3997, in a rat model of transient cerebral ischemia

Preliminary results demonstrate that the mixed adenosine A_{2A}/A_{2B} receptor agonist, MRS3997, chronically and systemically administered improves the neurological deficit and reduces the ischemic brain damage 7 days after tMCAo.

The chronic treatment with MRS3997 at the dose of 0.1 mg/kg protects from the neurological deficit 1 and 5 days after tMCAo, reduced the volume of the ischemic infarct in the cortex and tended to reduce the infarct volume in the ischemic striatum.

To date, no data are present in the literature regarding dual adenosine receptor ligands for the treatment of cerebral ischemia. MRS3997 has a high potency for both A_{2A} and A_{2B} adenosine receptors, and a low affinity for A_1 and A_3 receptor subtypes (Gao et al., 2014; Adachi et al., 2007).

Both A_{2A} (Melani et al., 2014) and A_{2B} (Dettori et al., 2020) receptor agonists proved protection *in vivo* against cerebral ischemia 7 days after tMCAo, when a defined neuroinflammation occurs.

However, the *in vivo* administration of a dual A_{2A}/A_{2B} receptor agonist, could offer several advantages respect to the simultaneous administration of the two A_{2A} and A_{2B} receptor agonists, CGS21680 and BAY60-6583, respectively. A mixed agonist may reach a uniformly distributed concentration in the plasma and in the tissues, respect to the two compound administered separately. This would guarantee the activation of both receptor subtypes. Furthermore, unlike BAY60-6583, which is a partial A_{2B} receptor agonist (Hinz et al., 2014), MRS3997 acts as a full agonist on A_{2B} receptors, thus offering the possibility of a correct interpretation of results. The compound MRS3997 does not present labile groups, and it has an increased half-life in comparison to CGS21680 and BAY60-6583. All these characteristics render the mixed A_{2A}/A_{2B} receptor agonist MRS3997 an interesting drug for the treatment of cerebral ischemia.

In this thesis it is reported that the adenosine A_{2A}/A_{2B} receptor agonist MRS3997, administered systemically and chronically, significantly decreased the infarct volume of the ischemic cortex and tended to reduce the volume of the ischemic infarct in the striatum 7 days after transient ischemia. This protective effect might be due to a synergic effect of A_{2A} and A_{2B} receptors, acting at peripheral and central level. Indeed, activation of central A_{2A} receptors is known to increase expression and release of neurotrophic factors (Sebastião & Ribeiro, 2009) such as NGF from microglia (Heese et al., 1997), BDNF from mice hippocampal neurons (Tebano et al., 2008) and from rat cortical neurons (Jeon et al., 2011). Moreover, selective activation of A_{2A} receptors directly on blood cells inhibits pro-inflammatory responses (Haskó et al., 2000; Lappas et al., 2006; Sitkovsky, 2003).

Centrally, A_{2B} receptors activation located on glial cells reduces TNF-α levels in primary microglia cultures (Merighi et al., 2015), augments the release of IL-10 by murine microglial cells (Koscsó et al., 2012), and stimulates the increase of IL-6 from astrocytes (Maeda et al., 1994). Moreover, A_{2B} receptor stimulation reduces vascular leak in a model of hypoxia in mice (Eckle et al., 2008).

Further experiments are needed to assess whether MRS3997 counteract neurodegeneration and neuroinflammation in ischemic areas. However, the simultaneous activation of A_{2A} and A_{2B} receptors located both on brain cells and on vascular endothelial and blood cells might potentially be more protective than A_{2A} and A_{2B} receptor agonists adminstered individually.

During the treatment of multifactorial pathologies such as neurodegenerative diseases and cerebral ischemia, the administration of several drugs in order to simultaneously act on multiple biological targets (combination therapy) often occurs. However, multi-target drugs, i.e. single molecules able to act on multiple targets and biochemical pathways, have recently been developed. These compounds seem to have more therapeutic potential. Indeed, a single drug having several pharmacological actions offers multiple advantages with respect to combination therapies, such as the absence of simultaneous administration of different active principles with different bioavailability, pharmacokinetic and metabolism. Moreover, a single drug would simplify the treatment regimen of patients after stroke. Therefore, a multi-target approach could be useful for the treatment of a complex pathology like brain ischemia.

References.

Abbasi, V., Salimi, M., & Amani, F. (2017). The effect of acetazolamide on intracerebral hemorrhage in stoke patients. *International Journal of Advances in Medicine*, *4*(1), 148–151. https://doi.org/10.18203/2349-3933.ijam20170099

Abbracchio, M. P., Brambilla, R., Ceruti, S., Kim, H. O., Von Lubitz, D. K. J. E., Jacobson, K. A., & Cattabeni, F. (1995). G protein-dependent activation of phospholipase C by adenosine A3 receptors in rat brain. *Molecular Pharmacology*, *48*(6). https://doi.org/10.1016/1043-6618(95)86932-8

Abdelrahman, A., Yerande, S. G., Namasivayam, V., Klapschinski, T. A., Alnouri, M. W., El-Tayeb, A., & Müller, C. E. (2020). Substituted 4-phenylthiazoles: Development of potent and selective A1, A3 and dual A1/A3 adenosine receptor antagonists. *European Journal of Medicinal Chemistry*, *186*. https://doi.org/10.1016/j.ejmech.2019.111879

Adachi, H., Palaniappan, K. K., Ivanov, A. A., Bergman, N., Gao, Z. G., & Jacobson, K. A. (2007). Structure-activity relationships of 2,N6,5'-substituted adenosine derivatives with potent activity at the A2B adenosine receptor. *Journal of Medicinal Chemistry*, *50*(8). https://doi.org/10.1021/jm061278q

Aggarwal, M., Kondeti, B., & McKenna, R. (2013). Anticonvulsant/antiepileptic carbonic anhydrase inhibitors: A patent review. *Expert Opinion on Therapeutic Patents*, Vol. 23, pp. 717–724. https://doi.org/10.1517/13543776.2013.782394

Albers, G. W., Goldstein, L. B., Hess, D. C., Wechsler, L. R., Furie, K. L., Gorelick, P. B., … Saver, J. L. (2011). Stroke treatment academic industry roundtable (STAIR) recommendations for maximizing the use of intravenous thrombolytics and expanding treatment options with intra-arterial and neuroprotective therapies. *Stroke*, Vol. 42. https://doi.org/10.1161/STROKEAHA.111.618850

Alessandrini, A., Namura, S., Moskowitz, M. A., & Bonventre, J. V. (1999). MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(22). https://doi.org/10.1073/pnas.96.22.12866

Allan, S. M., & Rothwell, N. J. (2001). Cytokines and acute neurodegeneration. *Nature Reviews Neuroscience*, Vol. 2. https://doi.org/10.1038/35094583

Almeida, T., Rodrigues, R. J., De Mendonça, A., Ribeiro, J. A., & Cunha, R. A. (2003). Purinergic P2 receptors trigger adenosine release leading to adenosine A2A receptor activation and facilitation of long-term potentiation in rat hippocampal slices. *Neuroscience*, *122*(1). https://doi.org/10.1016/S0306-4522(03)00523-2

Alnouri, M. W., Jepards, S., Casari, A., Schiedel, A. C., Hinz, S., & Müller, C. E. (2015). Selectivity is species-dependent: Characterization of standard agonists and antagonists at human, rat, and mouse adenosine receptors. *Purinergic Signalling*, *11*(3). https://doi.org/10.1007/s11302-015-9460-9

Ambrósio, A. F., Malva, J. O., Carvalho, A. P., & Carvalho, C. M. (1997). Inhibition of N-, P/Q- and other types of Ca2+ channels in rat hippocampal nerve terminals by the adenosine A1 receptor. *European Journal of Pharmacology*, *340*(2–3). https://doi.org/10.1016/S0014-2999(97)01451-9

Amelio, I., Landré, V., Knight, R. A., Lisitsa, A., Melino, G., & Antonov, A. V. (2015). Polypharmacology of small molecules targeting the ubiquitin-proteasome and ubiquitin-like systems. *Oncotarget*, *6*(12). https://doi.org/10.18632/oncotarget.3917

Anderson, C. M., Xiong, W., Young, J. D., Cass, C. E., & Parkinson, F. E. (1996). Demonstration of the existence of mRNAs encoding N1/cif and N2/cit sodium/nucleoside cotransporters in rat brain. *Molecular Brain Research*, *42*(2). https://doi.org/10.1016/S0169-328X(96)00244-6

Anderson, W. W., & Collingridge, G. L. (2007). Capabilities of the WinLTP data acquisition program extending beyond basic LTP experimental functions. *Journal of Neuroscience Methods*. https://doi.org/10.1016/j.jneumeth.2006.12.018

Andersson, M., Usiello, A., Borgkvist, A., Pozzi, L., Dominguez, C., Fienberg, A. A., … Fisone, G. (2005). Cannabinoid action depends on phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa at the protein kinase a site in striatal projection neurons. *Journal of Neuroscience*, *25*(37). https://doi.org/10.1523/JNEUROSCI.1289-05.2005

Andiné, P. (1993). Involvement of adenosine in ischemic and postischemic calcium regulation. *Molecular and Chemical Neuropathology*, *18*(1–2). https://doi.org/10.1007/BF03160021

Angeli, A., & Supuran, C. T. (2018). Treatment of sleep apnea with a combination of a carbonic anhydrase inhibitor and an aldosterone antagonist: a patent evaluation of CA2958110 and IN6616DEN2012. *Expert Opinion on Therapeutic Patents*, *28*(10). https://doi.org/10.1080/13543776.2018.1497160

Angiulli, F., Solesio, M., Debure, L., Cejudo, J., Wisniewski, T., & Fossati, S. (2018). Carbonic Anydrase Inhibitors ameliorate neurovascular dysfunction in a mouse model of cerebral amyloid angiopathy. *Alzheimer's & Dementia*, *14*(7S_Part_24). https://doi.org/10.1016/j.jalz.2018.06.1828

Anighoro, A., Bajorath, J., & Rastelli, G. (2014). Polypharmacology: Challenges and opportunities in drug discovery. *Journal of Medicinal Chemistry*, Vol. 57. https://doi.org/10.1021/jm5006463

Anrather, J., & Iadecola, C. (2016). Inflammation and Stroke: An Overview. *Neurotherapeutics*, Vol. 13. https://doi.org/10.1007/s13311-016-0483-x

Antonioli, L., Csóka, B., Fornai, M., Colucci, R., Kókai, E., Blandizzi, C., & Haskó, G. (2014). Adenosine and inflammation: What's new on the horizon? *Drug Discovery Today*, Vol. 19. https://doi.org/10.1016/j.drudis.2014.02.010

Asahi, M., Wang, X., Mori, T., Sumii, T., Jung, J. C., Moskowitz, M. A., … Lo, E. H. (2001). Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *Journal of Neuroscience*, *21*(19). https://doi.org/10.1523/jneurosci.21-19-07724.2001

Asiedu, M., Ossipov, M., Kaila, K., & Price, T. (2010). Acetazolamide and midazolam act synergistically to inhibit neuropathic pain. *Pain*, *148*(2), 302–308. https://doi.org/10.1016/j.pain.2009.11.015

Aspatwar, A., Tolvanen, M. E. E., Ortutay, C., & Parkkila, S. (2014). Carbonic anhydrase related proteins:

Molecular biology and evolution. *Sub-Cellular Biochemistry*, *75*. https://doi.org/10.1007/978-94-007-7359-2_8

Astrup, J., Siesjö, B. K., & Symon, L. (1981). Thresholds in cerebral ischemia — the ischemic penumbra. *Stroke*, Vol. 12. https://doi.org/10.1161/01.STR.12.6.723

Atack, J. R., Shook, B. C., Rassnick, S., Jackson, P. F., Rhodes, K., Drinkenburg, W. H., … Megens, A. A. H. P. (2014). JNJ-40255293, a novel adenosine A2A/A1 antagonist with efficacy in preclinical models of Parkinson's disease. *ACS Chemical Neuroscience*, *5*(10). https://doi.org/10.1021/cn5001606

Back, T, Kohno, K., & Hossmann, K. (1994). Cortical negative DC deflections following middle cerebral artery occlusion and KCl-induced spreading depression: Effect on blood flow, tissue oxygenation, and electroencephalogram. *Journal of Cerebral Blood Flow and Metabolism*, *14*(1), 12–19. https://doi.org/10.1038/jcbfm.1994.3

Back, Tobias, Hoehn, M., Mies, G., Busch, E., Schmitz, B., Kohno, K., & Hossmann, K. A. (2000). Penumbral tissue alkalosis in focal cerebral ischemia: Relationship to energy metabolism, blood flow, and steady potential. *Annals of Neurology*, *47*(4). https://doi.org/10.1002/1531-8249(200004)47:4<485::AID-ANA12>3.0.CO;2-8

Baldwin, S. A., Beal, P. R., Yao, S. Y. M., King, A. E., Cass, C. E., & Young, J. D. (2004). The equilibrative nucleoside transporter family, SLC29. *Pflugers Archiv European Journal of Physiology*, Vol. 447. https://doi.org/10.1007/s00424-003-1103-2

Baldwin, S. A., Mackey, J. R., Cass, C. E., & Young, J. D. (1999). Nucleoside transporters: Molecular biology and implications for therapeutic development. *Molecular Medicine Today*, Vol. 5. https://doi.org/10.1016/S1357-4310(99)01459-8

Barone, F. C., Irving, E. A., Ray, A. M., Lee, J. C., Kassis, S., Kumar, S., … Parsons, A. A. (2001). Inhibition of p38 mitogen-activated protein kinase provides neuroprotection in cerebral focal ischemia. *Medicinal Research Reviews*, Vol. 21. https://doi.org/10.1002/1098-1128(200103)21:2<129::AID-MED1003>3.0.CO;2-H

Barone, Frank C., & Feuerstein, G. Z. (1999). Inflammatory mediators and stroke: New opportunities for novel therapeutics. *Journal of Cerebral Blood Flow and Metabolism*, Vol. 19. https://doi.org/10.1097/00004647-199908000-00001

Becker, K. J. (2002). Anti-leukocyte antibodies: LeukArrest (Hu23F2G) and enlimomab (R6.5) in acute stroke. *Current Medical Research and Opinion*, *18*(SUPPL. 2). https://doi.org/10.1185/030079902125000688

Beckman, J. S. (1996). Oxidative damage and tyrosine nitration from peroxynitrite. *Chemical Research in Toxicology*, *9*(5). https://doi.org/10.1021/tx9501445

Bélanger, M., & Magistretti, P. J. (2009). The role of astroglia in neuroprotection. *Dialogues in Clinical Neuroscience*, Vol. 11, pp. 281–295. https://doi.org/10.31887/dcns.2009.11.3/mbelanger

Beppu, K., Sasaki, T., Tanaka, K., Yamanaka, A., Fukazawa, Y., Shigemoto, R., & Matsui, K. (2014).

Optogenetic countering of glial acidosis suppresses glial glutamate release and ischemic brain damage. *Neuron*, *81*(2), 314–320. https://doi.org/10.1016/j.neuron.2013.11.011

Beretta, S., Versace, A., Carone, D., Riva, M., Dell'Era, V., Cuccione, E., … Ferrarese, C. (2017). Cerebral collateral therapeutics in acute ischemic stroke: A randomized preclinical trial of four modulation strategies. *Journal of Cerebral Blood Flow and Metabolism*, *37*(10). https://doi.org/10.1177/0271678X16688705

Berrino, E., & Carta, F. (2019). Carbonic anhydrase inhibitors for the treatment of epilepsy and obesity. In *Carbonic Anhydrases: Biochemistry and Pharmacology of an Evergreen Pharmaceutical Target* (pp. 311–329). https://doi.org/10.1016/B978-0-12-816476-1.00015-0

Beukers, M. W., Chang, L. C. W., Von Frijtag Drabbe Künzel, J. K., Mulder-Krieger, T., Spanjersberg, R. F., Brussee, J., & Ijzerman, A. P. (2004). New, non-adenosine, high-potency agonists for the human adenosine A 2B receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine. *Journal of Medicinal Chemistry*, *47*(15). https://doi.org/10.1021/jm049947s

Bevan, N., Butchers, P. R., Cousins, R., Coates, J., Edgar, E. V., Morrison, V., … Wilson, D. J. (2007). Pharmacological characterisation and inhibitory effects of (2R,3R,4S,5R)-2-(6-amino-2-{[(1S)-2-hydroxy-1-(phenylmethyl)ethyl]amino} -9H-purin-9-yl)-5-(2-ethyl-2H-tetrazol-5-yl)tetrahydro-3,4-furandiol, a novel ligand that demonstrates both adenosine A2A receptor agonist and adenosine A3 receptor antagonist activity. *European Journal of Pharmacology*, *564*(1–3). https://doi.org/10.1016/j.ejphar.2007.01.094

Biber, K., Fiebich, B. L., Gebicke-Härter, P., & Van Calker, D. (1999). Carbamazepine-induced upregulation of adenosine A1-receptors in astrocyte cultures affects coupling to the phosphoinositol signaling pathway. *Neuropsychopharmacology*, *20*(3). https://doi.org/10.1016/S0893-133X(98)00059-1

Birnbaumer, L. (1992). Receptor-to-effector signaling through G proteins: Roles for βγ dimers as well as α subunits. *Cell*, Vol. 71. https://doi.org/10.1016/S0092-8674(05)80056-X

Block, M. L., Zecca, L., & Hong, J. S. (2007). Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms. *Nature Reviews Neuroscience*, Vol. 8. https://doi.org/10.1038/nrn2038

Boison, D., Chen, J. F., & Fredholm, B. B. (2010). Adenosine signaling and function in glial cells. *Cell Death and Differentiation*, Vol. 17. https://doi.org/10.1038/cdd.2009.131

Bolaños, J. P., & Almeida, A. (1999). Roles of nitric oxide in brain hypoxia-ischemia. *Biochimica et Biophysica Acta - Bioenergetics*, Vol. 1411. https://doi.org/10.1016/S0005-2728(99)00030-4

Borea, P. A., Gessi, S., Bar-Yehuda, S., & Fishman, P. (2009). A3 adenosine receptor: Pharmacology and role in disease. *Handbook of Experimental Pharmacology*, Vol. 193. https://doi.org/10.1007/978-3-540-89615-9_10

Borea, Pier Andrea, Gessi, S., Merighi, S., Vincenzi, F., & Varani, K. (2018). Pharmacology of adenosine

receptors: The state of the art. *Physiological Reviews*, Vol. 98. https://doi.org/10.1152/physrev.00049.2017

Borea, Pier Andrea, Varani, K., Vincenzi, F., Baraldi, P. G., Tabrizi, M. A., Merighi, S., & Gessi, S. (2015). The a3 adenosine receptor: History and perspectives. *Pharmacological Reviews*, *67*(1). https://doi.org/10.1124/pr.113.008540

Borsello, T., Clarkel, P. G. H., Hirt, L., Vercelli, A., Repici, M., Schorderet, D. F., … Bonny, C. (2003). A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nature Medicine*, *9*(9). https://doi.org/10.1038/nm911

Boswell-Casteel, R. C., & Hays, F. A. (2017). Equilibrative nucleoside transporters—A review. *Nucleosides, Nucleotides and Nucleic Acids*, Vol. 36. https://doi.org/10.1080/15257770.2016.1210805

Bot, I., De Vries, H., Korporaal, S. J. A., Foks, A. C., Bot, M., Van Veldhoven, J., … Ijzerman, A. P. (2012). Adenosine A 2B receptor agonism inhibits neointimal lesion development after arterial injury in apolipoprotein E-deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *32*(9). https://doi.org/10.1161/ATVBAHA.112.252924

Bowes, M. P., Rothlein, R., Fagan, S. C., & Zivin, J. A. (1995). Monoclonal antibodies preventing leukocyte activation reduce experimental neurologic injury and enhance efficacy of thrombolytic therapy. *Neurology*, *45*(4). https://doi.org/10.1212/WNL.45.4.815

Bralet, J., Schreiber, L., & Bouvier, C. (1992). Effect of acidosis and anoxia on iron delocalization from brain homogenates. *Biochemical Pharmacology*, *43*(5). https://doi.org/10.1016/0006-2952(92)90602-F

Brand, A., Vissiennon, Z., Eschke, D., & Nieber, K. (2001). Adenosine A1 and A3 receptors mediate inhibition of synaptic transmission in rat cortical neurons. *Neuropharmacology*, *40*(1). https://doi.org/10.1016/S0028-3908(00)00117-9

Braun, N., Lenz, C., Gillardon, F., Zimmermann, M., & Zimmermann, H. (1997). Focal cerebral ischemia enhances glial expression of ecto-5'- nucleotidase. *Brain Research*, *766*(1–2). https://doi.org/10.1016/S0006-8993(97)00559-3

Bridges, A. J., Bruns, R. F., Ortwine, D. F., Priebe, S. R., Szotek, D. L., & Trivedi, B. K. (1988). N6-[2-(3,5-Dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine and Its Uronamide Derivatives. Novel Adenosine Agonists with Both High Affinity and High Selectivity for the Adenosine A2 Receptor. *Journal of Medicinal Chemistry*, *31*(7). https://doi.org/10.1021/jm00402a004

Brodie, C., Blumberg, P. M., & Jacobson, K. A. (1998). Activation of the A(2A) adenosine receptor inhibits nitric oxide production in glial cells. *FEBS Letters*, *429*(2). https://doi.org/10.1016/S0014-5793(98)00556-0

Bruns, R. F., Fergus, J. H., Badger, E. W., Bristol, J. A., Santay, L. A., Hartman, J. D., … Huang, C. C. (1987). Binding of the A1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *335*(1).

https://doi.org/10.1007/BF00165037

Bua, S, Nocentini, A., & Supuran, C. (2019). Carbonic anhydrase inhibitors as diuretics. In *Carbonic Anhydrases: Biochemistry and Pharmacology of an Evergreen Pharmaceutical Target* (pp. 287–309). https://doi.org/10.1016/B978-0-12-816476-1.00014-9

Bua, Silvia, Di Cesare Mannelli, L., Vullo, D., Ghelardini, C., Bartolucci, G., Scozzafava, A., … Carta, F. (2017). Design and Synthesis of Novel Nonsteroidal Anti-Inflammatory Drugs and Carbonic Anhydrase Inhibitors Hybrids (NSAIDs-CAIs) for the Treatment of Rheumatoid Arthritis. *Journal of Medicinal Chemistry*, *60*(3). https://doi.org/10.1021/acs.jmedchem.6b01607

Bulli, I., Dettori, I., Coppi, E., Cherchi, F., Venturini, M., Mannelli, L. D. C., … Pedata, F. (2021). Role of carbonic anhydrase in cerebral ischemia and carbonic anhydrase inhibitors as putative protective agents. *International Journal of Molecular Sciences*, Vol. 22. https://doi.org/10.3390/ijms22095029

Burggraaff, L., Van Vlijmen, H. W. T., Ijzerman, A. P., & Van Westen, G. J. P. (2020). Quantitative prediction of selectivity between the A1 and A2A adenosine receptors. *Journal of Cheminformatics*, *12*(1). https://doi.org/10.1186/s13321-020-00438-3

Burke, S. P., & Nadler, J. V. (1988). Regulation of Glutamate and Aspartate Release from Slices of the Hippocampal CA1 Area: Effects of Adenosine and Baclofen. *Journal of Neurochemistry*, *51*(5). https://doi.org/10.1111/j.1471-4159.1988.tb01123.x

Burnstock, G., B. Fredholm, B., & Verkhratsky, A. (2011). Adenosine and ATP Receptors in the Brain. *Current Topics in Medicinal Chemistry*, *11*(8). https://doi.org/10.2174/156802611795347627

Burtscher, J., Gatterer, H., Faulhaber, M., & Burtscher, M. (2016). Acetazolamide pre-treatment before ascending to high altitudes: Sustained effect on systemic blood pressure during submaximal exercise. *International Journal of Clinical and Experimental Medicine*, *9*(3).

Busa, W. B., & Nuccitelli, R. (1984). Metabolic regulation via intracellular pH. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *15*(4). https://doi.org/10.1152/ajpregu.1984.246.4.r409

Calker, D. van, Müller, M., & Hamprecht, B. (1979). ADENOSINE REGULATES VIA TWO DIFFERENT TYPES OF RECEPTORS, THE ACCUMULATION OF CYCLIC AMP IN CULTURED BRAIN CELLS. *Journal of Neurochemistry*. https://doi.org/10.1111/j.1471-4159.1979.tb05236.x

Canazza, A., Minati, L., Boffano, C., Parati, E., & Binks, S. (2014). Experimental models of brain ischemia: A review of techniques, magnetic resonance imaging, and investigational cell-based therapies. *Frontiers in Neurology*. https://doi.org/10.3389/fneur.2014.00019

Cannizzaro, C., Monastero, R., Vacca, M., & Martire, M. (2003). [3H]-DA release evoked by low pH medium and internal H+ accumulation in rat hypothalamic synaptosomes: Involvement of calcium ions. *Neurochemistry International*, *43*(1), 9–17. https://doi.org/10.1016/S0197-0186(02)00211-5

Cao, J., Viholainen, J. I., Dart, C., Warwick, H. K., Leyland, M. L., & Courtney, M. J. (2005). The PSD95-nNOS

interface: A target for inhibition of excitotoxic p38 stress-activated protein kinase activation and cell death. *Journal of Cell Biology*, *168*(1). https://doi.org/10.1083/jcb.200407024

Capasso, C., & Supuran, C. T. (2015). Bacterial, fungal and protozoan carbonic anhydrases as drug targets. *Expert Opinion on Therapeutic Targets*, Vol. 19. https://doi.org/10.1517/14728222.2015.1067685

Capogrossi, M. C., Di Girolamo, M., Zimring, H., Crandall, D., Francendese, A., & Israili, Z. H. (1986). Adenosine Release from Isolated Rat Adipocytes: Influence of Fat Cell Concentration and Cell Size. *Proceedings of the Society for Experimental Biology and Medicine*, *182*(1). https://doi.org/10.3181/00379727-182-42303

Carbonell, T., & Rama, R. (2007). Iron, Oxidative Stress and Early Neurological Deterioration in Ischemic Stroke. *Current Medicinal Chemistry*, *14*(8). https://doi.org/10.2174/092986707780363014

Carta, F., & Supuran, C. T. (2013). Diuretics with carbonic anhydrase inhibitory action: A patent and literature review (2005-2013). *Expert Opinion on Therapeutic Patents*, Vol. 23. https://doi.org/10.1517/13543776.2013.780598

Carta, F., Supuran, C. T., & Scozzafava, A. (2012). Novel therapies for glaucoma: A patent review 2007 - 2011. *Expert Opinion on Therapeutic Patents*, Vol. 22. https://doi.org/10.1517/13543776.2012.649006

Casey, J. R., Grinstein, S., & Orlowski, J. (2010). Sensors and regulators of intracellular pH. *Nature Reviews Molecular Cell Biology*, Vol. 11. https://doi.org/10.1038/nrm2820

Cerbai, F., Lana, D., Nosi, D., Petkova-Kirova, P., Zecchi, S., Brothers, H. M., … Giovannini, M. G. (2012). The Neuron-Astrocyte-Microglia Triad in Normal Brain Ageing and in a Model of Neuroinflammation in the Rat Hippocampus. *PLoS ONE*, *7*(9). https://doi.org/10.1371/journal.pone.0045250

Chamorro, Á., Dirnagl, U., Urra, X., & Planas, A. M. (2016). Neuroprotection in acute stroke: Targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. *The Lancet Neurology*, Vol. 15. https://doi.org/10.1016/S1474-4422(16)00114-9

Chan, S. L., Sweet, J. G., Bishop, N., & Cipolla, M. J. (2016). Pial Collateral Reactivity during Hypertension and Aging: Understanding the Function of Collaterals for Stroke Therapy. *Stroke*, *47*(6). https://doi.org/10.1161/STROKEAHA.116.013392

Chang, X., Han, J., Zhao, Y., Yan, X., Sun, S., & Cui, Y. (2010). Increased expression of carbonic anhydrase i in the synovium of patients with ankylosing spondylitis. *BMC Musculoskeletal Disorders*, *11*. https://doi.org/10.1186/1471-2474-11-279

Chegwidden, W. R., & Carter, N. D. (2000). Introduction to the carbonic anhydrases. *EXS*. https://doi.org/10.1007/978-3-0348-8446-4_2

Chen, F., Qi, Z., Luo, Y., Hinchliffe, T., Ding, G., Xia, Y., & Ji, X. (2014). Non-pharmaceutical therapies for stroke: Mechanisms and clinical implications. *Progress in Neurobiology*, Vol. 115. https://doi.org/10.1016/j.pneurobio.2013.12.007

Chen, G. Y., & Nuñez, G. (2010). Sterile inflammation: Sensing and reacting to damage. *Nature Reviews*

Immunology, Vol. 10. https://doi.org/10.1038/nri2873

Chen, J., Sanberg, P. R., Li, Y., Wang, L., Lu, M., Willing, A. E., … Chopp, M. (2001). Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke*. https://doi.org/10.1161/hs1101.098367

Choi, D. W. (1990). Possible mechanisms limiting N-methyl-D-aspartate receptor overactivation and the therapeutic efficacy of N-methyl-D-aspartate antagonists. *Stroke*, *21*(11 SUPPL.).

Chopp, M., Li, Y., Jiang, N., Zhang, R. L., & Prostak, J. (1996). Antibodies against adhesion molecules reduce apoptosis after transient middle cerebral artery occlusion in rat brain. *Journal of Cerebral Blood Flow and Metabolism*, *16*(4). https://doi.org/10.1097/00004647-199607000-00007

Chopp, M., Zhang, R. L., Chen, H., Li, Y., Jiang, N., & Rusche, J. R. (1994). Postischemic administration of an anti-mac-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in rats. *Stroke*, *25*(4). https://doi.org/10.1161/01.STR.25.4.869

Choukèr, A., Thiel, M., Lukashev, D., Ward, J. M., Kaufmann, I., Apasov, S., … Ohta, A. (2008). Critical role of hypoxia and A2A adenosine receptors in liver tissue-protecting physiological anti-inflammatory pathway. *Molecular Medicine*, *14*(3–4). https://doi.org/10.2119/2007-00075.Chouker

Ciruela, F., Ferré, S., Casadó, V., Cortés, A., Cunha, R. A., Lluis, C., & Franco, R. (2006). Heterodimeric adenosine receptors: A device to regulate neurotransmitter release. *Cellular and Molecular Life Sciences*, Vol. 63. https://doi.org/10.1007/s00018-006-6216-2

Ciruela, Francisco, Casadó, V., Rodrigues, R. J., Luján, R., Burgueño, J., Canals, M., … Franco, R. (2006). Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. *Journal of Neuroscience*, *26*(7). https://doi.org/10.1523/JNEUROSCI.3574-05.2006

Clark, W. M., Lauten, J. D., Lessov, N., Woodward, W., & Coull, B. M. (1995). The influence of antiadhesion therapies on leukocyte subset accumulation in central nervous system ischemia in rats. *Journal of Molecular Neuroscience*, *6*(1). https://doi.org/10.1007/BF02736758

Clark, W. M., Lessov, N., Lauten, J. D., & Hazel, K. (1997). Doxycycline Treatment Reduces Ischemic Brain Damage in Transient Middle Cerebral Artery Occlusion in the Rat. *Journal of Molecular Neuroscience*, *9*(2). https://doi.org/10.1007/BF02736854

Clausen, B. H., Lundberg, L., Yli-Karjanmaa, M., Martin, N. A., Svensson, M., Alfsen, M. Z., … Lambertsen, K. L. (2017). Fumarate decreases edema volume and improves functional outcome after experimental stroke. *Experimental Neurology*, *295*. https://doi.org/10.1016/j.expneurol.2017.06.011

Connolly, E. S., Winfree, C. J., Springer, T. A., Naka, Y., Liao, H., Yan, S. Du, … Pinsky, D. J. (1996). Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *Journal of Clinical Investigation*, *97*(1). https://doi.org/10.1172/JCI118392

Cooper, A. J. L., & Kristal, B. S. (1997). Multiple roles of glutathione in the central nervous system. *Biological*

Chemistry, Vol. 378.

Coppi, E., Cherchi, F., Fusco, I., Failli, P., Vona, A., Dettori, I., … Pugliese, A. M. (2019). Adenosine A3 receptor activation inhibits pronociceptive N-type Ca2+ currents and cell excitability in dorsal root ganglion neurons. *Pain*, *160*(5). https://doi.org/10.1097/j.pain.0000000000001488

Coppi, E., Dettori, I., Cherchi, F., Bulli, I., Venturini, M., Lana, D., … Pugliese, A. M. (2020). A2b adenosine receptors: When outsiders may become an attractive target to treat brain ischemia or demyelination. *International Journal of Molecular Sciences*, Vol. 21. https://doi.org/10.3390/ijms21249697

Corradetti, R., Lo Conte, G., Moroni, F., Beatrice Passani, M., & Pepeu, G. (1984). Adenosine decreases aspartate and glutamate release from rat hippocampal slices. *European Journal of Pharmacology*, *104*(1–2). https://doi.org/10.1016/0014-2999(84)90364-9

Corti, F., Cellai, L., Melani, A., Donati, C., Bruni, P., & Pedata, F. (2013). Adenosine is present in rat brain synaptic vesicles. *NeuroReport*, *24*(17). https://doi.org/10.1097/WNR.0000000000000033

Costenla, A. R., Lopes, L. V., De Mendonça, A., & Ribeiro, J. A. (2001). A functional role for adenosine A3 receptors: Modulation of synaptic plasticity in the rat hippocampus. *Neuroscience Letters*, *302*(1). https://doi.org/10.1016/S0304-3940(01)01633-0

Crack, P. J., & Taylor, J. M. (2005). Reactive oxygen species and the modulation of stroke. *Free Radical Biology and Medicine*, Vol. 38. https://doi.org/10.1016/j.freeradbiomed.2005.01.019

Cristalli, G., Lambertucci, C., Marucci, G., Volpini, R., & Ben, D. (2008). A2A Adenosine Receptor and its Modulators: Overview on a Druggable GPCR and on Structure-Activity Relationship Analysis and Binding Requirements of Agonists and Antagonists. *Current Pharmaceutical Design*, *14*(15). https://doi.org/10.2174/138161208784480081

Cristóvão-Ferreira, S., Navarro, G., Brugarolas, M., Pérez-Capote, K., Vaz, S. H., Fattorini, G., … Sebastião, A. M. (2013). A1R-A2AR heteromers coupled to Gs and Gi/0 proteins modulate GABA transport into astrocytes. *Purinergic Signalling*, *9*(3). https://doi.org/10.1007/s11302-013-9364-5

Cunha, R. A. (2001). Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: Different roles, different sources and different receptors. *Neurochemistry International*, Vol. 38. https://doi.org/10.1016/S0197-0186(00)00034-6

Cunha, Rodrigo A., & Ribeiro, J. A. (2000). Purinergic modulation of [3H]GABA release from rat hippocampal nerve terminals. *Neuropharmacology*, *39*(7). https://doi.org/10.1016/S0028-3908(99)00237-3

Dai, S.-S., Zhou, Y.-G., Li, W., An, J.-H., Li, P., Yang, N., … Chen, J.-F. (2010). 2010 A - Local glutamate level dictates adenosine A2A receptor regulation of neuroinflammation and traumatic brain injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*. https://doi.org/10.1523/JNEUROSCI.0268-10.2010

Danner, H., & Pfister, C. (1981). [The cytoarchitecture of the rat globus pallidus]. *Journal Fur Hirnforschung*, *22*(1).

Davis, C., & Hackett, P. (2017). Advances in the Prevention and Treatment of High Altitude Illness. *Emergency Medicine Clinics of North America*, Vol. 35, pp. 241–260. https://doi.org/10.1016/j.emc.2017.01.002

de Mendonça, A., Sebastião, A. M., & Ribeiro, J. A. (1995). Inhibition of NMDA receptor-mediated currents in isolated rat hippocampal neurones by adenosine A1 receptor activation. *NeuroReport*, *6*(8). https://doi.org/10.1097/00001756-199505300-00006

Deckert, J., & Jorgensen, M. B. (1988). Evidence for pre- and postsynaptic localization of adenosine A1 receptors in the CA1 region of rat hippocampus: a quantitative autoradiographic study. *Brain Research*, *446*(1). https://doi.org/10.1016/0006-8993(88)91308-X

DeGraba, T. J. (1998). The role of inflammation after acute stroke: Utility of pursuing anti- adhesion molecule therapy. *Neurology*, *51*(3 SUPPL.). https://doi.org/10.1212/wnl.51.3_suppl_3.s62

Deitmer, J., Theparambil, S., Ruminot, I., Noor, S., & Becker, H. (2019). Energy Dynamics in the Brain: Contributions of Astrocytes to Metabolism and pH Homeostasis. *Frontiers in Neuroscience*. https://doi.org/10.3389/fnins.2019.01301

Del Prete, S., Vullo, D., Fisher, G. M., Andrews, K. T., Poulsen, S. A., Capasso, C., & Supuran, C. T. (2014). Discovery of a new family of carbonic anhydrases in the malaria pathogen Plasmodium falciparum - The η-carbonic anhydrases. *Bioorganic and Medicinal Chemistry Letters*, *24*(18), 4389–4396. https://doi.org/10.1016/j.bmcl.2014.08.015

Denes, A., Vidyasagar, R., Feng, J., Narvainen, J., McColl, B. W., Kauppinen, R. A., & Allan, S. M. (2007). Proliferating resident microglia after focal cerebral ischaemia in mice. *Journal of Cerebral Blood Flow and Metabolism*, *27*(12). https://doi.org/10.1038/sj.jcbfm.9600495

Dettori, I., Fusco, I., Bulli, I., Gaviano, L., Coppi, E., Cherchi, F., … Pedata, F. (2021). Protective effects of carbonic anhydrase inhibition in brain ischaemia in vitro and in vivo models. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *36*(1). https://doi.org/10.1080/14756366.2021.1907575

Dettori, I., Gaviano, L., Melani, A., Lucarini, L., Durante, M., Masini, E., & Pedata, F. (2018). A selective histamine H4 receptor antagonist, JNJ7777120, is protective in a rat model of transient cerebral ischemia. *Frontiers in Pharmacology*, *9*(OCT). https://doi.org/10.3389/fphar.2018.01231

Di Cesare Mannelli, L., Micheli, L., Carta, F., Cozzi, A., Ghelardini, C., & Supuran, C. T. (2016). Carbonic anhydrase inhibition for the management of cerebral ischemia: in vivo evaluation of sulfonamide and coumarin inhibitors. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *31*(6), 894–899. https://doi.org/10.3109/14756366.2015.1113407

Di Paola, R., Melani, A., Esposito, E., Mazzon, E., Paterniti, I., Bramanti, P., … Cuzzocrea, S. (2010). Adenosine a2a receptor-selective stimulation reduces signaling pathways involved in the development of intestine ischemia and reperfusion injury. *Shock*, *33*(5). https://doi.org/10.1097/SHK.0b013e3181c997dd

Diógenes, M. J., Fernandes, C. C., Sebastiâo, A. M., & Ribeiro, J. A. (2004). Activation of adenosine A2A receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices. *J Neurosci*. https://doi.org/24/12/2905 [pii] 10.1523/JNEUROSCI.4454-03.2004

Dirnagl, U., Iadecola, C., & Moskowitz, M. (1999). Pathobiology of ischaemic stroke: An integrated view. *Trends in Neurosciences*, Vol. 22, pp. 391–397. https://doi.org/10.1016/S0166-2236(99)01401-0

Dixon, A. K., Gubitz, A. K., Sirinathsinghji, D. J. S., Richardson, P. J., & Freeman, T. C. (1996). Tissue distribution of adenosine receptor mRNAs in the rat. *British Journal of Pharmacology*, *118*(6). https://doi.org/10.1111/j.1476-5381.1996.tb15561.x

Dobolyi, Á., Reichart, A., Szikra, T., Nyitrai, G., Kékesi, K. A., & Juhász, G. (2000). Sustained depolarisation induces changes in the extracellular concentrations of purine and pyrimidine nucleosides in the rat thalamus. *Neurochemistry International*, *37*(1). https://doi.org/10.1016/S0197-0186(99)00162-X

Dobrek, Ł., & Thor, P. (2011). Glutamate NMDA receptors in pathophysiology and pharmacotherapy of selected nervous system diseases. *Postepy Higieny i Medycyny Doswiadczalnej*, Vol. 65. https://doi.org/10.5604/17322693.946637

Domoki, F., Zimmermann, A., Tóth-Szuki, V., Busija, D., & Bari, F. (2008). Acetazolamide induces indomethacin and ischaemia-sensitive pial arteriolar vasodilation in the piglet. *Acta Paediatrica, International Journal of Paediatrics*, *97*(3), 280–284. https://doi.org/10.1111/j.1651-2227.2007.00615.x

Dong, Y., & Benveniste, E. N. (2001). Immune function of astrocytes. *GLIA*, *36*(2). https://doi.org/10.1002/glia.1107

Dostovic, Z., Dostovic, E., Smajlovic, D., Ibrahimagic, O., & Avdic, L. (2016). Brain Edema After Ischaemic Stroke. *Medical Archives (Sarajevo, Bosnia and Herzegovina)*, *70*(5), 339–341. https://doi.org/10.5455/medarh.2016.70.339-341

Doyle, K., Simon, R., & Stenzel-poore, M. (2008). Neuropharmacology – Special Issue on Cerebral Ischemia Mechanisms of Ischemic Brain Damage – Review Article. *Neuropharmacology*, *55*(3).

Drury, A. N., & Szent-Györgyi, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *The Journal of Physiology*, *68*(3). https://doi.org/10.1113/jphysiol.1929.sp002608

Dunwiddie, T. V. (1984). Interactions between the effects of adenosine and calcium on synaptic responses in rat hippocampus in vitro. *The Journal of Physiology*, *350*(1). https://doi.org/10.1113/jphysiol.1984.sp015217

DUNWIDDIE, T. V., & HOFFER, B. J. (1980). ADENINE NUCLEOTIDES AND SYNAPTIC TRANSMISSION IN THE in vitro RAT HIPPOCAMPUS. *British Journal of Pharmacology*, *69*(1). https://doi.org/10.1111/j.1476-5381.1980.tb10883.x

Dunwiddie, Thomas V. (1985). The Physiological Role of Adenosine In The Central Nervous System.

International Review of Neurobiology, *27*(C). https://doi.org/10.1016/S0074-7742(08)60556-5

Dunwiddie, Thomas V., & Masino, S. A. (2001). The Role and Regulation of Adenosine in the Central Nervous System. *Annual Review of Neuroscience*, *24*(1), 31–55. https://doi.org/10.1146/annurev.neuro.24.1.31

Dux, E., Fastbom, J., Ungerstedt, U., Rudolphi, K., & Fredholm, B. B. (1990). Protective effect of adenosine and a novel xanthine derivative propentofylline on the cell damage after bilateral carotid occlusion in the gerbil hippocampus. *Brain Research*, *516*(2). https://doi.org/10.1016/0006-8993(90)90925-2

Eckle, T., Faigle, M., Grenz, A., Laucher, S., Thompson, L. F., & Eltzschig, H. K. (2008). A2B adenosine receptor dampens hypoxia-induced vascular leak. *Blood*, *111*(4). https://doi.org/10.1182/blood-2007-10-117044

Eckle, T., Kewley, E. M., Brodsky, K. S., Tak, E., Bonney, S., Gobel, M., … Eltzschig, H. K. (2014). Identification of Hypoxia-Inducible Factor HIF-1A as Transcriptional Regulator of the A2B Adenosine Receptor during Acute Lung Injury. *The Journal of Immunology*, *192*(3). https://doi.org/10.4049/jimmunol.1100593

Eckle, T., Krahn, T., Grenz, A., Köhler, D., Mittelbronn, M., Ledent, C., … Eltzschig, H. K. (2007). Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. *Circulation*, *115*(12). https://doi.org/10.1161/CIRCULATIONAHA.106.669697

Ekdahl, C. T., Kokaia, Z., & Lindvall, O. (2009). Brain inflammation and adult neurogenesis: The dual role of microglia. *Neuroscience*, Vol. 158. https://doi.org/10.1016/j.neuroscience.2008.06.052

Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology*. https://doi.org/10.1080/01926230701320337

Eltzschig, H. K., Thompson, L. F., Karhausen, J., Cotta, R. J., Ibla, J. C., Robson, S. C., & Colgan, S. P. (2004). Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: Coordination by extracellular nucleotide metabolism. *Blood*, *104*(13). https://doi.org/10.1182/blood-2004-06-2066

Emsley, H. C.A., Smith, C. J., Georgiou, R. F., Vail, A., Hopkins, S. J., Rothwell, N. J., & Tyrrell, P. J. (2005). A randomised phase II study of interleukin-1 receptor antagonist in acute stroke patients. *Journal of Neurology, Neurosurgery and Psychiatry*, *76*(10). https://doi.org/10.1136/jnnp.2004.054882

Emsley, Hedley C.A., & Tyrrell, P. J. (2002). Inflammation and infection in clinical stroke. *Journal of Cerebral Blood Flow and Metabolism*, Vol. 22. https://doi.org/10.1097/01.WCB.0000037880.62590.28

Englert, M., Quitterer, U., & Klotz, K. N. (2002). Effector coupling of stably transfected human A3 adenosine receptors in CHO cells. *Biochemical Pharmacology*, *64*(1). https://doi.org/10.1016/S0006-2952(02)01071-7

Eric Thomas, W. (1992). Brain macrophages: evaluation of microglia and their functions. *Brain Research Reviews*, *17*(1). https://doi.org/10.1016/0165-0173(92)90007-9

F. Pedata, A.M. Pugliese, E. Coppi, P. Popoli, M. Morelli, M. A. Schwarzschild, & A. Melani. (2008). Adenosine in the Central Nervous System: Effects on Neurotransmission and Neuroprotection.

Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry, *7*(4). https://doi.org/10.2174/187152207781369832

Fang, Z. Z., Tosh, D. K., Tanaka, N., Wang, H., Krausz, K. W., O'Connor, R., … Gonzalez, F. J. (2015). Metabolic mapping of A3 adenosine receptor agonist MRS5980. *Biochemical Pharmacology*, *97*(2). https://doi.org/10.1016/j.bcp.2015.07.007

Farkas, E., Pratt, R., Sengpiel, F., & Obrenovitch, T. P. (2008). Direct, live imaging of cortical spreading depression and anoxic depolarisation using a fluorescent, voltage-sensitive dye. *Journal of Cerebral Blood Flow and Metabolism*, *28*(2). https://doi.org/10.1038/sj.jcbfm.9600569

Fausther, M., Lecka, J., Soliman, E., Kauffenstein, G., Pelletier, J., Sheung, N., … Sévigny, J. (2012). Coexpression of ecto-5'-nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *302*(4). https://doi.org/10.1152/ajpgi.00165.2011

Feoktistov, I., & Biaggioni, I. (1998). Pharmacological characterization of adenosine A2B receptors. *Biochemical Pharmacology*, *55*(5). https://doi.org/10.1016/s0006-2952(97)00512-1

Feoktistov, I., Ryzhov, S., Zhong, H., Goldstein, A. E., Matafonov, A., Zeng, D., & Biaggioni, I. (2004). Hypoxia modulates adenosine receptors in human endothelial and smooth muscle cells toward an A2B angiogenic phenotype. *Hypertension*, *44*(5). https://doi.org/10.1161/01.HYP.0000144800.21037.a5

Ferré, S., Karcz-Kubicha, M., Hope, B. T., Popoli, P., Burgueño, J., Gutiérrez, M. A., … Ciruela, F. (2002). Synergistic interaction between adenosine A2A and glutamate mGlu5 receptors: Implications for striatal neuronal function. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(18). https://doi.org/10.1073/pnas.172393799

Ferre, S., Quiroz, C., Woods, A., Cunha, R., Popoli, P., Ciruela, F., … Schiffmann, S. (2008). An Update on Adenosine A2A-Dopamine D2 Receptor Interactions: Implications for the Function of G Protein-Coupled Receptors. *Current Pharmaceutical Design*, *14*(15). https://doi.org/10.2174/138161208784480108

Ferre, Sergi, Von Euler, G., Johansson, B., Fredholm, B. B., & Fuxe, K. (1991). Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(16). https://doi.org/10.1073/pnas.88.16.7238

Fiebich, B. L., Biber, K., Lieb, K., Van Calker, D., Berger, M., Bauer, J., & Gebicke-Haerter, P. J. (1996). Cyclooxygenase-2 expression in rat microglia is induced by adenosine A2a-receptors. *GLIA*, *18*(2). https://doi.org/10.1002/(sici)1098-1136(199610)18:2<152::aid-glia7>3.0.co;2-2

Fink, J. S., Weaver, D. R., Rivkees, S. A., Peterfreund, R. A., Pollack, A. E., Adler, E. M., & Reppert, S. M. (1992). Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Molecular Brain Research*, *14*(3). https://doi.org/10.1016/0169-

328X(92)90173-9

Fontinha, B. M., Diógenes, M. J., Ribeiro, J. A., & Sebastião, A. M. (2008). Enhancement of long-term potentiation by brain-derived neurotrophic factor requires adenosine A2A receptor activation by endogenous adenosine. *Neuropharmacology*. https://doi.org/10.1016/j.neuropharm.2008.01.011

Fossati, S., Giannoni, P., Solesio, M. E., Cocklin, S. L., Cabrera, E., Ghiso, J., & Rostagno, A. (2016). The carbonic anhydrase inhibitor methazolamide prevents amyloid beta-induced mitochondrial dysfunction and caspase activation protecting neuronal and glial cells in vitro and in the mouse brain. *Neurobiology of Disease*, *86*, 29–40. https://doi.org/10.1016/j.nbd.2015.11.006

Fredholm, B. B., & Dunwiddie, T. V. (1988). How does adenosine inhibit transmitter release? *Trends in Pharmacological Sciences*, Vol. 9. https://doi.org/10.1016/0165-6147(88)90194-0

Fredholm, B. B., Ijzerman, A. P., Jacobson, K. A., Klotz, K. N., & Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacological Reviews*, Vol. 53.

FREDHOLM, B. B., LINDGREN, E., LINDSTRÖM, K., & NORDSTEDT, C. (1987). α-Adrenoceptor stimulation, but not muscarinic stimulation, increases cyclic AMP accumulation in brain slices due to protein kinase C mediated enhancement of adenosine receptor effects. *Acta Physiologica Scandinavica*, *131*(4). https://doi.org/10.1111/j.1748-1716.1987.tb08274.x

Fredholm, B., Cunha, R., & Svenningsson, P. (2005). Pharmacology of Adenosine A2A Receptors and Therapeutic Applications. *Current Topics in Medicinal Chemistry*, *3*(4). https://doi.org/10.2174/1568026033392200

Fredholm, Bertil B., Arslan, G., Halldner, L., Kull, B., Schulte, G., & Wasserman, W. (2000). Structure and function of adenosine receptors and their genes. *Naunyn-Schmiedeberg's Archives of Pharmacology*, Vol. 362. https://doi.org/10.1007/s002100000313

Fredholm, Bertil B., Chen, J. F., Cunha, R. A., Svenningsson, P., & Vaugeois, J. M. (2005). Adenosine and Brain Function. *International Review of Neurobiology*, *63*. https://doi.org/10.1016/S0074-7742(05)63007-3

Fredholm, Bertil B., Chern, Y., Franco, R., & Sitkovsky, M. (2007). Aspects of the general biology of adenosine A2A signaling. *Progress in Neurobiology*, Vol. 83. https://doi.org/10.1016/j.pneurobio.2007.07.005

Fredholm, Bertil B., & Svenningsson, P. (2003). Adenosine-dopamine interactions: Development of a concept and some comments on therapeutic possibilities. *Neurology*, Vol. 61. https://doi.org/10.1212/01.wnl.0000095204.89871.ff

Frenguelli, B. G., Wigmore, G., Llaudet, E., & Dale, N. (2007). Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. *Journal of Neurochemistry*, *101*(5). https://doi.org/10.1111/j.1471-4159.2006.04425.x

Furukawa, K., Fu, W., Li, Y., Witke, W., Kwiatkowski, D. J., & Mattson, M. P. (1997). The actin-severing protein gelsolin modulates calcium channel and NMDA receptor activities and vulnerability to excitotoxicity in hippocampal neurons. *The Journal of Neuroscience*.

Fusco, I., Ugolini, F., Lana, D., Coppi, E., Dettori, I., Gaviano, L., … Pugliese, A. M. (2018). The selective antagonism of adenosine A2B receptors reduces the synaptic failure and neuronal death induced by oxygen and glucose deprivation in rat CA1 hippocampus in vitro. *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2018.00399

Gao, B. B., Clermont, A., Rook, S., Fonda, S. J., Srinivasan, V. J., Wojtkowski, M., … Feener, E. P. (2007). Extracellular carbonic anhydrase mediates hemorrhagic retinal and cerebral vascular permeability through prekallikrein activation. *Nature Medicine*, *13*(2), 181–188. https://doi.org/10.1038/nm1534

Gao, Z. G., Balasubramanian, R., Kiselev, E., Wei, Q., & Jacobson, K. A. (2014). Probing biased/partial agonism at the G protein-coupled A2B adenosine receptor. *Biochemical Pharmacology*, *90*(3). https://doi.org/10.1016/j.bcp.2014.05.008

Gao, Z. G., Kim, S. K., Biadatti, T., Chen, W., Lee, K., Barak, D., … Jacobson, K. A. (2002). Structural determinants of A3 adenosine receptor activation: Nucleoside ligands at the agonist/antagonist boundary. *Journal of Medicinal Chemistry*, *45*(20). https://doi.org/10.1021/jm020211+

Garau, A., Bertini, R., Colotta, F., Casilli, F., Bigini, P., Cagnotto, A., … Villa, P. (2005). Neuroprotection with the CXCL8 inhibitor repertaxin in transient brain ischemia. *Cytokine*, *30*(3). https://doi.org/10.1016/j.cyto.2004.12.014

Garcia, J. H., Liu, K. F., & Ho, K. L. (1995). Neuronal necrosis after middle cerebral artery occlusion in wistar rats progresses at different time intervals in the caudoputamen and the cortex. *Stroke*, *26*(4). https://doi.org/10.1161/01.str.26.4.636

Gelderblom, M., Leypoldt, F., Steinbach, K., Behrens, D., Choe, C. U., Siler, D. A., … Magnus, T. (2009). Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke*, *40*(5). https://doi.org/10.1161/STROKEAHA.108.534503

Genovese, T., Melani, A., Esposito, E., Mazzon, E., Di Paola, R., Bramanti, P., … Cuzzocrea, S. (2009). The selective adenosine A2A receptor agonist CGS 21680 reduces JNK MAPK activation in oligodendrocytes in injured spinal cord. *Shock*, *32*(6). https://doi.org/10.1097/SHK.0b013e3181a20792

Gessi, S., Varani, K., Merighi, S., Cattabriga, E., Pancaldi, C., Szabadkai, Y., … Borea, P. A. (2005). Expression, pharmacological profile, and functional coupling of A 2b receptors in a recombinant system and in peripheral blood cells using a novel selective antagonist radioligand, [3H]MRE 2029-F20. *Molecular Pharmacology*, *67*(6). https://doi.org/10.1124/mol.104.009225

Gharibi, B., Abraham, A. A., Ham, J., & Evans, B. A. J. (2012). Contrasting effects of A1 and A2b adenosine receptors on adipogenesis. *International Journal of Obesity*, *36*(3).

https://doi.org/10.1038/ijo.2011.129

Gibson, C. L. (2013). Cerebral ischemic stroke: Is gender important? *Journal of Cerebral Blood Flow and Metabolism*, Vol. 33, pp. 1355–1361. https://doi.org/10.1038/jcbfm.2013.102

Giffard, R. G., & Swanson, R. A. (2005). Ischemia-induced programmed cell death in astrocytes. *GLIA*, Vol. 50. https://doi.org/10.1002/glia.20167

Giffard, R., Monyer, H., & Choi, D. (1990). Selective vulnerability of cultured cortical glia to injury by extracellular acidosis. *Brain Research*, *530*(1), 138–141. https://doi.org/10.1016/0006-8993(90)90670-7

Giovannini, M. G., Scali, C., Prosperi, C., Bellucci, A., Vannucchi, M. G., Rosi, S., … Casamenti, F. (2002). β-amyloid-induced inflammation and cholinergic hypofunction in the rat brain in vivo: Involvement of the p38MAPK pathway. *Neurobiology of Disease*, *11*(2). https://doi.org/10.1006/nbdi.2002.0538

Gnad, T., Navarro, G., Lahesmaa, M., Reverte-Salisa, L., Copperi, F., Cordomi, A., … Pfeifer, A. (2020). Adenosine/A2B Receptor Signaling Ameliorates the Effects of Aging and Counteracts Obesity. *Cell Metabolism*, *32*(1). https://doi.org/10.1016/j.cmet.2020.06.006

Gomes, C. A. R. V., Vaz, S. H., Ribeiro, J. A., & Sebastião, A. M. (2006). Glial cell line-derived neurotrophic factor (GDNF) enhances dopamine release from striatal nerve endings in an adenosine A2A receptor-dependent manner. *Brain Research*, *1113*(1). https://doi.org/10.1016/j.brainres.2006.07.025

Gomes, C., Ferreira, R., George, J., Sanches, R., Rodrigues, D. I., Gonçalves, N., & Cunha, R. A. (2013). Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia. *Journal of Neuroinflammation*, *10*. https://doi.org/10.1186/1742-2094-10-16

Gomes, J. I., Farinha-Ferreira, M., Rei, N., Gonçalves-Ribeiro, J., Ribeiro, J. A., Sebastião, A. M., & Vaz, S. H. (2021). Of adenosine and the blues: The adenosinergic system in the pathophysiology and treatment of major depressive disorder. *Pharmacological Research*, *163*. https://doi.org/10.1016/j.phrs.2020.105363

Gonçalves, M. L., Cunha, R. A., & Ribeiro, J. A. (1997). Adenosine A(2A) receptors facilitate 45Ca2+ uptake through class A calcium channels in rat hippocampal CA3 but not CA1 synaptosomes. *Neuroscience Letters*, *238*(1–2). https://doi.org/10.1016/S0304-3940(97)00803-3

Gordon, A., & Price, L. H. (1999). Mood stabilization and weight loss with topiramate [2]. *American Journal of Psychiatry*, Vol. 156. https://doi.org/10.1176/ajp.156.6.968a

Gray, J. H., Owen, R. P., & Giacomini, K. M. (2004). The concentrative nucleoside transporter family, SLC28. *Pflugers Archiv European Journal of Physiology*, Vol. 447. https://doi.org/10.1007/s00424-003-1107-y

Greene, R. W., & Haas, H. L. (1991). The electrophysiology of adenosine in the mammalian central nervous system. *Progress in Neurobiology*, Vol. 36. https://doi.org/10.1016/0301-0082(91)90005-L

Grysiewicz, R. A., Thomas, K., & Pandey, D. K. (2008). Epidemiology of Ischemic and Hemorrhagic Stroke: Incidence, Prevalence, Mortality, and Risk Factors. *Neurologic Clinics*, Vol. 26. https://doi.org/10.1016/j.ncl.2008.07.003

Gu, L. Z., Huang, B. S., Shen, W., Gao, L., Ding, Z. Z., Wu, H. W., & Guo, J. (2013). Early activation of nSMase2/ceramide pathway in astrocytes is involved in ischemia-associated neuronal damage via inflammation in rat hippocampi. *Journal of Neuroinflammation*, *10*. https://doi.org/10.1186/1742-2094-10-109

Gubitz, A. K., Widdowson, L., Kurokawa, M., Kirkpatrick, K. A., & Richardson, P. J. (1996). Dual signalling by the adenosine A2a receptor involves activation of both N- and P-type calcium channels by different G proteins and protein kinases in the same striatal nerve terminals. *Journal of Neurochemistry*, *67*(1). https://doi.org/10.1046/j.1471-4159.1996.67010374.x

Guo, F., Hua, Y., Wang, J., Keep, R., & Xi, G. (2012). Inhibition of Carbonic Anhydrase Reduces Brain Injury After Intracerebral Hemorrhage. *Translational Stroke Research*, *3*(1), 130–137. https://doi.org/10.1007/s12975-011-0106-0

Hagberg, H., Andersson, P., Lacarewicz, J., Jacobson, I., Butcher, S., & Sandberg, M. (1987). Extracellular Adenosine, Inosine, Hypoxanthine, and Xanthine in Relation to Tissue Nucleotides and Purines in Rat Striatum During Transient Ischemia. *Journal of Neurochemistry*, *49*(1). https://doi.org/10.1111/j.1471-4159.1987.tb03419.x

Halassa, M. M., Fellin, T., & Haydon, P. G. (2009). Tripartite synapses: Roles for astrocytic purines in the control of synaptic physiology and behavior. *Neuropharmacology*, Vol. 57. https://doi.org/10.1016/j.neuropharm.2009.06.031

Hallenbeck, J. M. (1996). Significance of the Inflammatory Response in Brain Ischemia. *Acta Neurochirurgica, Supplement*, *1996*(66). https://doi.org/10.1007/978-3-7091-9465-2_5

Halliwell, B. (2006). Oxidative stress and neurodegeneration: Where are we now? *Journal of Neurochemistry*, Vol. 97. https://doi.org/10.1111/j.1471-4159.2006.03907.x

Halmi, P., Parkkila, S., & Honkaniemi, J. (2006). Expression of carbonic anhydrases II, IV, VII, VIII and XII in rat brain after kainic acid induced status epilepticus. *Neurochemistry International*, *48*(1). https://doi.org/10.1016/j.neuint.2005.08.007

Han, B. H., & Holtzman, D. M. (2000). BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. *Journal of Neuroscience*, *20*(15). https://doi.org/10.1523/jneurosci.20-15-05775.2000

Han, M., Kwon, I., Ha, J., Kim, J., Cha, M. J., Kim, Y. D., … Nam, H. S. (2020). Collateral augmentation treatment with a combination of acetazolamide and head-down tilt in a rat ischemic stroke model. *Journal of Clinical Neuroscience*, *73*, 252–258. https://doi.org/10.1016/j.jocn.2020.01.079

Hanson, M., Nye, P., & Torrance, R. (1981). The location of carbonic anhydrase in relation to the blood-

brain barrier at the medullary chemoreceptors of the cat. *The Journal of Physiology*, *320*(1), 113–125. https://doi.org/10.1113/jphysiol.1981.sp013938

Harms, H. H., Wardeh, G., & Mulder, A. H. (1979). Effects of adenosine on depolarization-induced release of various radiolabelled neurotransmitters from slices of rat corpus striatum. *Neuropharmacology*, *18*(7). https://doi.org/10.1016/0028-3908(79)90107-2

Harms, Herman H., Wardeh, G., & Mulder, A. H. (1978). Adenosine modulates depolarization-induced release of 3H-noradrenaline from slices of rat brain neocortex. *European Journal of Pharmacology*, *49*(3). https://doi.org/10.1016/0014-2999(78)90107-3

Hartmann, M., Heumann, R., & Lessmann, V. (2001). Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. *EMBO Journal*, *20*(21). https://doi.org/10.1093/emboj/20.21.5887

Haskó, G., Kuhel, D. G., Chen, J.-F., Schwarzschild, M. A., Deitch, E. A., Mabley, J. G., … Szabó, C. (2000). Adenosine inhibits IL-12 and TNF-α production via adenosine A 2a receptor-dependent and independent mechanisms . *The FASEB Journal*, *14*(13). https://doi.org/10.1096/fj.99-0508com

Haskó, G., Linden, J., Cronstein, B., & Pacher, P. (2008). Adenosine receptors: Therapeutic aspects for inflammatory and immune diseases. *Nature Reviews Drug Discovery*, Vol. 7. https://doi.org/10.1038/nrd2638

Heales, D. S. (1999). pH AND BRAIN FUNCTION. *Brain*, *122*(9). https://doi.org/10.1093/brain/122.9.1794

Heese, K., Fiebich, B. L., Bauer, J., & Otten, U. (1997). Nerve growth factor (NGF) expression in rat microglia is induced by adenosine A(2a)-receptors. *Neuroscience Letters*, *231*(2). https://doi.org/10.1016/S0304-3940(97)00545-4

Hettinger, B. D., Lee, A., Linden, J., & Rosin, D. L. (2001). Ultrastructural localization of adenosine A2A receptors suggests multiple cellular sites for modulation of GABAergic neurons in rat striatum. *Journal of Comparative Neurology*, *431*(3). https://doi.org/10.1002/1096-9861(20010312)431:3<331::AID-CNE1074>3.0.CO;2-W

Hewett-Emmett, D. (2000). Evolution and distribution of the carbonic anhydrase gene families. *EXS*, pp. 26–76. https://doi.org/10.1007/978-3-0348-8446-4_3

Hinz, S., Lacher, S. K., Seibt, B. F., & Müller, C. E. (2014). BAY60-6583 acts as a partial agonist at adenosine A2B receptors. *Journal of Pharmacology and Experimental Therapeutics*, *349*(3). https://doi.org/10.1124/jpet.113.210849

Hladky, S. B., & Barrand, M. A. (2016). Fluid and ion transfer across the blood-brain and blood-cerebrospinal fluid barriers; a comparative account of mechanisms and roles. *Fluids and Barriers of the CNS*, Vol. 13. https://doi.org/10.1186/s12987-016-0040-3

Hollingsworth, E. B., Sears, E. B., & Daly, J. W. (1985). An activator of protein kinase C (phorbol-12-myristate-13-acetate) augments 2-chloroadenosine-elicited accumulation of cyclic AMP in guinea pig

cerebral cortical particulate preparations. *FEBS Letters*, *184*(2). https://doi.org/10.1016/0014-5793(85)80634-7

Hossmann, K. -A. (1994). Viability thresholds and the penumbra of focal ischemia. *Annals of Neurology*, *36*(4). https://doi.org/10.1002/ana.410360404

Hossmann, K. A. (2006). Pathophysiology and therapy of experimental stroke. *Cellular and Molecular Neurobiology*, Vol. 26. https://doi.org/10.1007/s10571-006-9008-1

Huang, J., Agus, D. B., Winfree, C. J., Kiss, S., Mack, W. J., McTaggart, R. A., … Connolly, E. S. (2001). Dehydroascorbic acid, a blood-brain barrier transportable form of vitamin C, mediates potent cerebroprotection in experimental stroke. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(20). https://doi.org/10.1073/pnas.171325998

Huang, J., Choudhri, T. F., Winfree, C. J., McTaggart, R. A., Kiss, S., Mocco, J., … Connolly, E. S. (2000). Postischemic cerebrovascular E-selectin expression mediates tissue injury in murine stroke. *Stroke*, *31*(12). https://doi.org/10.1161/01.STR.31.12.3047

Huang, J., Upadhyay, U. M., & Tamargo, R. J. (2006). Inflammation in stroke and focal cerebral ischemia. *Surgical Neurology*, *66*(3). https://doi.org/10.1016/j.surneu.2005.12.028

Hulse, R. E., Winterfield, J., Kunkler, P. E., & Kraig, R. P. (2001). Astrocytic clasmatodendrosis in hippocampal organ culture. *GLIA*, *33*(2). https://doi.org/10.1002/1098-1136(200102)33:2<169::AID-GLIA1016>3.0.CO;2-B

Hunsucker, S. A., Mitchell, B. S., & Spychala, J. (2005). The 5'-nucleotidases as regulators of nucleotide and drug metabolism. *Pharmacology and Therapeutics*, Vol. 107. https://doi.org/10.1016/j.pharmthera.2005.01.003

Iadecola, C., & Anrather, J. (2011). Stroke research at a crossroad: Asking the brain for directions. *Nature Neuroscience*, *14*(11). https://doi.org/10.1038/nn.2953

Intiso, D., Zarrelli, M. M., Lagioia, G., Di Rienzo, F., Checchia De Ambrosio, C., Simone, P., … Cioffi, R. P. (2004). Tumor necrosis factor alpha serum levels and inflammatory response in acute ischemic stroke patients. *Neurological Sciences*, *24*(6). https://doi.org/10.1007/s10072-003-0194-z

Irving, E. A., & Bamford, M. (2002). Role of mitogen- and stress-activated kinases in ischemic injury. *Journal of Cerebral Blood Flow and Metabolism*, Vol. 22. https://doi.org/10.1097/00004647-200206000-00001

Irving, E. A., Barone, F. C., Reith, A. D., Hadingham, S. J., & Parsons, A. A. (2000). Differential activation of MAPK/ERK and p38/SAPK in neurones and glia following focal cerebral ischaemia in the rat. *Molecular Brain Research*, *77*(1). https://doi.org/10.1016/S0169-328X(00)00043-7

J. Geldenhuys, W., & J. Van der Schyf, C. (2013). Rationally Designed Multi-Targeted Agents Against Neurodegenerative Diseases. *Current Medicinal Chemistry*, *20*(13). https://doi.org/10.2174/09298673113209990112

Jacobson, K. A., Nikodijevic, O., Ji, X. duo, Berkich, D. A., Eveleth, D., Dean, R. L., … Maillard, M. (1992).

Synthesis and Biological Activity of N6-(p-Sulfophenyl)alkyl and N6-Sulfoalkyl Derivatives of Adenosine: Water-Soluble and Peripherally Selective Adenosine Agonists. *Journal of Medicinal Chemistry*, *35*(22). https://doi.org/10.1021/jm00100a020

Jacobson, K. A., Nikodijević, O., Padgett, W. L., Gallo-Rodriguez, C., Maillard, M., & Daly, J. W. (1993). 8-(3-Chlorostyryl)caffeine (CSC) is a selective A2-adenosine antagonist in vitro and in vivo. *FEBS Letters*, *323*(1–2). https://doi.org/10.1016/0014-5793(93)81466-D

Jalencas, X., & Mestres, J. (2013). On the origins of drug polypharmacology. *MedChemComm*, Vol. 4. https://doi.org/10.1039/c2md20242e

James, S., & Richardson, P. J. (1993). Production of Adenosine from Extracellular ATP at the Striatal Cholinergic Synapse. *Journal of Neurochemistry*, *60*(1). https://doi.org/10.1111/j.1471-4159.1993.tb05841.x

Jarvis, C. R., Anderson, T. R., & Andrew, R. D. (2001). Anoxic depolarization mediates acute damage independent of glutamate in neocortical brain slices. *Cerebral Cortex*, *11*(3). https://doi.org/10.1093/cercor/11.3.249

Jarvis, M. F., Schulz, R., Hutchison, A. J., Do, U. H., Sills, M. A., & Williams, M. (1989). [3H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. *Journal of Pharmacology and Experimental Therapeutics*, *251*(3).

Jensen, E., Clement, R., Kosta, A., Maberly, S., & Gontero, B. (2019). A new widespread subclass of carbonic anhydrase in marine phytoplankton. *ISME Journal*, *13*(8), 2094–2106. https://doi.org/10.1038/s41396-019-0426-8

Jeon, S. J., Rhee, S. Y., Ryu, J. H., Cheong, J. H., Kwon, K., Yang, S. Il, … Shin, C. Y. (2011). Activation of adenosine A2A receptor up-regulates BDNF expression in rat primary cortical neurons. *Neurochemical Research*, *36*(12). https://doi.org/10.1007/s11064-011-0550-y

Jeong, L. S., Jin, D. Z., Kim, H. O., Shin, D. H., Moon, H. R., Gunaga, P., … Jacobson, K. A. (2003). N6-substituted D-4′-thioadenosine-5′-methyluronamides: Potent and selective agonists at the human A3 adenosine receptor. *Journal of Medicinal Chemistry*, *46*(18). https://doi.org/10.1021/jm034098e

Ji, X. D., Gallorodriguez, C., & Jacobson, K. A. (1994). A selective agonist affinity label for A3 adenosine receptors. *Biochemical and Biophysical Research Communications*, *203*(1). https://doi.org/10.1006/bbrc.1994.2220

Jiang, X., & Wang, X. (2004). Cytochrome C-mediated apoptosis. *Annual Review of Biochemistry*, Vol. 73. https://doi.org/10.1146/annurev.biochem.73.011303.073706

Jickling, G. C., & Sharp, F. R. (2011). Blood Biomarkers of Ischemic Stroke. *Neurotherapeutics*, Vol. 8. https://doi.org/10.1007/s13311-011-0050-4

Jin, R., Liu, L., Zhang, S., Nanda, A., & Li, G. (2013). Role of inflammation and its mediators in acute ischemic stroke. *Journal of Cardiovascular Translational Research*, *6*(5). https://doi.org/10.1007/s12265-013-

9508-6

Jin, R., Yang, G., & Li, G. (2010). Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *Journal of Leukocyte Biology*, *87*(5). https://doi.org/10.1189/jlb.1109766

Jones, P. A., Smith, R. A., & Stone, T. W. (1998). Protection against kainate-induced excitotoxicity by adenosine A(2A) receptor agonists and antagonists. *Neuroscience*, *85*(1). https://doi.org/10.1016/S0306-4522(97)00613-1

Justicia, C., Panés, J., Solé, S., Cervera, Á., Deulofeu, R., Chamorro, Á., & Planas, A. M. (2003). Neutrophil Infiltration Increases Matrix Metalloproteinase-9 in the Ischemic Brain after Occlusion/Reperfusion of the Middle Cerebral Artery in Rats. *Journal of Cerebral Blood Flow and Metabolism*, *23*(12). https://doi.org/10.1097/01.WCB.0000090680.07515.C8

Kamegawa, A., Hiroaki, Y., Tani, K., & Fujiyoshi, Y. (2016, April 1). Two-dimensional crystal structure of aquaporin-4 bound to the inhibitor acetazolamide. *Microscopy (Oxford, England)*, Vol. 65, pp. 177–184. https://doi.org/10.1093/jmicro/dfv368

Katsura -i., K., Kristian, T., & Siesjo, B. K. (1994). Energy metabolism, ion homeostasis, and cell damage in the brain. *Biochemical Society Transactions*, *22*(4). https://doi.org/10.1042/bst0220991

Katsura, K. I., Kurihara, J., Siesjö, B. K., & Wieloch, T. (1999). Acidosis enhances translocation of protein kinase C but not Ca2+/calmodulin-dependent protein kinase II to cell membranes during complete cerebral ischemia. *Brain Research*, *849*(1–2). https://doi.org/10.1016/S0006-8993(99)02072-7

Katzman, R., Clasen, R., Klatzo, I., Meyer, J. S., Pappius, H. M., & Waltz, A. G. (1977). Report of Joint Committee for Stroke Resources. IV. Brain edema in stroke. *Stroke*, *8*(4). https://doi.org/10.1161/01.str.8.4.512

Kerr, J. F. R., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, *26*(4). https://doi.org/10.1038/bjc.1972.33

Kessey, K., Trommer, B. L., Overstreet, L. S., Ji, T., & Mogul, D. J. (1997). A role for adenosine A2 receptors in the induction of long-term potentiation in the CA1 region of rat hippocampus. *Brain Research*, *756*(1–2). https://doi.org/10.1016/S0006-8993(97)00148-0

Khalifah, R. G. (1971). The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *Journal of Biological Chemistry*, *246*(8). https://doi.org/10.1016/S0021-9258(18)62326-9

Kikutani, S., Nakajima, K., Nagasato, C., Tsuji, Y., Miyatake, A., & Matsuda, Y. (2016). Thylakoid luminal Θ-carbonic anhydrase critical for growth and photosynthesis in the marine diatom Phaeodactylum tricornutum. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(35), 9828–9833. https://doi.org/10.1073/pnas.1603112113

Kim, B. M., Baek, J. H., Heo, J. H., Nam, H. S., Kim, Y. D., Yoo, J., … Bang, O. Y. (2018). Collateral status affects

the onset-to-reperfusion time window for good outcome. *Journal of Neurology, Neurosurgery and Psychiatry*, *89*(9). https://doi.org/10.1136/jnnp-2017-317627

Kim, M., Spelta, V., Sim, J., North, R. A., & Surprenant, A. (2001). Differential Assembly of Rat Purinergic P2X7 Receptor in Immune Cells of the Brain and Periphery. *Journal of Biological Chemistry*, *276*(26). https://doi.org/10.1074/jbc.M102253200

Kiss, B., Dallinger, S., Findl, O., Rainer, G., Eichler, H. G., & Schmetterer, L. (1999). Acetazolamide-induced cerebral and ocular vasodilation in humans is independent of nitric oxide. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *276*(6(45-6)). https://doi.org/10.1152/ajpregu.1999.276.6.r1661

Kitagawa, H., Mori, A., Shimada, J., Mitsumoto, Y., & Kikuchi, T. (2002). Intracerebral adenosine infusion improves neurological outcome after transient focal ischemia in rats. *Neurological Research*, *24*(3). https://doi.org/10.1179/016164102101199819

Kitakaze, M., Minamino, T., Node, K., Komamura, K., & Hori, M. (1996). Activation of ecto-5'-nucleotidase and cardioprotection by ischemic preconditioning. *Basic Research in Cardiology*, *91*(1). https://doi.org/10.1007/978-3-642-53793-6_42

Klatzo, I. (1985). Brain oedema following brain ischaemia and the influence of therapy. *British Journal of Anaesthesia*, *57*(1), 18–22. https://doi.org/10.1093/bja/57.1.18

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., & Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: A primary site for Bcl- 2 regulation of apoptosis. *Science*, *275*(5303). https://doi.org/10.1126/science.275.5303.1132

Kniep, E. M., Roehlecke, C., Özkucur, N., Steinberg, A., Reber, F., Knels, L., & Funk, R. H. W. (2006). Inhibition of apoptosis and reduction of intracellular pH decrease in retinal neural cell cultures by a blocker of carbonic anhydrase. *Investigative Ophthalmology and Visual Science*, *47*(3), 1185–1192. https://doi.org/10.1167/iovs.05-0555

Koeppen, M., Eckle, T., & Eltzschig, H. K. (2011). Interplay of Hypoxia and A 2B Adenosine Receptors in Tissue Protection. In *Advances in Pharmacology* (Vol. 61). https://doi.org/10.1016/B978-0-12-385526-8.00006-0

Koistinaho, M., & Koistinaho, J. (2002). Role of p38 and p44/42 mitogen-activated protein kinases in microglia. *GLIA*, Vol. 40. https://doi.org/10.1002/glia.10151

Kolaczkowska, E., & Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology*, Vol. 13. https://doi.org/10.1038/nri3399

Kondej, M., Stępnicki, P., & Kaczor, A. A. (2018). Multi-target approach for drug discovery against schizophrenia. *International Journal of Molecular Sciences*, Vol. 19. https://doi.org/10.3390/ijms19103105

Kong, T., Westerman, K. A., Faigle, M., Eltzschig, H. K., & Colgan, S. P. (2006). HIF-dependent induction of

adenosine A2B receptor in hypoxia. *The FASEB Journal*, *20*(13). https://doi.org/10.1096/fj.06-6419com

König, B. F. R., KLIPPEL, R. A., & ZEMAN, W. (1964). The Rat Brain. A Stereotaxic Atlas. *Journal of Neuropathology and Experimental Neurology*, *23*(4). https://doi.org/10.1097/00005072-196410000-00012

Konrad, F. M., Witte, E., Vollmer, I., Stark, S., & Reutershan, J. (2012). Adenosine receptor A 2b on hematopoietic cells mediates LPS-induced migration of PMNs into the lung interstitium. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *303*(5). https://doi.org/10.1152/ajplung.00387.2011

Koscsó, B., Csóka, B., Selmeczy, Z., Himer, L., Pacher, P., Virág, L., & Haskó, G. (2012). Adenosine Augments IL-10 Production by Microglial Cells through an A 2B Adenosine Receptor-Mediated Process . *The Journal of Immunology*, *188*(1). https://doi.org/10.4049/jimmunol.1101224

Kostopoulos, G. K., Limacher, J. J., & Phillis, J. W. (1975). Action of various adenine derivatives on cerebellar Purkinje cells. *Brain Research*, *88*(1). https://doi.org/10.1016/0006-8993(75)90966-X

Krams, M., Lees, K. R., Hacke, W., Grieve, A. P., Orgogozo, J. M., & Ford, G. A. (2003). Acute Stroke Therapy by Inhibition of Neutrophils (ASTIN): An Adaptive Dose-Response Study of UK-279,276 in Acute Ischemic Stroke. *Stroke*, *34*(11). https://doi.org/10.1161/01.STR.0000092527.33910.89

Kreutzberg, G. W. (1996). Microglia: A sensor for pathological events in the CNS. *Trends in Neurosciences*, Vol. 19. https://doi.org/10.1016/0166-2236(96)10049-7

Kreutzberg, G. W., & Barron, K. D. (1978). 5'-Nucleotidase of microglial cells in the facial nucleus during axonal reaction. *Journal of Neurocytology*, *7*(5). https://doi.org/10.1007/BF01260892

Kriz, J. (2006). Inflammation in ischemic brain injury: Timing is important. *Critical Reviews in Neurobiology*, Vol. 18. https://doi.org/10.1615/CritRevNeurobiol.v18.i1-2.150

Kulesskaya, N., Võikar, V., Peltola, M., Yegutkin, G. G., Salmi, M., Jalkanen, S., & Rauvala, H. (2013). CD73 Is a Major Regulator of Adenosinergic Signalling in Mouse Brain. *PLoS ONE*, *8*(6). https://doi.org/10.1371/journal.pone.0066896

Kuroda, Y., Saito, M., & Kobayashi, K. (1976). Concomitant changes in cyclic AMP level and postsynaptic potentials of olfactory cortex slices induced by adenosine derivatives. *Brain Research*, *109*(1). https://doi.org/10.1016/0006-8993(76)90393-0

Lai, A. Y., & Todd, K. G. (2006). Microglia in cerebral ischemia: Molecular actions and interactions. *Canadian Journal of Physiology and Pharmacology*, *84*(1). https://doi.org/10.1139/y05-143

Lai, T. W., Zhang, S., & Wang, Y. T. (2014). Excitotoxicity and stroke: Identifying novel targets for neuroprotection. *Progress in Neurobiology*, Vol. 115. https://doi.org/10.1016/j.pneurobio.2013.11.006

Lakhan, S. E., Kirchgessner, A., Tepper, D., & Leonard, A. (2013). Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke. *Frontiers in Neurology*, *4 APR*.

https://doi.org/10.3389/fneur.2013.00032

Lalancette-Hébert, M., Gowing, G., Simard, A., Yuan, C. W., & Kriz, J. (2007). Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. *Journal of Neuroscience*, *27*(10). https://doi.org/10.1523/JNEUROSCI.5360-06.2007

Lana, D., Melani, A., Pugliese, A. M., Cipriani, S., Nosi, D., Pedata, F., & Giovannini, M. G. (2014). The neuron-astrocyte-microglia triad in a rat model of chronic cerebral hypoperfusion: Protective effect of dipyridamole. *Frontiers in Aging Neuroscience*, *6*(NOV). https://doi.org/10.3389/fnagi.2014.00322

Lana, D., Ugolini, F., Melani, A., Nosi, D., Pedata, F., & Giovannini, M. G. (2017). The neuron-astrocyte-microglia triad in CA3 after chronic cerebral hypoperfusion in the rat: Protective effect of dipyridamole. *Experimental Gerontology*, *96*. https://doi.org/10.1016/j.exger.2017.06.006

Lappas, C. M., Day, Y. J., Marshall, M. A., Engelhard, V. H., & Linden, J. (2006). Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation. *Journal of Experimental Medicine*, *203*(12). https://doi.org/10.1084/jem.20061097

Latini, S., Corsi, C., Pedata, F., & Pepeu, G. (1996). The source of brain adenosine outflow during ischemia and electrical stimulation. *Neurochemistry International*, *28*(1). https://doi.org/10.1016/0197-0186(95)00062-D

Latini, Serena, Pazzagli, M., Pepeu, G., & Pedata, F. (1996). A2 adenosine receptors: Their presence and neuromodulatory role in the central nervous system. *General Pharmacology*, Vol. 27. https://doi.org/10.1016/0306-3623(96)00044-4

Latini, Serena, & Pedata, F. (2001). Adenosine in the central nervous system: Release mechanisms and extracellular concentrations. *Journal of Neurochemistry*, Vol. 79. https://doi.org/10.1046/j.1471-4159.2001.00607.x

Ledoux, S., Runembert, I., Koumanov, K., Michel, J. B., Trugnan, G., & Friedlander, G. (2003). Hypoxia enhances Ecto-5'-nucleotidase activity and cell surface expression in endothelial cells: Role of membrane lipids. *Circulation Research*, *92*(8). https://doi.org/10.1161/01.RES.0000069022.95401.FE

Lee, Y. C., Chien, C. L., Sun, C. N., Huang, C. L., Huang, N. K., Chiang, M. C., … Chern, Y. (2003). Characterization of the rat A2A adenosine receptor gene: A 4.8-kb promoter-proximal DNA fragment confers selective expression in the central nervous system. *European Journal of Neuroscience*, *18*(7). https://doi.org/10.1046/j.1460-9568.2003.02907.x

Lennmyr, F., Karlsson, S., Gerwins, P., Ata, K. A., & Terént, A. (2002). Activation of mitogen-activated protein kinases in experimental cerebral ischemia. *Acta Neurologica Scandinavica*, *106*(6). https://doi.org/10.1034/j.1600-0404.2002.01313.x

Levine, S. R. (2004). Pathophysiology and therapeutic targets for ischemic stroke. *Clinical Cardiology*, Vol. 27. https://doi.org/10.1002/clc.4960271404

Li, A. H., Moro, S., Melman, N., Ji, X. D., & Jacobson, K. A. (1998). Structure-activity relationships and

molecular modeling of 3,5-diacyl- 2,4-dialkylpyridine derivatives as selective A3 adenosine receptor antagonists. *Journal of Medicinal Chemistry*, *41*(17). https://doi.org/10.1021/jm980093j

Li, Q., Han, X., Lan, X., Hong, X., Li, Q., Gao, Y., … Wang, J. (2017). Inhibition of tPA-induced hemorrhagic transformation involves adenosine A2b receptor activation after cerebral ischemia. *Neurobiology of Disease*. https://doi.org/10.1016/j.nbd.2017.08.011

Li, Y., Oskouian, R. J., Day, Y. J., Rieger, J. M., Liu, L., Kern, J. A., & Linden, J. (2006). Mouse spinal cord compression injury is reduced by either activation of the adenosine A2A receptor on bone marrow-derived cells or deletion of the A2A receptor on non-bone marrow-derived cells. *Neuroscience*, *141*(4). https://doi.org/10.1016/j.neuroscience.2006.05.014

Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., … Veltkamp, R. (2009). Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nature Medicine*, *15*(2). https://doi.org/10.1038/nm.1927

Lin, M. P., Brott, T. G., Liebeskind, D. S., Meschia, J. F., Sam, K., & Gottesman, R. F. (2020). Collateral Recruitment Is Impaired by Cerebral Small Vessel Disease. *Stroke*. https://doi.org/10.1161/STROKEAHA.119.027661

Linden, J. (1994). Cloned adenosine A3 receptors: Pharmacological properties, species differences and receptor functions. *Trends in Pharmacological Sciences*, Vol. 15. https://doi.org/10.1016/0165-6147(94)90011-6

Linden, J. (2005). Adenosine in tissue protection and tissue regeneration. *Molecular Pharmacology*, Vol. 67. https://doi.org/10.1124/mol.105.011783

Lisk, C., McCord, J., Bose, S., Sullivan, T., Loomis, Z., Nozik-Grayck, E., … Irwin, D. C. (2013). Nrf2 activation: A potential strategy for the prevention of acute mountain sickness. *Free Radical Biology and Medicine*, *63*, 264–273. https://doi.org/10.1016/j.freeradbiomed.2013.05.024

Liu, T., Clark, R. K., McDonnell, P. C., Young, P. R., White, R. F., Barone, F. C., & Feuerstein, G. Z. (1994). 1994-Liu-TNF alpha expression in ischemic neurons.pdf. *Stroke*, *25*(7).

Liu, X., Zhu, F., H. Ma, X., Shi, Z., Y. Yang, S., Q. Wei, Y., & Z. Chen, Y. (2013). Predicting Targeted Polypharmacology for Drug Repositioning and Multi- Target Drug Discovery. *Current Medicinal Chemistry*, *20*(13). https://doi.org/10.2174/0929867311320130005

Liu, Xiaochen, Lu, D., Bowser, R., & Liu, J. (2016). Expression of carbonic anhydrase I in motor neurons and alterations in ALS. *International Journal of Molecular Sciences*, *17*(11). https://doi.org/10.3390/ijms17111820

Loddick, S. A., Turnbull, A. V., & Rothwell, N. J. (1998). Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. *Journal of Cerebral Blood Flow and Metabolism*, *18*(2). https://doi.org/10.1097/00004647-199802000-00008

Longa, E. Z., Weinstein, P. R., Carlson, S., & Cummins, R. (1989). Reversible middle cerebral artery occlusion

without craniectomy in rats. *Stroke*, *20*(1). https://doi.org/10.1161/01.STR.20.1.84

Lopes, L. V., Cunha, R. A., Kull, B., Fredholm, B. B., & Ribeiro, J. A. (2002). Adenosine A2A receptor facilitation of hippocampal synaptic transmission is dependent on tonic A1 receptor inhibition. *Neuroscience*, *112*(2). https://doi.org/10.1016/S0306-4522(02)00080-5

Lopes, Luísa V., Rebola, N., Pinheiro, P. C., Richardson, P. J., Oliveira, C. R., & Cunha, R. A. (2003). Adenosine A3 receptors are located in neurons of the rat hippocampus. *NeuroReport*, *14*(12). https://doi.org/10.1097/00001756-200308260-00021

Lou, Y., McDonald, P. C., Oloumi, A., Chia, S., Ostlund, C., Ahmadi, A., … Dedhar, S. (2011). Targeting tumor hypoxia: Suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Research*, *71*(9). https://doi.org/10.1158/0008-5472.CAN-10-4261

Lu, X. C. M., Williams, A. J., Wagstaff, J. D., Tortella, F. C., & Hartings, J. A. (2005). Effects of delayed intrathecal infusion of an NMDA receptor antagonist on ischemic injury and peri-infarct depolarizations. *Brain Research*, *1056*(2). https://doi.org/10.1016/j.brainres.2005.07.041

Lucas, S. M., Rothwell, N. J., & Gibson, R. M. (2006). The role of inflammation in CNS injury and disease. *British Journal of Pharmacology*, Vol. 147. https://doi.org/10.1038/sj.bjp.0706400

Ma, Y., Wang, J., Wang, Y., & Yang, G. Y. (2017). The biphasic function of microglia in ischemic stroke. *Progress in Neurobiology*, Vol. 157. https://doi.org/10.1016/j.pneurobio.2016.01.005

MacRae, I. (2011). Preclinical stroke research - Advantages and disadvantages of the most common rodent models of focal ischaemia. *British Journal of Pharmacology*, Vol. 164. https://doi.org/10.1111/j.1476-5381.2011.01398.x

Macrez, R., Ali, C., Toutirais, O., Le Mauff, B., Defer, G., Dirnagl, U., & Vivien Denis, D. (2011). Stroke and the immune system: From pathophysiology to new therapeutic strategies. *The Lancet Neurology*, Vol. 10. https://doi.org/10.1016/S1474-4422(11)70066-7

Maeda, Y., Matsumoto, M., Hori, O., Kuwabara, K., Ogawa, S., Yan, S. Du, … Kamada, T. (1994). Hypoxia/reoxygenation-mediated induction of astrocyte interleukin 6: A paracrine mechanism potentially enhancing neuron survival. *Journal of Experimental Medicine*, *180*(6). https://doi.org/10.1084/jem.180.6.2297

Magistretti, P. J., & Pellerin, L. (1996). Cellular mechanisms of brain energy metabolism: Relevance to functional brain imaging and to neurodegenerative disorders. *Annals of the New York Academy of Sciences*, *777*. https://doi.org/10.1111/j.1749-6632.1996.tb34449.x

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis: An overview of cell death. *American Journal of Pathology*, Vol. 146.

Makhoba, X. H., Viegas, C., Mosa, R. A., Viegas, F. P. D., & Pooe, O. J. (2020). Potential impact of the multi-target drug approach in the treatment of some complex diseases. *Drug Design, Development and Therapy*, *14*. https://doi.org/10.2147/DDDT.S257494

Maren, T. H. (1967). Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiological Reviews*, Vol. 47. https://doi.org/10.1152/physrev.1967.47.4.595

Margheri, F., Ceruso, M., Carta, F., Laurenzana, A., Maggi, L., Lazzeri, S., … Cimaz, R. (2016). Overexpression of the transmembrane carbonic anhydrase isoforms IX and XII in the inflamed synovium. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *31*. https://doi.org/10.1080/14756366.2016.1217857

Maria Pugliese, A., Coppi, E., Volpini, R., Cristalli, G., Corradetti, R., Jeong, L. S., … Pedata, F. (2007). Role of adenosine A3 receptors on CA1 hippocampal neurotransmission during oxygen-glucose deprivation episodes of different duration. *Biochemical Pharmacology*, *74*(5). https://doi.org/10.1016/j.bcp.2007.06.003

Markus, H. S. (2004). Cerebral perfusion and stroke. *Journal of Neurology, Neurosurgery and Psychiatry*, Vol. 75. https://doi.org/10.1136/jnnp.2003.025825

Martin, R. L., Lloyd, H. G. E., & Cowan, A. I. (1994). The early events of oxygen and glucose deprivation: setting the scene for neuronal death? *Trends in Neurosciences*, *17*(6). https://doi.org/10.1016/0166-2236(94)90008-6

Martínez-Fábregas, J., Díaz-Moreno, I., González-Arzola, K., Janochas, S., Navarro, J. A., Hervás, M., … De La Rosa, M. A. (2014). Structural and functional analysis of novel human cytochrome c targets in apoptosis. *Molecular and Cellular Proteomics*, *13*(6). https://doi.org/10.1074/mcp.M113.034322

Matsumoto, K., Graf, R., Rosner, G., Shimada, N., & Heiss, W. D. (1992). Flow thresholds for extracellular purine catabolite elevation in cat focal ischemia. *Brain Research*, *579*(2). https://doi.org/10.1016/0006-8993(92)90066-I

Mazzon, E., Esposito, E., Impellizzeri, D., Di Paola, R., Melani, A., Bramanti, P., … Cuzzocrea, S. (2011). CGS 21680, an Agonist of the Adenosine (A2A) receptor, reduces progression of murine type II collagen-induced arthritis. *Journal of Rheumatology*, *38*(10). https://doi.org/10.3899/jrheum.110111

Medina-Pulido, L., Molina-Arcas, M., Justicia, C., Soriano, E., Burgaya, F., Planas, A. M., & Pastor-Anglada, M. (2013). Hypoxia and P1 receptor activation regulate the high-affinity concentrative adenosine transporter CNT2 in differentiated neuronal PC12 cells. *Biochemical Journal*, *454*(3). https://doi.org/10.1042/BJ20130231

Meghji, P. (1993). Storage, release, uptake, and inactivation of purines. *Drug Development Research*, *28*(3). https://doi.org/10.1002/ddr.430280305

Meibom, D., Albrecht-Küpper, B., Diedrichs, N., Hübsch, W., Kast, R., Krämer, T., … Zimmermann, K. (2017). Neladenoson Bialanate Hydrochloride: A Prodrug of a Partial Adenosine A1 Receptor Agonist for the Chronic Treatment of Heart Diseases. *ChemMedChem*, *12*(10). https://doi.org/10.1002/cmdc.201700151

Melani, A., Cipriani, S., Vannucchi, M. G., Nosi, D., Donati, C., Bruni, P., … Pedata, F. (2009). Selective adenosine A2a receptor antagonism reduces JNK activation in oligodendrocytes after cerebral

ischaemia. *Brain*, *132*(6). https://doi.org/10.1093/brain/awp076

Melani, A., Corti, F., Cellai, L., Giuliana Vannucchi, M., & Pedata, F. (2014). Low doses of the selective adenosine A2A receptor agonist CGS21680 are protective in a rat model of transient cerebral ischemia. *Brain Research*. https://doi.org/10.1016/j.brainres.2014.01.014

Melani, A., Corti, F., Stephan, H., Müller, C. E., Donati, C., Bruni, P., … Pedata, F. (2012). Ecto-ATPase inhibition: ATP and adenosine release under physiological and ischemic in vivo conditions in the rat striatum. *Experimental Neurology*, *233*(1). https://doi.org/10.1016/j.expneurol.2011.09.036

Melani, A., Dettori, I., Corti, F., Cellai, L., & Pedata, F. (2015). Time-course of protection by the selective A2A receptor antagonist SCH58261 after transient focal cerebral ischemia. *Neurological Sciences*, *36*(8). https://doi.org/10.1007/s10072-015-2160-y

Melani, A., Gianfriddo, M., Vannucchi, M. G., Cipriani, S., Baraldi, P. G., Giovannini, M. G., & Pedata, F. (2006). The selective A2A receptor antagonist SCH 58261 protects from neurological deficit, brain damage and activation of p38 MAPK in rat focal cerebral ischemia. *Brain Research*. https://doi.org/10.1016/j.brainres.2005.12.010

Melani, A., Pantoni, L., Corsi, C., Bianchi, L., Monopoli, A., Bertorelli, R., … Pedata, F. (1999). Striatal Outflow of Adenosine, Excitatory Amino Acids, γ-Aminobutyric Acid, and Taurine in Awake Freely Moving Rats After Middle Cerebral Artery Occlusion. *Stroke*. https://doi.org/10.1161/01.str.30.11.2448

Mena, F. V., Baab, P. J., Zielke, C. L., & Zielke, H. R. (2000). In vivo glutamine hydrolysis in the formation of extracellular glutamate in the injured rat brain. *Journal of Neuroscience Research*, *60*(5). https://doi.org/10.1002/(SICI)1097-4547(20000601)60:5<632::AID-JNR8>3.0.CO;2-3

Mercatelli, R., Lana, D., Bucciantini, M., Giovannini, M. G., Cerbai, F., Quercioli, F., … Nosi, D. (2016). Clasmatodendrosis and b-amyloidosis in aging hippocampus. *FASEB Journal*, *30*(4). https://doi.org/10.1096/fj.15-275503

Merighi, S., Borea, P. A., Stefanelli, A., Bencivenni, S., Castillo, C. A., Varani, K., & Gessi, S. (2015). A2a and a2b adenosine receptors affect HIF-1α signaling in activated primary microglial cells. *GLIA*, *63*(11). https://doi.org/10.1002/glia.22861

Meyerhof, W., Müller-Brechlin, R., & Richter, D. (1991). Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Letters*, *284*(2). https://doi.org/10.1016/0014-5793(91)80674-R

Michaelis, M. L., Michaelis, E. K., & Myers, S. L. (1979). Adenosine modulation of synaptosomal dopamine release. *Life Sciences*, *24*(22). https://doi.org/10.1016/0024-3205(79)90082-1

Mies, G., Iijima, T., & Hossmann, K. A. (1993). Correlation between peri-infarct dc shifts and ischaemic neuronal damage in rat. *NeuroReport*, *4*(6). https://doi.org/10.1097/00001756-199306000-00027

Mincione, F., Scozzafava, A., & Supuran, C. (2007). The Development of Topically Acting Carbonic Anhydrase Inhibitors as Antiglaucoma Agents. *Current Topics in Medicinal Chemistry*, *7*(9).

https://doi.org/10.2174/156802607780636735

Mirabet, M., Mallol, J., Lluis, C., & Franco, R. (1997). Calcium mobilization in Jurkat cells via A(2b) adenosine receptors. *British Journal of Pharmacology*, *122*(6). https://doi.org/10.1038/sj.bjp.0701495

Mishiro, K., Ishiguro, M., Suzuki, Y., Tsuruma, K., Shimazawa, M., & Hara, H. (2012). A broad-spectrum matrix metalloproteinase inhibitor prevents hemorrhagic complications induced by tissue plasminogen activator in mice. *Neuroscience*, *205*. https://doi.org/10.1016/j.neuroscience.2011.12.042

Mishra, C., Tiwari, M., & Supuran, C. (2020). Progress in the development of human carbonic anhydrase inhibitors and their pharmacological applications: Where are we today? *Medicinal Research Reviews*, Vol. 40, pp. 2485–2565. https://doi.org/10.1002/med.21713

Mogul, D. J., Adams, M. E., & Fox, A. P. (1993). Differential activation of adenosine receptors decreases N-type but potentiates P-type Ca2+ current in hippocampal CA3 neurons. *Neuron*, *10*(2). https://doi.org/10.1016/0896-6273(93)90322-I

Montaner, J., Alvarez-Sabín, J., Molina, C., Anglés, A., Abilleira, S., Arenillas, J., … Monasterio, J. (2001). Matrix metalloproteinase expression after human cardioembolic stroke: Temporal profile and relation to neurological impairment. *Stroke*, *32*(8). https://doi.org/10.1161/01.STR.32.8.1759

Moreau, J. L., & Huber, G. (1999). Central adenosine A(2A) receptors: An overview. *Brain Research Reviews*, Vol. 31. https://doi.org/10.1016/S0165-0173(99)00059-4

Morphy, R., Kay, C., & Rankovic, Z. (2004). From magic bullets to designed multiple ligands. *Drug Discovery Today*, Vol. 9. https://doi.org/10.1016/S1359-6446(04)03163-0

Mozaffarian, D., Benjamin, E. J., Go, A. S., Arnett, D. K., Blaha, M. J., Cushman, M., … Turner, M. B. (2015). Heart Disease and Stroke Statistics—2015 Update. *Circulation*, *131*(4), 29–39. https://doi.org/10.1161/cir.0000000000000152

Müller, C. E., & Jacobson, K. A. (2011). Recent developments in adenosine receptor ligands and their potential as novel drugs. *Biochimica et Biophysica Acta - Biomembranes*, Vol. 1808. https://doi.org/10.1016/j.bbamem.2010.12.017

Murray, P. S., & Holmes, P. V. (2011). An overview of brain-derived neurotrophic factor and implications for excitotoxic vulnerability in the hippocampus. *International Journal of Peptides*. https://doi.org/10.1155/2011/654085

Nadareishvili, Z. G., Li, H., Wright, V., Maric, D., Warach, S., Hallenbeck, J. M., … Baird, A. E. (2004). Elevated pro-inflammatory CD4+CD28- lymphocytes and stroke recurrence and death. *Neurology*, *63*(8). https://doi.org/10.1212/01.WNL.0000142260.61443.7C

Naito, Y., & Lowenstein, J. M. (1985). 5'-Nucleotidase from rat heart membranes. Inhibition by adenine nucleotides and related compounds. *Biochemical Journal*, *226*(3). https://doi.org/10.1042/bj2260645

Nallet, H., MacKenzie, E., & Roussel, S. (1999). The nature of penumbral depolarizations following focal

cerebral ischemia in the rat. *Brain Research*, *842*(1), 148–158. https://doi.org/10.1016/S0006-8993(99)01859-4

Navarro, G., Aymerich, M. S., Marcellino, D., Cortés, A., Casadó, V., Mallol, J., … Franco, R. (2009). Interactions between calmodulin, adenosine A2A, and dopamine D2 receptors. *Journal of Biological Chemistry*, *284*(41). https://doi.org/10.1074/jbc.M109.034231

Nedergaard, M., Vorstrup, S., & Astrup, J. (1986). Cell density in the border zone around old small human brain infarcts. *Stroke*, *17*(6), 1129–1137. https://doi.org/10.1161/01.STR.17.6.1129

Neri, D., & Supuran, C. T. (2011). Interfering with pH regulation in tumours as a therapeutic strategy. *Nature Reviews Drug Discovery*, Vol. 10. https://doi.org/10.1038/nrd3554

Nishizaki, T., Nagai, K., Nomura, T., Tada, H., Kanno, T., Tozaki, H., … Saito, N. (2002). A new neuromodulatory pathway with a glial contribution mediated via A2a adenosine receptors. *GLIA*, *39*(2). https://doi.org/10.1002/glia.10100

Nocentini, A., Angeli, A., Carta, F., Winum, J. Y., Zalubovskis, R., Carradori, S., … Supuran, C. T. (2021). Reconsidering anion inhibitors in the general context of drug design studies of modulators of activity of the classical enzyme carbonic anhydrase. *Journal of Enzyme Inhibition and Medicinal Chemistry*, Vol. 36. https://doi.org/10.1080/14756366.2021.1882453

Nocentini, A., Ferraroni, M., Carta, F., Ceruso, M., Gratteri, P., Lanzi, C., … Supuran, C. T. (2016). Benzenesulfonamides Incorporating Flexible Triazole Moieties Are Highly Effective Carbonic Anhydrase Inhibitors: Synthesis and Kinetic, Crystallographic, Computational, and Intraocular Pressure Lowering Investigations. *Journal of Medicinal Chemistry*, *59*(23), 10692–10704. https://doi.org/10.1021/acs.jmedchem.6b01389

Nonaka, H., Ichimura, M., Takeda, M., Nonaka, Y., Shimada, J., Suzuki, F., … Kase, H. (1994). KF17837 ((E)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxanthine) a potent and selective adenosine A2 receptor antagonist. *European Journal of Pharmacology: Molecular Pharmacology*, *267*(3). https://doi.org/10.1016/0922-4106(94)90159-7

Noshita, N., Sugawara, T., Fujimura, M., Morita-Fujimura, Y., & Chan, P. H. (2001). Manganese superoxide dismutase affects cytochrome c release and caspase-9 activation after transient focal cerebral ischemia in mice. *Journal of Cerebral Blood Flow and Metabolism*, *21*(5). https://doi.org/10.1097/00004647-200105000-00010

Nozaki, K., Nishimura, M., & Hashimoto, N. (2001). Mitogen-activated protein kinases and cerebral ischemia. *Molecular Neurobiology*, Vol. 23. https://doi.org/10.1385/mn:23:1:01

Nuñez-Figueredo, Y., Ramírez-Sánchez, J., Hansel, G., Simões Pires, E. N., Merino, N., Valdes, O., … Pardo-Andreu, G. L. (2014). A novel multi-target ligand (JM-20) protects mitochondrial integrity, inhibits brain excitatory amino acid release and reduces cerebral ischemia injury in vitro and in vivo. *Neuropharmacology*, *85*. https://doi.org/10.1016/j.neuropharm.2014.06.009

Obara, M., Szeliga, M., & Albrecht, J. (2008). Regulation of pH in the mammalian central nervous system under normal and pathological conditions: Facts and hypotheses. *Neurochemistry International*, Vol. 52, pp. 905–919. https://doi.org/10.1016/j.neuint.2007.10.015

Odashima, M., Bamias, G., Rivera-Nieves, J., Linden, J., Nast, C. C., Moskaluk, C. A., … Cominelli, F. (2005). Activation of A2A adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease. *Gastroenterology*, *129*(1). https://doi.org/10.1053/j.gastro.2005.05.032

Ohta, K., Graf, R., Rosner, G., & Heiss, W. D. (2001). Calcium ion transients in peri-infarct depolarizations may deteriorate ion homeostasis and expand infarction in focal cerebral ischemia in cats. *Stroke*, *32*(2). https://doi.org/10.1161/01.STR.32.2.535

Okada, M., Nutt, D. J., Murakami, T., Zhu, G., Kamata, A., Kawata, Y., & Kaneko, S. (2001). Adenosine receptor subtypes modulate two major functional pathways for hippocampal serotonin release. *Journal of Neuroscience*, *21*(2). https://doi.org/10.1523/jneurosci.21-02-00628.2001

Okada, Y., Copeland, B. R., Mori, E., Tung, M. M., Thomas, W. S., & del Zoppo, G. J. (1994). P-Selectin and intercellular adhesion Molecule-1 expression after focal brain ischemia and reperfusion. *Stroke*, *25*(1). https://doi.org/10.1161/01.str.25.1.202

Okasa, Y., & Ozawa, S. (1980). Inhibitory action of adenosine on synaptic transmission in the hippocampus of the guinea pig in vitro. *European Journal of Pharmacology*, *68*(4). https://doi.org/10.1016/0014-2999(80)90424-0

Ongini, E., Adami, M., Ferri, C., & Bertorelli, R. (1997). Adenosine A(2A) receptors and neuroprotection. *Annals of the New York Academy of Sciences*, *825*. https://doi.org/10.1111/j.1749-6632.1997.tb48412.x

Ozkan, O. V., Yuzbasioglu, M. F., Ciralik, H., Kurutas, E. B., Yonden, Z., Aydin, M., … Duran, N. (2009). Resveratrol, a natural antioxidant attenuates intestinal ischemia/reperfusion injury in rats. *Tohoku Journal of Experimental Medicine*, *218*(3). https://doi.org/10.1620/tjem.218.251

Pak, M. A., Haas, H. L., Decking, U. K. M., & Schrader, J. (1994). Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. *Neuropharmacology*, *33*(9). https://doi.org/10.1016/0028-3908(94)90142-2

Papadopoulos, M. C., & Verkman, A. S. (2007). Aquaporin-4 and brain edema. *Pediatric Nephrology*, Vol. 22, pp. 778–784. https://doi.org/10.1007/s00467-006-0411-0

Patel, A., Craig, R. H., Daluge, S. M., & Linden, J. (1988). 125I-BW-A844U, an antagonist radioligand with high affinity and selectivity for adenosine A1 receptors, and 125I-azido-BW-A844U, a photoaffinity label. *Molecular Pharmacology*, *33*(6).

Paterniti, I., Melani, A., Cipriani, S., Corti, F., Mello, T., Mazzon, E., … Pedata, F. (2011). Selective adenosine A2Areceptor agonists and antagonists protect against spinal cord injury through peripheral and central effects. *Journal of Neuroinflammation*, *8*. https://doi.org/10.1186/1742-2094-8-31

Pazzagli, M., Pedata, F., & Pepeu, G. (1993). Effect of K+ depolarization, tetrodotoxin, and NMDA receptor inhibition on extracellular adenosine levels in rat striatum. *European Journal of Pharmacology*, *234*(1). https://doi.org/10.1016/0014-2999(93)90706-N

Peakman, M. -C, & Hill, S. J. (1994). Adenosine A2B-receptor-mediated cyclic AMP accumulation in primary rat astrocytes. *British Journal of Pharmacology*, *111*(1). https://doi.org/10.1111/j.1476-5381.1994.tb14043.x

Pearson, T., Damian, K., Lynas, R. E., & Frenguelli, B. G. (2006). Sustained elevation of extracellular adenosine and activation of A 1 receptors underlie the post-ischaemic inhibition of neuronal function in rat hippocampus in vitro. *Journal of Neurochemistry*, *97*(5). https://doi.org/10.1111/j.1471-4159.2006.03823.x

Pedata, F., Dettori, I., Coppi, E., Melani, A., Fusco, I., Corradetti, R., & Pugliese, A. M. (2016). Purinergic signalling in brain ischemia. *Neuropharmacology*. https://doi.org/10.1016/j.neuropharm.2015.11.007

Pedata, F., Latini, S., Pugliese, A. M., & Pepeu, G. (1993). Investigations into the Adenosine Outflow from Hippocampal Slices Evoked by Ischemia-Like Conditions. *Journal of Neurochemistry*, *61*(1), 284–289. https://doi.org/10.1111/j.1471-4159.1993.tb03566.x

Pedata, F., Pazzagli, M., Tilli, S., & Pepeu, G. (1990). Regional differences in the electrically stimulated release of endogenous and radioactive adenosine and purine derivatives from rat brain slices. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *342*(4). https://doi.org/10.1007/BF00169463

Pedata, F., Pepeu, G., & Spignoli, G. (1984). Biphasic effect of methylxanthines on acetylcholine release from electrically-stimulated brain slices. *British Journal of Pharmacology*, *83*(1). https://doi.org/10.1111/j.1476-5381.1984.tb10120.x

Pedata, F., Pugliese, A. M., Coppi, E., Dettori, I., Maraula, G., Cellai, L., & Melani, A. (2014). Adenosine A 2A receptors modulate acute injury and neuroinflammation in brain ischemia. *Mediators of Inflammation*. https://doi.org/10.1155/2014/805198

Pedata, F., Pugliese, A. M., Sebastião, A. M., & Ribeiro, J. A. (2010). Adenosine A3 receptor signaling in the central nervous system. In *A3 Adenosine Receptors from Cell Biology to Pharmacology and Therapeutics*. https://doi.org/10.1007/978-90-481-3144-0_9

Pei, Z., Pang, S. F., & Cheung, R. T. F. (2002). Pretreatment with melatonin reduces volume of cerebral infarction in a rat middle cerebral artery occlusion stroke model. *Journal of Pineal Research*, *32*(3). https://doi.org/10.1034/j.1600-079x.2002.1o847.x

Pettersen, E. O., Ebbesen, P., Gieling, R. G., Williams, K. J., Dubois, L., Lambin, P., … Cojocari, D. (2015). Targeting tumour hypoxia to prevent cancer metastasis. From biology, biosensing and technology to drug development: The METOXIA consortium. *Journal of Enzyme Inhibition and Medicinal Chemistry*, Vol. 30, pp. 689–721. https://doi.org/10.3109/14756366.2014.966704

Phillis, J. W., Edstrom, J. P., Kostopoulos, G. K., & Kirkpatrick, J. R. (1979). Effects of adenosine and adenine

nucleotides on synaptic transmission in the cerebral cortex. *Canadian Journal of Physiology and Pharmacology*, *57*(11). https://doi.org/10.1139/y79-194

Phillis, J. W., Smith-Barbour, M., O'Regan, M. H., & Perkins, L. M. (1994). Amino acid and purine release in rat brain following temporary middle cerebral artery occlusion. *Neurochemical Research*, *19*(9). https://doi.org/10.1007/BF00965145

Phillis, John W. (2004). Adenosine and adenine nucleotides as regulators of cerebral blood flow: Roles of acidosis, cell swelling, and KATP channels. *Critical Reviews in Neurobiology*, Vol. 16. https://doi.org/10.1615/CritRevNeurobiol.v16.i4.20

Phillis, John W., O'Regan, M. H., & Perkins, L. M. (1993). Effect of adenosine receptor agonists on spontaneous and K+-evoked acetylcholine release from the in vivo rat cerebral cortex. *Brain Research*, *605*(2). https://doi.org/10.1016/0006-8993(93)91753-F

Piao, C. S., Kim, J. Bin, Han, P. L., & Lee, J. K. (2003). Administration of the p38 MAPK inhibitor SB203580 affords brain protection with a wide therapeutic window against focal ischemic insult. *Journal of Neuroscience Research*, *73*(4). https://doi.org/10.1002/jnr.10671

Pickkers, P., Hughes, A. D., Russel, F. G. M., Thien, T., & Smits, P. (2001). In vivo evidence for KCa channel opening properties of acetazolamide in the human vasculature. *British Journal of Pharmacology*, *132*(2), 443–450. https://doi.org/10.1038/sj.bjp.0703825

Pinard, M. A., Boone, C. D., Rife, B. D., Supuran, C. T., & McKenna, R. (2013). Structural study of interaction between brinzolamide and dorzolamide inhibition of human carbonic anhydrases. *Bioorganic and Medicinal Chemistry*, *21*(22). https://doi.org/10.1016/j.bmc.2013.08.033

Pinto-Duarte, A., Coelho, J. E., Cunha, R. A., Ribeiro, J. A., & Sebastião, A. M. (2005). Adenosine A2A receptors control the extracellular levels of adenosine through modulation of nucleoside transporters activity in the rat hippocampus. *Journal of Neurochemistry*, *93*(3). https://doi.org/10.1111/j.1471-4159.2005.03071.x

Pintor, A., Galluzzo, M., Grieco, R., Pèzzola, A., Reggio, R., & Popoli, P. (2004). Adenosine A2A receptor antagonists prevent the increase in striatal glutamate levels induced by glutamate uptake inhibitors. *Journal of Neurochemistry*, *89*(1). https://doi.org/10.1111/j.1471-4159.2003.02306.x

Pittaluga, A., Segantini, D., Feligioni, M., & Raiteri, M. (2005). Extracellular protons differentially potentiate the responses of native AMPA receptor subtypes regulating neurotransmitter release. *British Journal of Pharmacology*, *144*(2), 293–299. https://doi.org/10.1038/sj.bjp.0705960

Plum, F. (1983). What causes infarction in ischemic brain?: The Robert Wartenberg Lecture. *Neurology*, *33*(2), 222–233. https://doi.org/10.1212/wnl.33.2.222

Porter, N. A. (1984). [32] Chemistry of Lipid Peroxidation. *Methods in Enzymology*, *105*(C). https://doi.org/10.1016/S0076-6879(84)05035-7

Press, N. J., Taylor, R. J., Fullerton, J. D., Tranter, P., McCarthy, C., Keller, T. H., … Fozard, J. R. (2005). A new

orally bioavailable dual adenosine A2B/A3 receptor antagonist with therapeutic potential. *Bioorganic and Medicinal Chemistry Letters*, *15*(12). https://doi.org/10.1016/j.bmcl.2005.04.021

Price, T., Sheibani, N., & Shah, G. (2017). Regulation of high glucose-induced apoptosis of brain pericytes by mitochondrial CA VA: A specific target for prevention of diabetic cerebrovascular pathology. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1863*(4), 929–935. https://doi.org/10.1016/j.bbadis.2017.01.025

Prince, D. A., & Stevens, C. F. (1992). Adenosine decreases neurotransmitter release at central synapses. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(18). https://doi.org/10.1073/pnas.89.18.8586

Proctor, W. R., & Dunwiddie, T. V. (1987). Pre- and postsynaptic actions of adenosine in the in vitro rat hippocampus. *Brain Research*, *426*(1). https://doi.org/10.1016/0006-8993(87)90441-0

Provensi, G., Carta, F., Nocentini, A., Supuran, C. T., Casamenti, F., Passani, M. B., & Fossati, S. (2019). A new kid on the block? Carbonic anhydrases as possible new targets in alzheimer's disease. *International Journal of Molecular Sciences*, Vol. 20, p. 4724. https://doi.org/10.3390/ijms20194724

Puffinbarger, N. K., Hansen, K. R., Resta, R., Laurent, A. B., Knudsen, T. B., Madara, J. L., & Thompson, L. F. (1995). Production and characterization of multiple antigenic peptide antibodies to the adenosine A(2b) receptor. *Molecular Pharmacology*, *47*(6).

Pugliese, A. M., Traini, C., Cipriani, S., Gianfriddo, M., Mello, T., Giovannini, M. G., … Pedata, F. (2009). The adenosine A 2A receptor antagonist ZM241385 enhances neuronal survival after oxygen-glucose deprivation in rat CA1 hippocampal slices. *British Journal of Pharmacology*, *157*(5). https://doi.org/10.1111/j.1476-5381.2009.00218.x

Pugliese, Anna Maria, Coppi, E., Spalluto, G., Corradetti, R., & Pedata, F. (2006). A 3 adenosine receptor antagonists delay irreversible synaptic failure caused by oxygen and glucose deprivation in the rat CA1 hippocampus in vitro. *British Journal of Pharmacology*, *147*(5), 524–532. https://doi.org/10.1038/sj.bjp.0706646

Pugliese, Anna Maria, Latini, S., Corradetti, R., & Pedata, F. (2003). Brief, repeated, oxygen-glucose deprivation episodes protect neurotransmission from a longer ischemic episode in the in vitro hippocampus: Role of adenosine receptors. *British Journal of Pharmacology*, *140*(2). https://doi.org/10.1038/sj.bjp.0705442

Pugliese, Anna Maria, Trincavelli, M. L., Lecca, D., Coppi, E., Fumagalli, M., Ferrario, S., … Abbracchio, M. P. (2009). Functional characterization of two isoforms of the P2Y-like receptor GPR17: [35S]GTPγS binding and electrophysiological studies in 1321N1 cells. *American Journal of Physiology - Cell Physiology*, *297*(4). https://doi.org/10.1152/ajpcell.00658.2008

Pullagurla, S. R., Baird, A. E., Adamski, M. G., & Soper, S. A. (2015). Current and future bioanalytical approaches for stroke assessment. *Bioanalysis*, Vol. 7. https://doi.org/10.4155/bio.15.40

Qin, A. P., Liu, C. F., Qin, Y. Y., Hong, L. Z., Xu, M., Yang, L., … Zhang, H. L. (2010). Autophagy was activated in injured astrocytes and mildly decreased cell survival following glucose and oxygen deprivation and focal cerebral ischemia. *Autophagy*, *6*(6). https://doi.org/10.4161/auto.6.6.12573

Rabinstein, A. A. (2020). Update on Treatment of Acute Ischemic Stroke. *CONTINUUM Lifelong Learning in Neurology*, Vol. 26, pp. 268–286. https://doi.org/10.1212/CON.0000000000000840

Rama, R., & Garcia Rodriguez, J. C. (2012). Excitotoxicity and Oxidative Stress in Acute Ischemic Stroke. In *Acute Ischemic Stroke*. https://doi.org/10.5772/28300

Ramsay, R. R., Popovic-Nikolic, M. R., Nikolic, K., Uliassi, E., & Bolognesi, M. L. (2018). A perspective on multi-target drug discovery and design for complex diseases. *Clinical and Translational Medicine*, *7*(1). https://doi.org/10.1186/s40169-017-0181-2

Rasmussen, J. K., & Boedtkjer, E. (2018). Carbonic anhydrase inhibitors modify intracellular pH transients and contractions of rat middle cerebral arteries during CO 2 /HCO 3– fluctuations. *Journal of Cerebral Blood Flow and Metabolism*, *38*(3), 492–505. https://doi.org/10.1177/0271678X17699224

Rebola, N., Lujan, R., Cunha, R. A., & Mulle, C. (2008). Adenosine A2A Receptors Are Essential for Long-Term Potentiation of NMDA-EPSCs at Hippocampal Mossy Fiber Synapses. *Neuron*, *57*(1). https://doi.org/10.1016/j.neuron.2007.11.023

Rebola, N., Oliveira, C. R., & Cunha, R. A. (2002). Transducing system operated by adenosine A2A receptors to facilitate acetylcholine release in the rat hippocampus. *European Journal of Pharmacology*, *454*(1). https://doi.org/10.1016/S0014-2999(02)02475-5

Regli, F., Yamaguchi, T., & Walz, A. (1971). Effects of Acetazolamide on Cerebral Ischemia and Infarction After Experimental Occlusion of Middle Cerebral Artery. *Stroke*, *2*(5), 456–460. https://doi.org/10.1161/01.STR.2.5.456

Resta, R., Yamashita, Y., & Thompson, L. F. (1998). Ecto-enzyme and signaling functions of lymphocyte CD73. *Immunological Reviews*, Vol. 161. https://doi.org/10.1111/j.1600-065X.1998.tb01574.x

Ribeiro, J. A., Sebastião, A. M., & De Mendonça, A. (2002). Adenosine receptors in the nervous system: Pathophysiological implications. *Progress in Neurobiology*, Vol. 68. https://doi.org/10.1016/S0301-0082(02)00155-7

Rivkees, S. A., Price, S. L., & Zhou, F. C. (1995). Immunohistochemical detection of A1 adenosine receptors in rat brain with emphasis on localization in the hippocampal formation, cerebral cortex, cerebellum, and basal ganglia. *Brain Research*, *677*(2). https://doi.org/10.1016/0006-8993(95)00062-U

Rogato, A., Del Prete, S., Nocentini, A., Carginale, V., Supuran, C. T., & Capasso, C. (2019). Phaeodactylum tricornutum as a model organism for testing the membrane penetrability of sulphonamide carbonic anhydrase inhibitors. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *34*(1), 510–518. https://doi.org/10.1080/14756366.2018.1559840

Rosin, D. L., Hettinger, B. D., Lee, A., & Linden, J. (2003). Anatomy of adenosine A2A receptors in brain:

Morphological substrates for integration of striatal function. *Neurology*, *61*(11 SUPPL. 6). https://doi.org/10.1212/01.wnl.0000095205.33940.99

Rosin, D. L., Robeva, A., Woodard, R. L., Guyenet, P. G., & Linden, J. (1998). Immunohistochemical localization of adenosine A2A receptors in the rat central nervous system. *The Journal of Comparative Neurology*, *401*(2). https://doi.org/10.1002/(sici)1096-9861(19981116)401:2<163::aid-cne2>3.3.co;2-4

Roth, B. L., Sheffer, D. J., & Kroeze, W. K. (2004). Magic shotguns versus magic bullets: Selectively non-selective drugs for mood disorders and schizophrenia. *Nature Reviews Drug Discovery*, Vol. 3. https://doi.org/10.1038/nrd1346

Röther, J., De Crespigny, A. J., D'Arceuil, H., & Moseley, M. E. (1996). MR detection of cortical spreading depression immediately after focal ischemia in the rat. *Journal of Cerebral Blood Flow and Metabolism*, *16*(2), 214–220. https://doi.org/10.1097/00004647-199603000-00005

Rousselet, E., Kriz, J., & Seidah, N. G. (2012). Mouse model of intraluminal MCAO: cerebral infarct evaluation by cresyl violet staining. *Journal of Visualized Experiments : JoVE*, (69). https://doi.org/10.3791/4038

Ruehl, M. L., Orozco, J. A., Stoker, M. B., McDonagh, P. F., Coull, B. M., & Ritter, L. S. (2002). Protective effects of inhibiting both blood and vascular selectins after stroke and reperfusion. *Neurological Research*, *24*(3). https://doi.org/10.1179/016164102101199738

Sacco, R. L., Ellenberg, J. H., Mohr, J. P., Tatemichi, T. K., Hier, D. B., Price, T. R., & Wolf, P. A. (1989). Infarcts of undetermined cause: The NINCDS stroke data bank. *Annals of Neurology*, *25*(4). https://doi.org/10.1002/ana.410250410

Sairanen, T., Carpén, O., Karjalainen-Lindsberg, M. L., Paetau, A., Turpeinen, U., Kaste, M., & Lindsberg, P. J. (2001). Evolution of cerebral tumor necrosis factor-α production during human ischemic stroke. *Stroke*, *32*(8). https://doi.org/10.1161/01.str.32.8.1750

Saito, A., Hayashi, T., Okuno, S., Nishi, T., & Chan, P. H. (2004). Modulation of the Omi/HtrA2 signaling pathway after transient focal cerebral ischemia in mouse brains that overexpress SOD1. *Molecular Brain Research*, *127*(1–2). https://doi.org/10.1016/j.molbrainres.2004.05.012

Sala-Newby, G. B., Freeman, N. V. E., Skladanowski, A. C., & Newby, A. C. (2000). Distinct roles for recombinant cytosolic 5'-nucleotidase-I and -II in AMP and IMP catabolism in COS-7 and H9c2 rat myoblast cell lines. *Journal of Biological Chemistry*, *275*(16). https://doi.org/10.1074/jbc.275.16.11666

Sala-Newby, G. B., & Newby, A. C. (2001). Cloning of a mouse cytosolic 5'-nucleotidase-I identifies a new gene related to human autoimmune infertility-related protein. *Biochimica et Biophysica Acta - Gene Structure and Expression*, *1521*(1–3). https://doi.org/10.1016/S0167-4781(01)00278-0

Salameh, T., Shah, G., Price, T., Hayden, M., & Banks, W. (2016). Blood-brain barrier disruption and neurovascular unit dysfunction in diabetic mice: Protection with the mitochondrial carbonic

anhydrase inhibitor topiramate. *Journal of Pharmacology and Experimental Therapeutics*, *359*(3), 452–459. https://doi.org/10.1124/jpet.116.237057

Sánchez-Moreno, C., Dashe, J. F., Scott, T., Thaler, D., Folstein, M. F., & Martin, A. (2004). Decreased Levels of Plasma Vitamin C and Increased Concentrations of Inflammatory and Oxidative Stress Markers after Stroke. *Stroke*, *35*(1). https://doi.org/10.1161/01.STR.0000105391.62306.2E

Sarges, R., Howard, H. R., Browne, R. G., Lebel, L. A., Seymour, P. A., & Koe, B. K. (1990). 4-Amino[1,2,4]triazolo[4,3-a]quinoxalines. A Novel Class of Potent Adenosine Receptor Antagonists and Potential Rapid-Onset Antidepressants. *Journal of Medicinal Chemistry*, *33*(8). https://doi.org/10.1021/jm00170a031

Sattin, A., & Rall, T. W. (1970). The effect of adenosine and adenine nucleotides on the cyclic adenosine 3', 5'-phosphate content of guinea pig cerebral cortex slices. *Molecular Pharmacology*, *6*(1).

Schiffmann, S. N., Libert, F., Vassart, G., & Vanderhaeghen, J. J. (1991). Distribution of adenosine A2 receptor mRNA in the human brain. *Neuroscience Letters*, *130*(2). https://doi.org/10.1016/0304-3940(91)90391-6

Schindler, M., Harris, C. A., Hayes, B., Papotti, M., & Humphrey, P. P. A. (2001). Immunohistochemical localization of adenosine A1 receptors in human brain regions. *Neuroscience Letters*, *297*(3). https://doi.org/10.1016/S0304-3940(00)01643-8

Schoen, S. W., Kreutzberg, G. W., & Singer, W. (1993). Cytochemical Redistribution of 5'-Nucleotidase in the Developing Cat Visual Cortex. *European Journal of Neuroscience*, *5*(3). https://doi.org/10.1111/j.1460-9568.1993.tb00487.x

Schulte, G., & Fredholm, B. B. (2003). Signalling from adenosine receptors to mitogen-activated protein kinases. *Cellular Signalling*, Vol. 15. https://doi.org/10.1016/S0898-6568(03)00058-5

Schwabe, U., Ebert, R., & Erbler, H. C. (1975). Adenosine release from fat cells: effect on cyclic AMP levels and hormone actions. *Advances in Cyclic Nucleotide Research*, *5*.

Schwartz, M. (2003). Macrophages and microglia in central nervous system injury: Are they helpful or harmful? *Journal of Cerebral Blood Flow and Metabolism*, Vol. 23. https://doi.org/10.1097/01.WCB.0000061881.75234.5E

Schwarzschild, M. A., Chen, J. F., & Ascherio, A. (2002). Caffeinated clues and the promise of adenosine A2a antagonists in PD. *Neurology*, Vol. 58. https://doi.org/10.1212/WNL.58.8.1154

Sciotti, V. M., Roche, F. M., Grabb, M. C., & Van Wylen, D. G. L. (1992). Adenosine receptor blockade augments interstitial fluid levels of excitatory amino acids during cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism*, *12*(4). https://doi.org/10.1038/jcbfm.1992.89

Sebastião, A. M., & Ribeiro, J. A. (1996). Adenosine A2 receptor-mediated excitatory actions on the nervous system. *Progress in Neurobiology*, Vol. 48. https://doi.org/10.1016/0301-0082(95)00035-6

Sebastião, Ana M., & Ribeiro, J. A. (2009). Triggering neurotrophic factor actions through adenosine A2A

receptor activation: Implications for neuroprotection. *British Journal of Pharmacology*, Vol. 158. https://doi.org/10.1111/j.1476-5381.2009.00157.x

Shneyvays, V., Leshem, D., Zinman, T., Mamedova, L. K., Jacobson, K. A., & Shainberg, A. (2005). Role of adenosine A1 and A3 receptors in regulation of cardiomyocyte homeostasis after mitochondrial respiratory chain injury. *American Journal of Physiology - Heart and Circulatory Physiology*, *288*(6 57-6). https://doi.org/10.1152/ajpheart.01157.2004

Shneyvays, V., Zinman, T., & Shainberg, A. (2004). Analysis of calcium responses mediated by the A3 adenosine receptor in cultured newborn rat cardiac myocytes. *Cell Calcium*, *36*(5). https://doi.org/10.1016/j.ceca.2004.03.004

Shook, B. C., Rassnick, S., Osborne, M. C., Davis, S., Westover, L., Boulet, J., … Jackson, P. F. (2010). In vivo characterization of a dual adenosine A2A/A1 receptor antagonist in animal models of Parkinson's disease. *Journal of Medicinal Chemistry*, *53*(22). https://doi.org/10.1021/jm100971t

Shyu, K. G., Chang, H., & Lin, C. C. (1997). Serum levels of intercellular adhesion molecule-1 and E-selectin in patients with acute ischaemic stroke. *Journal of Neurology*, *244*(2). https://doi.org/10.1007/s004150050055

Silberstein, S. D. (2017). Topiramate in Migraine Prevention: A 2016 Perspective. *Headache*, *57*(1), 165–178. https://doi.org/10.1111/head.12997

Silverman, D. N., & Lindskog, S. (1988). The Catalytic Mechanism of Carbonic Anhydrase: Implications of a Rate-Limiting Protolysis of Water. *Accounts of Chemical Research*, *21*(1). https://doi.org/10.1021/ar00145a005

Sitkovsky, M. V. (2003). Use of the A 2A adenosine receptor as a physiological immunosuppressor and to engineer inflammation in vivo. *Biochemical Pharmacology*, Vol. 65. https://doi.org/10.1016/S0006-2952(02)01548-4

Sitkovsky, M. V., Lukashev, D., Apasov, S., Kojima, H., Koshiba, M., Caldwell, C., … Thiel, M. (2004). Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annual Review of Immunology*, Vol. 22. https://doi.org/10.1146/annurev.immunol.22.012703.104731

Sollevi, A. (1986). Cardiovascular effects of adenosine in man; possible clinical implications. *Progress in Neurobiology*, Vol. 27. https://doi.org/10.1016/0301-0082(86)90005-5

Somjen, G. G. (2001). Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. *Physiological Reviews*, Vol. 81. https://doi.org/10.1152/physrev.2001.81.3.1065

Spera, P. A., Ellison, J. A., Feuerstein, G. Z., & Barone, F. C. (1998). IL-10 reduces rat brain injury following focal stroke. *Neuroscience Letters*, *251*(3). https://doi.org/10.1016/S0304-3940(98)00537-0

Spignoli, G., Pedata, F., & Pepeu, G. (1984). A1 and A2 adenosine receptors modulate acetylcholine release from brain slices. *European Journal of Pharmacology*, *97*(3–4). https://doi.org/10.1016/0014-

2999(84)90475-8

Spychala, J. (2000). Tumor-promoting functions of adenosine. *Pharmacology and Therapeutics*, *87*(2–3). https://doi.org/10.1016/S0163-7258(00)00053-X

Stanimirovic, D., & Satoh, K. (2000). Inflammatory mediators of cerebral endothelium: A role in ischemic brain inflammation. *Brain Pathology*, *10*(1). https://doi.org/10.1111/j.1750-3639.2000.tb00248.x

Stevens, B., Porta, S., Haak, L. L., Gallo, V., & Fields, R. D. (2002). Adenosine: A neuron-glial transmitter promoting myelination in the CNS in response to action potentials. *Neuron*, *36*(5). https://doi.org/10.1016/S0896-6273(02)01067-X

Stewart, M., Steinig, A. G., Ma, C., Song, J. P., McKibben, B., Castelhano, A. L., & MacLennan, S. J. (2004). [3H]OSIP339391, a selective, novel, and high affinity antagonist radioligand for adenosine A2B receptors. *Biochemical Pharmacology*, *68*(2). https://doi.org/10.1016/j.bcp.2004.03.026

Stiehl, D. P., Wirthner, R., Köditz, J., Spielmann, P., Camenisch, G., & Wenger, R. H. (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels: Evidence for an autoregulatory oxygen-sensing system. *Journal of Biological Chemistry*, *281*(33), 23482–23491. https://doi.org/10.1074/jbc.M601719200

Stoll, G., Jander, S., & Schroeter, M. (1998). Inflammation and glial responses in ischemic brain lesions. *Progress in Neurobiology*, *56*(2). https://doi.org/10.1016/S0301-0082(98)00034-3

Stone, T. W., & Behan, W. M. H. (2007). Interleukin-1β but not tumor necrosis factor-α potentiates neuronal damage by quinolinic acid: protection by an adenosine A2A receptor antagonist. *Journal of Neuroscience Research*, *85*(5). https://doi.org/10.1002/jnr.21212

Strong, A. J., Harland, S. P., Meldrum, B. S., & Whittington, D. J. (1996). The use of in vivo fluorescence image sequences to indicate the occurrence and propagation of transient focal depolarizations in cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism*, *16*(3), 367–377. https://doi.org/10.1097/00004647-199605000-00003

Sudlow, C. L. M., & Warlow, C. P. (1997). Comparable studies of the incidence of stroke and its pathological types: Results from an international collaboration. *Stroke*, *28*(3). https://doi.org/10.1161/01.STR.28.3.491

Suen, D. F., Norris, K. L., & Youle, R. J. (2008). Mitochondrial dynamics and apoptosis. *Genes and Development*, Vol. 22. https://doi.org/10.1101/gad.1658508

Sugawara, T., Noshita, N., Lewen, A., Gasche, Y., Ferrand-Drake, M., Fujimura, M., … Chan, P. H. (2002). Overexpression of copper/zinc superoxide dismutase in transgenic rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. *Journal of Neuroscience*, *22*(1). https://doi.org/10.1523/jneurosci.22-01-00209.2002

Sughrue, M. E., Mehra, A., Connolly, E. S., & D'Ambrosio, A. L. (2004). Anti-adhesion molecule strategies as potential neuroprotective agents in cerebral ischemia: A critical review of the literature. *Inflammation*

Research, Vol. 53. https://doi.org/10.1007/s00011-004-1282-0

Sugino, T., Nozaki, K., & Hashimoto, N. (2000). Activation of mitogen-activated protein kinases in gerbil hippocampus with ischemic tolerance induced by 3-nitropropionic acid. *Neuroscience Letters*, *278*(1–2). https://doi.org/10.1016/S0304-3940(99)00906-4

Sun, Y., & Huang, P. (2016). Adenosine A2B receptor: From cell biology to human diseases. *Frontiers in Chemistry*, Vol. 4. https://doi.org/10.3389/fchem.2016.00037

Supuran, C. (2007). Carbonic Anhydrases as Drug Targets - An Overview. *Current Topics in Medicinal Chemistry*, *7*(9). https://doi.org/10.2174/156802607780636690

Supuran, C. T. (2008). Carbonic anhydrases: Novel therapeutic applications for inhibitors and activators. *Nature Reviews Drug Discovery*, Vol. 7. https://doi.org/10.1038/nrd2467

Supuran, C. T. (2015). Acetazolamide for the treatment of idiopathic intracranial hypertension. *Expert Review of Neurotherapeutics*. https://doi.org/10.1586/14737175.2015.1066675

Supuran, C. T. (2016a). Carbonic anhydrase inhibition and the management of neuropathic pain. *Expert Review of Neurotherapeutics*, pp. 961–968. https://doi.org/10.1080/14737175.2016.1193009

Supuran, C. T. (2016b). How many carbonic anhydrase inhibition mechanisms exist? *Journal of Enzyme Inhibition and Medicinal Chemistry*, Vol. 31. https://doi.org/10.3109/14756366.2015.1122001

Supuran, C. T. (2018a). Applications of carbonic anhydrases inhibitors in renal and central nervous system diseases. *Expert Opinion on Therapeutic Patents*, pp. 1744–7674. https://doi.org/10.1080/13543776.2018.1519023

Supuran, C. T. (2018b). Carbonic anhydrase inhibitors and their potential in a range of therapeutic areas. *Expert Opinion on Therapeutic Patents*, pp. 709–712. https://doi.org/10.1080/13543776.2018.1523897

Supuran, C. T., Altamini, A., & Carta, F. (2019). Carbonic anhydrase inhibition and the management of glaucoma: a literature and patent review 2013-2019. *Expert Opinion on Therapeutic Patents*, Vol. 29, pp. 781–792. https://doi.org/10.1080/13543776.2019.1679117

Supuran, C. T., & Scozzafava, A. (2002). Applications of carbonic anhydrase inhibitors and activators in therapy. *Expert Opinion on Therapeutic Patents*, Vol. 12. https://doi.org/10.1517/13543776.12.2.217

Svenningsson, P., Hall, H., Sedvall, G., & Fredholm, B. B. (1997). Distribution of adenosine receptors in the postmortem human brain: An extended autoradiographic study. *Synapse*, *27*(4). https://doi.org/10.1002/(SICI)1098-2396(199712)27:4<322::AID-SYN6>3.0.CO;2-E

Svenningsson, P., Le Moine, C., Fisone, G., & Fredholm, B. B. (1999). Distribution, biochemistry and function of striatal adenosine A(2A) receptors. *Progress in Neurobiology*, Vol. 59. https://doi.org/10.1016/S0301-0082(99)00011-8

Svichar, N., Esquenazi, S., Waheed, A., Sly, W. S., & Chesler, M. (2006). Functional demonstration of surface carbonic anhydrase IV activity on rat astrocytes. *GLIA*, *53*(3), 241–247.

https://doi.org/10.1002/glia.20277

Swanson, T. H., Drazba, J. A., & Rivkees, S. A. (1995). Adenosine A1 receptors are located predominantly on axons in the rat hippocampal formation. *Journal of Comparative Neurology*, *363*(4). https://doi.org/10.1002/cne.903630402

Swietach, P., Vaughan-Jones, R. D., & Harris, A. L. (2007). Regulation of tumor pH and the role of carbonic anhydrase 9. *Cancer and Metastasis Reviews*, Vol. 26. https://doi.org/10.1007/s10555-007-9064-0

Szabó, C., Scott, G. S., Virág, L., Egnaczyk, G., Salzman, A. L., Shanley, T. P., & Haskó, G. (1998). Suppression of macrophage inflammatory protein (MIP)-1α production and collagen-induced arthritis by adenosine receptor agonists. *British Journal of Pharmacology*, *125*(2). https://doi.org/10.1038/sj.bjp.0702040

Szigeti, K., Horváth, I., Veres, D. S., Martinecz, B., Lénárt, N., Kovács, N., … Dénes, Á. (2015). A novel SPECT-based approach reveals early mechanisms of central and peripheral inflammation after cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism*, *35*(12). https://doi.org/10.1038/jcbfm.2015.174

Takagi, Y., Nozaki, K., Sugino, T., Hattori, I., & Hashimoto, N. (2000). Phosphorylation of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase after transient forebrain ischemia in mice. *Neuroscience Letters*, *294*(2). https://doi.org/10.1016/S0304-3940(00)01552-4

Takahashi, M., Fujita, M., Asai, N., Saki, M., & Mori, A. (2018). Safety and effectiveness of istradefylline in patients with Parkinson's disease: interim analysis of a post-marketing surveillance study in Japan. *Expert Opinion on Pharmacotherapy*, *19*(15). https://doi.org/10.1080/14656566.2018.1518433

Takigawa, T., & Alzheimer, C. (1999). G protein-activated inwardly rectifying K+ (GIRK) currents in dendrites of rat neocortical pyramidal cells. *Journal of Physiology*, *517*(2). https://doi.org/10.1111/j.1469-7793.1999.0385t.x

Takigawa, T., & Alzheimer, C. (2002). Phasic and tonic attenuation of EPSPs by inward rectifier K+ channels in rat hippocampal pyramidal cells. *Journal of Physiology*, *539*(1). https://doi.org/10.1113/jphysiol.2001.012883

Tan, D. X., Manchester, L. C., Reiter, R. J., Qi, W. B., Karbownik, M., & Calvoa, J. R. (2000). Significance of melatonin in antioxidative defense system: Reactions and products. *NeuroSignals*, *9*(3–4). https://doi.org/10.1159/000014635

Tanaka, E., Yamamoto, S., Kudo, Y., Mihara, S., & Higashi, H. (1997). Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro. *Journal of Neurophysiology*, *78*(2), 891–902. https://doi.org/10.1152/jn.1997.78.2.891

Tebano, M. T., Martire, A., Potenza, R. L., Grò, C., Pepponi, R., Armida, M., … Popoli, P. (2008). Adenosine A2A receptors are required for normal BDNF levels and BDNF-induced potentiation of synaptic transmission in the mouse hippocampus. *Journal of Neurochemistry*, *104*(1).

https://doi.org/10.1111/j.1471-4159.2007.05046.x

Tendera, M., Gaszewska-Zurek, E., Parma, Z., Ponikowski, P., Jankowska, E., Kawecka-Jaszcz, K., … Agrawal, R. (2012). The new oral adenosine A1 receptor agonist capadenoson in male patients with stable angina. *Clinical Research in Cardiology*, *101*(7). https://doi.org/10.1007/s00392-012-0430-8

Thauerer, B., Zur Nedden, S., & Baier-Bitterlich, G. (2012). Purine nucleosides: Endogenous neuroprotectants in hypoxic brain. *Journal of Neurochemistry*, Vol. 121. https://doi.org/10.1111/j.1471-4159.2012.07692.x

Thiry, A., Dogne, J.-M., Supuran, C., & Masereel, B. (2007). Carbonic Anhydrase Inhibitors as Anticonvulsant Agents. *Current Topics in Medicinal Chemistry*, *7*(9), 855–864. https://doi.org/10.2174/156802607780636726

Tibbles, L. A., & Woodgett, J. R. (1999). The stress-activated protein kinase pathways. *Cellular and Molecular Life Sciences*, Vol. 55. https://doi.org/10.1007/s000180050369

Tong, C., Brion, L., Suarez, C., & Chesler, M. (2000). Interstitial carbonic anhydrase (CA) activity in brain is attributable to membrane-bound CA type IV. *Journal of Neuroscience*, *20*(22), 8247–8253. https://doi.org/10.1523/jneurosci.20-22-08247.2000

Tosh, D. K., Finley, A., Paoletta, S., Moss, S. M., Gao, Z. G., Gizewski, E. T., … Jacobson, K. A. (2014). In vivo phenotypic screening for treating chronic neuropathic pain: Modification of C2-arylethynyl group of conformationally constrained A3 adenosine receptor agonists. *Journal of Medicinal Chemistry*, *57*(23). https://doi.org/10.1021/jm501021n

Traut, T. W. (1994). Physiological concentrations of purines and pyrimidines. *Molecular and Cellular Biochemistry*, Vol. 140. https://doi.org/10.1007/BF00928361

Trincavelli, M. L., Marroni, M., Tuscano, D., Ceruti, S., Mazzola, A., Mitro, N., … Martini, C. (2004). Regulation of A2B adenosine receptor functioning by tumour necrosis factor a in human astroglial cells. *Journal of Neurochemistry*, *91*(5). https://doi.org/10.1111/j.1471-4159.2004.02793.x

Trincavelli, M. L., Melani, A., Guidi, S., Cuboni, S., Cipriani, S., Pedata, F., & Martini, C. (2008). Regulation of A2A adenosine receptor expression and functioning following permanent focal ischemia in rat brain. *Journal of Neurochemistry*, *104*(2). https://doi.org/10.1111/j.1471-4159.2007.04990.x

Tsuji, K., Aoki, T., Tejima, E., Arai, K., Lee, S. R., Atochin, D. N., … Lo, E. H. (2005). Tissue plasminogen activator promotes matrix metalloproteinase-9 upregulation after focal cerebral ischemia. *Stroke*, *36*(9). https://doi.org/10.1161/01.STR.0000177517.01203.eb

Tuettenberg, J., Heimann, A., & Kempski, O. (2001). Nitric oxide modulates cerebral blood flow stimulation by acetazolamide in the rat cortex: A laser doppler scanning study. *Neuroscience Letters*, *315*(1–2), 65–68. https://doi.org/10.1016/S0304-3940(01)02325-4

Uldall, M., Botfield, H., Jansen-Olesen, I., Sinclair, A., & Jensen, R. (2017). Acetazolamide lowers intracranial pressure and modulates the cerebrospinal fluid secretion pathway in healthy rats. *Neuroscience*

Letters, *645*, 33–39. https://doi.org/10.1016/j.neulet.2017.02.032

Van Westen, G. J. P., Van Den Hoven, O. O., Van Der Pijl, R., Mulder-Krieger, T., De Vries, H., Wegner, J. K., … Bender, A. (2012). Identifying novel adenosine receptor ligands by simultaneous proteochemometric modeling of rat and human bioactivity data. *Journal of Medicinal Chemistry*, *55*(16). https://doi.org/10.1021/jm3003069

Varani, K., Gessi, S., Dionisotti, S., Ongini, E., & Borea, P. A. (1998). [3H]-SCH 58261 labelling of functional A(2A) adenosine receptors in human neutrophil membranes. *British Journal of Pharmacology*, *123*(8). https://doi.org/10.1038/sj.bjp.0701758

Velíš̌ek, L., Moshé, S., & Stanton, P. (1995). Resistance of hippocampal synaptic transmission to hypoxia in carbonic anhydrase II-deficient mice. *Brain Research*, *671*(2), 245–253. https://doi.org/10.1016/0006-8993(94)01336-G

Virani, S. S., Alonso, A., Aparicio, H. J., Benjamin, E. J., Bittencourt, M. S., Callaway, C. W., … Tsao, C. W. (2021). Heart Disease and Stroke Statistics-2021 Update: A Report From the American Heart Association. *Circulation*, *143*(8). https://doi.org/10.1161/CIR.0000000000000950

von Lubitz, D. K. (1999). Adenosine and cerebral ischemia: therapeutic future or death of a brave concept? *European Journal of Pharmacology*, *371*(1). https://doi.org/10.1016/S0014-2999(99)00135-1

Von Lubitz, D. K. J. E., Lin, R. C. S., Melman, N., Xiao-duo Ji, Carter, M. F., & Jacobson, K. A. (1994). Chronic administration of selective adenosine A1 receptor agonist or antagonist in cerebral ischemia. *European Journal of Pharmacology*, *256*(2). https://doi.org/10.1016/0014-2999(94)90241-0

Voors, A. A., Düngen, H. D., Senni, M., Nodari, S., Agostoni, P., Ponikowski, P., … Gheorghiade, M. (2017). Safety and Tolerability of Neladenoson Bialanate, a Novel Oral Partial Adenosine A1 Receptor Agonist, in Patients With Chronic Heart Failure. *Journal of Clinical Pharmacology*, *57*(4). https://doi.org/10.1002/jcph.828

Vorstrup, S., Henriksen, L., & Paulson, O. B. (1984). Effect of acetazolamide on cerebral blood flow and cerebral metabolic rate for oxygen. *Journal of Clinical Investigation*, *74*(5), 1634–1639. https://doi.org/10.1172/JCI111579

Wakai, A., Wang, J. H., Winter, D. C., Street, J. T., O'Sullivan, R. G., & Redmond, H. P. (2001). Adenosine inhibits neutrophil vascular endothelial growth factor release and transendothelial migration via A2B receptor activation. *Shock*, *15*(4). https://doi.org/10.1097/00024382-200115040-00008

Wang, T., Liu, X. huan, Guan, J., Ge, S., Wu, M. Bin, Lin, J. ping, & Yang, L. rong. (2019). Advancement of multi-target drug discoveries and promising applications in the field of Alzheimer's disease. *European Journal of Medicinal Chemistry*, *169*. https://doi.org/10.1016/j.ejmech.2019.02.076

Wei, W., Du, C., Lv, J., Zhao, G., Li, Z., Wu, Z., … Xie, X. (2013). Blocking A 2B Adenosine Receptor Alleviates Pathogenesis of Experimental Autoimmune Encephalomyelitis via Inhibition of IL-6 Production and Th17 Differentiation . *The Journal of Immunology*, *190*(1). https://doi.org/10.4049/jimmunol.1103721

Williams, T. C., & Jarvis, S. M. (1991). Multiple sodium-dependent nucleoside transport systems in bovine renal brush-border membrane vesicles. *Biochemical Journal*, *274*(1). https://doi.org/10.1042/bj2740027

Williamson, M. R., Wilkinson, C. M., Dietrich, K., & Colbourne, F. (2019). Acetazolamide Mitigates Intracranial Pressure Spikes Without Affecting Functional Outcome After Experimental Hemorrhagic Stroke. *Translational Stroke Research*, *10*(4), 428–439. https://doi.org/10.1007/s12975-018-0663-6

Wirkner, K., Gerevich, Z., Krause, T., Günther, A., Köles, L., Schneider, D., … Illes, P. (2004). Adenosine A2A receptor-induced inhibition of NMDA and GABA A receptor-mediated synaptic currents in a subpopulation of rat striatal neurons. *Neuropharmacology*, *46*(7). https://doi.org/10.1016/j.neuropharm.2004.01.008

Wood, P. L. (1995). Microglia as a unique cellular target in the treatment of stroke: Potential neurotoxic mediators produced by activated microglia. *Neurological Research*, Vol. 17. https://doi.org/10.1080/01616412.1995.11740321

Woodruff, T. M., Thundyil, J., Tang, S. C., Sobey, C. G., Taylor, S. M., & Arumugam, T. V. (2011). Pathophysiology, treatment, and animal and cellular models of human ischemic stroke. *Molecular Neurodegeneration*, Vol. 6. https://doi.org/10.1186/1750-1326-6-11

Wu, L. G., & Saggau, P. (1994). Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus. *Neuron*, *12*(5). https://doi.org/10.1016/0896-6273(94)90321-2

Wykoff, C. C., Beasley, N. J. P., Watson, P. H., Turner, K. J., Pastorek, J., Sibtain, A., … Harris, A. L. (2000). Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Research*, *60*(24), 7075–7083.

Xu, J., Guo, S., Jia, Z., Ma, S., Li, Z., & Xue, R. (2013). Additive effect of prostaglandin E2 and adenosine in mouse experimental autoimmune encephalomyelitis. *Prostaglandins and Other Lipid Mediators*, *100–101*(1). https://doi.org/10.1016/j.prostaglandins.2012.11.004

Xu, K., Bastia, E., & Schwarzschild, M. (2005). Therapeutic potential of adenosine A2A receptor antagonists in Parkinson's disease. *Pharmacology and Therapeutics*, Vol. 105. https://doi.org/10.1016/j.pharmthera.2004.10.007

Yamagata, K., Ichinose, S., Miyashita, A., & Tagami, M. (2008). Protective effects of ebselen, a seleno-organic antioxidant on neurodegeneration induced by hypoxia and reperfusion in stroke-prone spontaneously hypertensive rat. *Neuroscience*, *153*(2). https://doi.org/10.1016/j.neuroscience.2008.02.028

Yang, D., Chen, H., Koupenova, M., Carroll, S. H., Eliades, A., Freedman, J. E., … Ravid, K. (2010). A new role for the A2b adenosine receptor in regulating platelet function. *Journal of Thrombosis and Haemostasis*, *8*(4). https://doi.org/10.1111/j.1538-7836.2010.03769.x

Yang, Dan, Zhang, Y., Nguyen, H. G., Koupenova, M., Chauhan, A. K., Makitalo, M., … Ravid, K. (2006). The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. *Journal of Clinical Investigation*, *116*(7). https://doi.org/10.1172/JCI27933

Yang, H., Avila, M. Y., Peterson-Yantorno, K., Coca-Prados, M., Stone, R. A., Jacobson, K. A., & Civan, M. M. (2005). The cross-species A3 adenosine-receptor antagonist MRS 1292 inhibits adenosine-triggered human nonpigmented ciliary epithelial cell fluid release and reduces mouse intraocular pressure. *Current Eye Research*, *30*(9). https://doi.org/10.1080/02713680590953147

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., … Wang, X. (1997). Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. *Science*, *275*(5303). https://doi.org/10.1126/science.275.5303.1129

Yawo, H., & Chuhma, N. (1993). Preferential inhibition of oω-conotoxin-sensitive presynaptic Ca 2+ channels by adenosine autoreceptors. *Nature*, *365*(6443). https://doi.org/10.1038/365256a0

Yenari, M. A., Kunis, D., Sun, G. H., Onley, D., Watson, L., Turner, S., … Steinberg, G. K. (1998). Hu23F2G, an antibody recognizing the leukocyte CD 11/CD 18 integrin, reduces injury in a rabbit model of transient focal cerebral ischemia. *Experimental Neurology*, *153*(2). https://doi.org/10.1006/exnr.1998.6876

Yepes, M., Roussel, B. D., Ali, C., & Vivien, D. (2009). Tissue-type plasminogen activator in the ischemic brain: more than a thrombolytic. *Trends in Neurosciences*, Vol. 32. https://doi.org/10.1016/j.tins.2008.09.006

Yilmaz, G., Arumugam, T. V., Stokes, K. Y., & Granger, D. N. (2006). Role of T lymphocytes and interferon-γ in ischemic stroke. *Circulation*, *113*(17). https://doi.org/10.1161/CIRCULATIONAHA.105.593046

Young, J. D., Yao, S. Y. M., Baldwin, J. M., Cass, C. E., & Baldwin, S. A. (2013). The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29. *Molecular Aspects of Medicine*, Vol. 34. https://doi.org/10.1016/j.mam.2012.05.007

Yu, L., Huang, Z., Mariani, J., Wang, Y., Moskowitz, M., & Chen, J. F. (2004). Selective inactivation or reconstitution of adenosine A2A receptors in bone marrow cells reveals their significant contribution to the development of ischemic brain injury. *Nature Medicine*, *10*(10). https://doi.org/10.1038/nm1103

Zetterström, T., & Fillenz, M. (1990). Adenosine agonists can both inhibit and enhance in vivo striatal dopamine release. *European Journal of Pharmacology*, *180*(1). https://doi.org/10.1016/0014-2999(90)90601-2

Zhang, R. L., Chopp, M., Jiang, N., Tang, W. X., Prostak, J., Manning, A. M., & Anderson, D. C. (1995). Anti-intercellular adhesion molecule-1 antibody reduces ischemic cell damage after transient but not permanent middle cerebral artery occlusion in the wistar rat. *Stroke*, *26*(8). https://doi.org/10.1161/01.str.26.8.1438

Zhao, Z., Francis, C. E., & Ravid, K. (1997). An A3-subtype adenosine receptor is highly expressed in rat

vascular smooth muscle cells: Its role in attenuating adenosine-induced increase in cAMP. *Microvascular Research*, *54*(3). https://doi.org/10.1006/mvre.1997.2044

Zhu, Y., Yang, G. Y., Ahlemeyer, B., Pang, L., Che, X. M., Culmsee, C., … Krieglstein, J. (2002). Transforming Growth Factor-β1 Increases Bad Phosphorylation and Protects Neurons Against Damage. *Journal of Neuroscience*, *22*(10). https://doi.org/10.1523/jneurosci.22-10-03898.2002

Zimmermann, H. (1992). 5'-Nucleotidase: Molecular structure and functional aspects. *Biochemical Journal*, Vol. 285. https://doi.org/10.1042/bj2850345

Zimmermann, H. (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn-Schmiedeberg's Archives of Pharmacology*, Vol. 362. https://doi.org/10.1007/s002100000309