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DOTTORATO DI RICERCA TOSCANO IN NEUROSCIENZE

CICLO XXXI

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Protection by adenosinergic and histaminergic drugs from ischemic damage induced by middle cerebral artery occlusion in the rat.

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Index……

Introduction……..…….. 1

Cerebral Ischemia 2

1. Pathobiology of ischemia…..…….…….… 4

1.1 Glutamate-mediated excitotoxicity……..……..… 5

1.2 Oxydative stress……..…..…….……. 6

1.3 Stress signalling in response to ischemia….……..……..… 7

1.4 Neuroinflammation……...…..…….. 8

1.5 Cell death in cerebral ischemia.....….……..… 9

2. Animal models……..…..…….……..… 11

2.1 Stroke model selection...…..……...…….. 11

2.2 The focal ischemia models...…….. 12

2.2.1 Thromboembolic model.....……...……... 12

2.2.2 Endothelin-1 model……...……... 12

2.2.3 Photochemical model……....……... 13

2.2.4 Intraluminal suture Middle Cerebral Artery occlusion (MCAo) model…….…….……. 13

2.2.5 Tamura model (Craniectomy model)…… 14

Histaminergic System 15

3. Histamine: synthesis, regulation and metabolism….……..……. 15

3.1 The role of histamine in the periphery…….…….……...… 16

3.2 The role of histamine in the CNS…….……... 18

3.3 Histamine receptors……...…….…….……..... 19

3.3.1 H_1 receptor: distribution, peripheral and central functions..... 20

3.3.2 H_2 receptor: distribution, peripheral and central functions……... 22

3.3.3 H_3 receptor: distribution and central functions……..... 23

3.3.4 H_4 receptor: distribution, peripheral and central functions..... 24

Histamine and Cerebral Ischemia 28

4. Protective effects of histamine in cerebral ischemia…….…….. 29

4.1 Histamine and the regulation of excitotoxicity in the acute period after ischemia……..…….. 29

4.2 Histamine and the regulation of inflammation in the subacute period after ischemia ..……..……... 30

4.3 Histamine and the regulation of glial scar formation in the late period after ischemia....……..…….. 31

4.4 Role of histamine H_3 receptor in cerebral ischemia……..…….. 31

4.5 Role of histamine H_4 receptor in cerebral ischemia…..……..….. 32

The Adenosinergic System 33

5 Adenosine formation……...……..…….. 34

5.1 5'-endonucleotidases……...……..…….. 35

5.2 5'-ectonucleotidases.……...……..…….. 36

6. Adenosine release….……...……..…….. 37

7. Adenosine catabolism...……...….…….…….. 38

8. Adenosine receptors.……...….……... 40

8.1 A_1 receptor: molecular structure, localization and function….……... 42

8.2 A_{2A} receptor: molecular structure, localization and function….……. 45

8.3 A_{2B} receptor: molecular structure, localization and function……... 49

8.4 A_3 receptor: molecular structure, localization and function….……... 51

Role of Adenosine A_{2A} and A_{2B} Receptors in Cerebral Ischemia 54

9. Adenosine in cerebral ischemia…….…….. 54

9.1 Adenosine A_{2A} receptor antagonists protect against primary ischemic injury.….……..……. 55

9.1.1 Adenosine A_{2A} receptor antagonists are protective against ischemic damage……..……... 55

9.1.2 A_{2A} receptor antagonists protect from the increase of glutammatergic excitatory transmission…..…….. 56

9.1.3 A_{2A} receptor antagonists protect from ischemia-induced activation of intracellular pathways.…..…….…….. 58

9.2 Adenosine A_{2A} receptor agonists protects against secondary injury…...…. 59

9.2.1 Adenosine A_{2A} receptor agonists are protective against ischemic damage..…….…….…….. 59

9.2.2 A_{2A} receptors and neuroinflammation...…..…….. 60

9.3 Adenosine A_{2B} receptors in brain ischemia…..……..……. 62

Aims of the Research…….……. 65

Materials and Methods…..……. 69

1. Animals……...…..……. 70

2. Surgery.…..……..……. 70

3. Treatment and experimental groups……..……. 71

3.1 Chronic treatment with histamine H_4 receptor antagonist, JNJ7777120…. 71

3.1.1 Drug administration…….. 71

3.1.2 Experimental groups……...…….. 72

3.2 Chronic treatment with adenosine A_{2A} receptor antagonist, SCH58261..... 72

3.2.1 Drug administration…….. 72

3.2.2 Experimental groups……...…….. 73

3.3 Chronic treatment with adenosine A_{2A} receptor agonist, CGS21680…..... 73

3.3.1 Drug administration…….. 73

3.3.2 Experimental groups……...…….. 74

3.4 Chronic treatment with adenosine A_{2B} receptor agonist, BAY 60-6583..... 74

3.4.1 Drug administration…….. 74

3.4.2 Experimental groups……...…….. 74

4. Neurological deficit……..... 75

5. Body weight evaluation……..…..... 76

6. Ischemic brain damage ……..... 77

7. Blood cell infiltration and gliosis……..... 77

8. MAG staining …….……..... 78

9. Determination of TNF-α, IL-1β and IL-10..……..... 79

10. Platelet aggregation assay…..……..... 79

11. Statistical analysis…….……..... 79

Results……...……. 80

The protective effects of the selective histamine H_4 receptor antagonist, JNJ7777120, in a rat model of transient cerebral ischemia.……..……... 81

1. Effect of treatment with the histamine H_4 receptor antagonist on neurological deficit after tMCAo……. 81

2. Effect of treatment with the histamine H_4 receptor antagonist on body weight loss after tMCAo…….…….. 81

3. Effect of treatment with the histamine H_4 receptor antagonist on brain ischemic damage after tMCAo...…….. 83

4. Effect of treatment with the histamine H_4 receptor antagonist on gliosis after tMCAo.....……..……. 86

5. Effect of treatment with the histamine H_4 receptor antagonist on cytokine plasma levels after tMCAo……..…….. 89

6. Effect of treatment with the histamine H_4 receptor antagonist on blood cell infiltration after tMCAo.…….…..…….. 89

Time-course of protection by the selective A_{2A} receptor antagonist, SCH58261, after transient focal cerebral ischemia……...…….. 91

1. Effect of treatment with the adenosine A_{2A} receptor antagonist on neurological deficit after tMCAo…….…..…….. 91

2. Effect of treatment with the adenosine A_{2A} receptor antagonist on body weight loss after tMCAo.…….…..…….. 91

3. Effect of treatment with the adenosine A_{2A} receptor antagonist on brain ischemic damage after tMCAo……. 93

4. Effect of treatment with the adenosine A_{2A} receptor antagonist on blood cell infiltration after tMCAo...……..……..…….. 96

Effects of chronic treatment with the adenosine A_{2A} receptor agonist, CGS21680, on platelet aggregation.……...……...…….. 97

1. Adenosine A_{2A} receptor agonist, CGS21680, does not modify platelet aggregation....……..……..……..…….. 97

Adenosine A_{2B} receptor agonist, BAY 60-6583, protects from behavioral and histological damage after focal transient brain ischemia in the rat.……..……...……...…….. 98

1. Effect of treatment with the adenosine A_{2B} receptor agonist on neurological deficit after tMCAo……..……..…….. 98

2. Effect of treatment with the adenosine A_{2B} receptor agonist on body weight loss after tMCAo.……..……...…….. 99

3. Effect of treatment with the adenosine A_{2B} receptor agonist on brain ischemic damage after tMCAo..……..…….. 100

Discussion……...……..…….. 103

References……...……..…….. 113

Introduction.

Cerebral Ischemia

Stroke is today evaluated as the second most common cause of death and a major cause of long-term disabilities. Ischemic stroke commonly accounts for approximately 80% of all stroke cases and is caused from occlusion of a major cerebral artery by a thrombus or an embolism, which leads to loss of cerebral blood flow, a condition of hypoxia and glucose deprivation and subsequently tissue damage in the affected region (Gibson, 2013). Brain injury results from a complex sequence of pathophysiological events consequent to the ischemic insult, that evolve over time (Figure 1).

At cellular level, the reduction of cerebral blood flow and the subsequent oxygen depletion trigger biochemical events resulting in anaerobic metabolism. The anaerobic glycolysis is insufficient and determines the depletion of ATP, accumulation of lactic acid (Wyatt et al., 1989), calcium (Ca^{2+}) and water that leads to edema. Further, the depolarization of the cell plasma membrane and the consequent release of excitatory neurotransmitters, particularly glutamate, bring to primary acute mechanisms of excitotoxicity that cause cell death.

After ischemia the early massive increase of extracellular glutamate is followed by activation of resident immune cells, i.e microglia, and production or activation of inflammation mediators (Dirnagl et al., 1999). Proinflammatory cytokines, that upregulate cell adhesion molecules, exert an important role in promoting neutrophil infiltration and accumulation in brain parenchyma (Stoll et al., 1998; Huang et al., 2006). Although after ischemia precocious activation of immune cells may be neuroprotective and supportive for regeneration, protracted neuroinflammation is now recognized as the predominant mechanism of secondary brain injury progression.

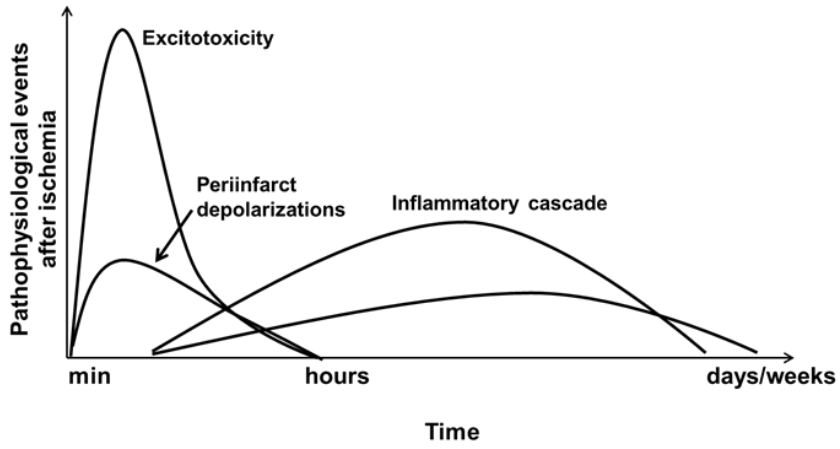

Figure 1: Cascade of pathogenetic mechanisms after ischemia. Primary mechanisms of excitotoxicity lead to acute cell death. Depolarization spreads in the perinfarct areas. In the following several hours, activation of resident immune cells, i.e. microglia and a cascade of production of inflammation mediators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

Neuropathological analysis reveals that brain ischemia leads to two distinct areas of ischemia: the *core* zone which is an area of severe ischemia, and the *penumbra* zone, a term used to describe ischemic, but still viable cerebral tissue. In the *core* zone, severe reduction of blood supply to the brain causes cell death in few minutes. Meanwhile, in the *penumbra* zone an hypoperfusion persists resulting in neurophysiological function loss, but the cellular metabolism and structure are preserved (Sharp et al., 2000; Hossmann, 2009) (Figure 2).

It is estimated that initially, the area of *penumbra* corresponds to 50% of the tissue that later will progress into infarct (Dirnagl et al., 1999). The understanding of mechanisms occurring in the *penumbra* zone is essential to preserve function and promote the survival of nervous cells after reperfusion. The main goal of a neuroprotective pharmacological treatment is to recover the area of ischemic *penumbra* from an irreversible neurodegeneration.

The extent of ischemic damage depends on the duration of ischemia, but also reperfusion plays a prominent role in the setting of the damage. Hence, paradoxally, reperfusion initiates an inflammatory cascade with free radicals, released from dead cells, worsening the ischemic injury.

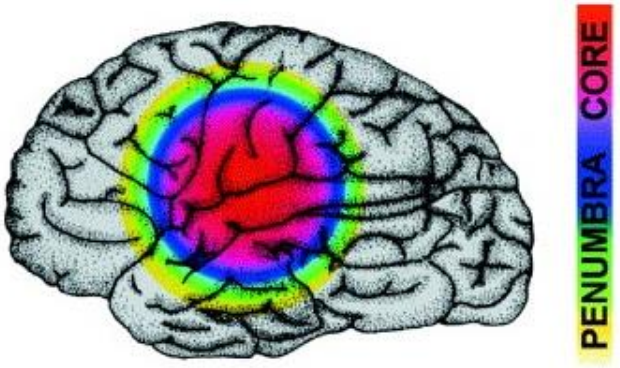

Figure 2 Ischemic *core* and *penumbra*: the red area on the figure marks the infarcted *core*, a brain region of low perfusion in which cells have lost their membrane potential. It is surrounded by a *penumbra* area, marked by the remaining colors, in which intermediate perfusion prevails and identified as the salvageble brain area. Figure modified from (Dirnagl et al., 1999).

In the last years basic research yeldied 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. However, drugs have failed to be efficacious during clinical trials and the only successful pharmacological treatment approved to date is tissue plasminogen activator (tPA) that aims to decrease ischemia-associated thrombosis risk. Yet, because of the narrow therapeutic time-window involved, thrombolytic application is very restricted in clinical settings (Chen et al., 2014a). Aspirin, other antiplatelets, and anticoagulants are used as preventive therapy of stroke (Albers et al., 2011; Macrez et al., 2011).

1. Pathobiology of ischemia

Cerebral ischemia triggers a series of haemodynamic, biochemical and behavioural disorders. Initially, due to the decrease in blood flow, neurological functions are affected. Subsequently, as a result of ischemia progress, the metabolic activity is suppressed in order to maintain the structural integrity of the cells (Hossmann, 1998). This leads to activation of several signalling 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 Glutamate-mediated excitotoxicity

In physiological condition extracellular glutamate concentration is maintained in the micromolar range. During cerebral ischemia, blood flow is severely diminished along with energy failure, collapse of the ATP-dependent cell metabolism, and of ionic channels function (Kimelberg and Mongin, 1998). Depolarization of the cell membrane brings to Ca^{2+} entry and release of the glutamate retained in the intracellular vesicles (Mena et al., 2000). Moreover, the glutamate transporters present on astrocytes operate in the inverse direction due to the disruption of the electrochemical gradient. Therefore, an excessive release of glutamate occurs (Budd, 1998) concomitant to an insufficient uptake of glutamate by astrocytes (Mori et al., 2004; Giffard and Swanson, 2005).

Glutamate is responsible for the generation of post-synaptic signals by acting through ionotropic receptors (NMDA, AMPA, kainate) whose activation leads to an increased permeability to Na^+, K^+ and Ca^{2+} ions, and through metabotropic receptors that mediate their effects via G proteins that cause the mobilization of Ca^{2+} from intracellular stores. It is thought that most of the neuronal degeneration is due to the intense activation of NMDA receptors. 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 and Thor, 2011).

The perinfarct depolarization, one of the early events after ischemia (see Figure 1), consists of spontaneous depolarization waves that propagate from the *core* toward the *penumbra* area and may be triggered by the glutamate accumulation that leads to an eccessive influx of Na^+ and Ca^{2+} together with water, resulting in cytotoxic edema (Furukawa et al., 1997). 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, 1996; Ohta et al., 2001). 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 (Ohta et al., 2001; Lu et al., 2005).

1.2. Oxydative stress

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

The nervous tissue is too vulnerable to the action of free radicals because of some peculiarities, such as being rich in lipids and unsaturated fatty acids which may react with ROS to form peroxyl radicals that determine the lipid oxidation of the membrane of neurons (Porter, 1984). Additionally, the brain has low to moderate catalase, and glutathione activity which eliminate the hydrogen peroxidase (H_2O_2) reducing oxidation (Cooper and Kristal, 1997). 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 over-expression attenuates apoptosis (Saito et al., 2004). Also, melatonin is known for its neuroprotective effect in models of ischemia, due to its anti-oxidant properties and capability in eliminating free radicals (Tan et al., 2000; Pei et al., 2002).

The free radical, NO, is synthesised by the enzyme NO synthase (NOS) that is heavily activated during ischemia, as well as during reperfusion. The NO has neuroprotective and neurotoxic properties. The activation of NOS during the ischemia causes the death of neurons by combining with a superoxide yielding peroxynitrite ($ONOO^-$), a potent radical which is an irreversible inhibitor of mitochondrial function in addition to being a pro-oxidant that damages lipids, proteins and DNA and increases glutamate release determining neuronal cell death (Radi et al., 1991). 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 increase of blood flow in the affected cerebral region (Bolaños and Almeida, 1999).

1.3. Stress signalling in response to ischemia

Cerebral ischemia triggers a series of signalling cascades crucial for a faith of cell survival of damage, such as mitogen-activated protein kinase (MAPK) cascade (Nozaki et al., 2001). The MAPK enzymatic family consists of three members: ERK, p38 and JNK, activated under stress conditions (Sugino et al., 2000) (Figure 3). For instance, free radicals, generated following oxidative stress triggers MAPK activation (Cao et al., 2005).

In response to ischemia, ERK activation exerts neuroprotection mediated by neurotrophins and in particular by the brain-derived neurotrophic factor (BDNF) (Han and Holtzman, 2000), which affects neuronal differentiation and survival and it is also involved in the short- and long-term plasticity at glutamatergic synapses (Hartmann et al., 2001). On the contrary, p38 and JNK kinases act as mediators of cellular stress in ischemia (Alessandrini et al., 1999; Irving and Bamford, 2002), indeed their inhibition has a neuroprotective role in cerebral ischemia (Barone et al., 2001; Borsello et al., 2003).

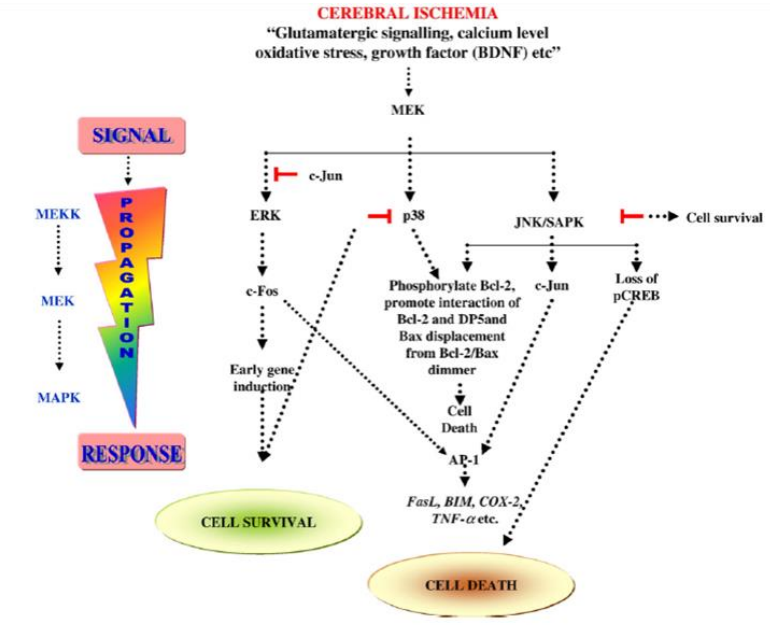

Figure 3 Representation of the MAPK cascade (ERK, p38 and JNK) in cerebral ischemia and their role in the mechanisms of cell survival/death. In the diagram the MAPK activators (MEK and MEKK) are represented, the three core members of the MAPKs family and relationships among them. The signal is propagated through a phosphorylative series that activates MAPKs that phosphorylate intracellular enzymes and transcription factors.

1.4. Neuroinflammation

A few hours after the ischemia onset, a switch from the ischemic damage to the inflammatory damage occurs. The inflammatory response is characterized by the activation of endogenous microglia and influx of peripheral leukocytes (neutrophils, lymphocytes and monocytes) in the brain parenchyma promoted by alteration of the blood brain barrier (BBB) permeability (Iadecola and 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. Adhesion molecules play a key role in the process of leukocyte infiltration into the brain parenchyma after ischemia (Sughrue et al., 2004). The interaction between leukocytes and the vascular endothelium is mediated by three groups of adhesion molecules: the selectins (P, E and L), the intracellular adhesion, (ICAM-1,2) and vascular cell adhesion (VCAM-1) molecules and the integrins (CD11a-c) (DeGraba, 1998; Emsley and Tyrrell, 2002). After ischemia and reperfusion, the expression of selectins (Okada et al., 1994; Huang et al., 2000) and of the ICAM and VCAM is upregulated (Zhang et al., 1995; Shyu et al., 1997) and it has been shown that the blockade of adhesion molecules leads to a reduction of neurological damage (Clark et al., 1995, 1997). Cytokines are mediators of inflammation and they are expressed by cells of the immune system but also by resident cells of the Central Nervous System (CNS) such as glial cells and neurons. In the ischemic brain their 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 and Rothwell, 2001). The Ca^{2+} accumulation that occurs following the over-activation of NMDA receptor results in the activation of phospholipase A2 (PLA2) and the linked arachidonic acid pathway (Stanimirovic and Satoh, 2000), whose metabolites are potent mediators of the post-ischemic cerebral inflammation (Sánchez-Moreno et al., 2004).

Neutrophils are the first leukocyte subpopulation to be recruited into the ischemic brain and may exacerbate the damage following the release of deleterious substances or of inflammatory mediators (Hallenbeck, 1996). Therefore, the inhibition of neutrophil infiltration can significantly reduce the infarct volume after ischemia (Bowes et al., 1995; Clark et al., 1995; Chopp et al., 1996; Connolly et al., 1996; Yenari et al., 1998; Garau et al., 2005). Following permanent middle cerebral artery occlusion (MCAo) in

rats, lymphocytes represent the second most abundant leukocyte subtype, after neutrophils, infiltrated in the ischemic lesion (Stevens et al., 2002) and exert pro-inflammatory actions, harmful for the cerebral tissue (Nadareishvili et al., 2004).

Microglial cells represent the local phagocytic immune cells in the CNS (Kreutzberg, 1996). Following ischemia microglia gets activated, thus undergoes to proliferation, chemotaxis, morphological changes and release of several substances, both cytotoxic and cytoprotective (Wood, 1995). Astrocytes are known for their ability to express different kinds of inflammatory mediators such as cytokines, chemokines, and the inducible NOS (iNOS) (Dong and 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 (MHC) and by stimulating the expression of cytokines such as IL-2 (Dong and Beneveniste, 2001).

1.5. Cell death in cerebral ischemia

After ischemia, nervous cells undergo death, due to a process of necrosis or apoptosis following an excessive activation of glutamate receptors, that leads to an activation of a cascade mechanism culminating in cell death that comprises: increase of intracellular Ca^{2+} concentration, production of free radicals, damage to the mitochondria or to the DNA (Figure 4). The ischemia severity and duration will determine the kind of the cell death: a rapid death due to necrotic lysis, the main death mechanism in the acute phase, or a slow programmed death by apoptosis that occurs mainly in the *penumbra*.

The necrotic process is due to the loss of the osmotic homeostasis in response to a trauma, such as acute anoxia, a sudden lack of nutrients or an extreme physical or chemical damage. In the ischemic *core*, cells undergo necrosis and thus appear characterized by edema, rupture of cytoplasmic organelles, loss of the membrane integrity. Lysis of the neuronal cells activates the inflammatory process (Shalak and Perlman, 2004).

The apoptotic process is a programmed cell death, a mechanism strictly regulated and highly efficient, crucial for tissue homeostasis. This process proceeds without any loss of intracellular content. The enzymes involved in apoptosis are the caspases, whose gene is highly expressed during the infarction (Dirnagl et al., 1999). Caspases activation

occurs through two alternative pathways: extrinsic or intrinsic. Post-ischemic apoptosis occurs via intrinsic (or mitochondrial) activation, that is initiated by high levels of glutamate, intracellular Ca^{2+}, ROS, and DNA damage. When the mitochondria receive proper apoptotic signalling or suffer an irreversible damage, release pro-apoptotic molecules such as cytochrome C to the cytoplasm leading to the activation of caspase-9. Caspase-9 activates, among others, the caspase-3 which initiates the cell death by apoptosis (Carbonell and Rama, 2007).

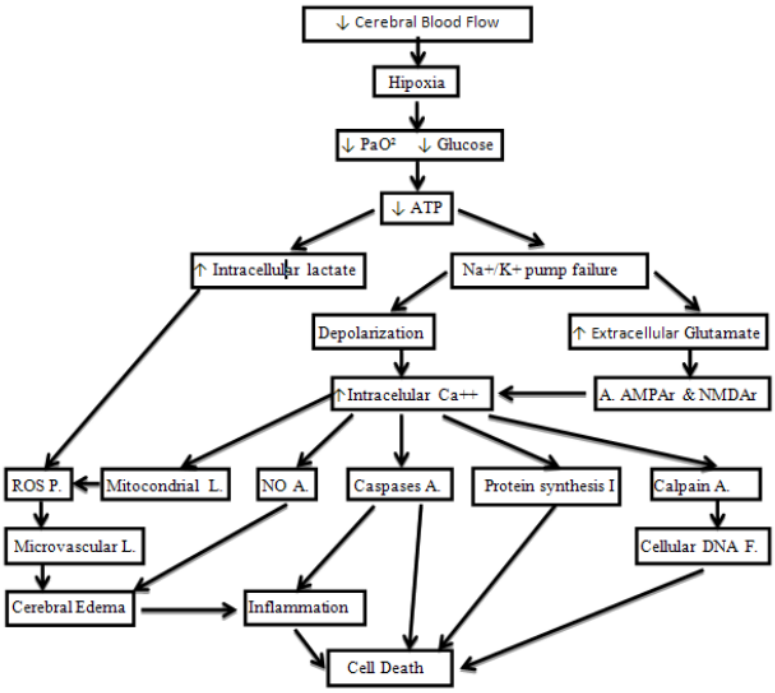

Figure 4 Physiopathological evolution of cerebral ischemia. (P) Production. (L) Lesion. (A) Activation. (I) Interruption. (F) Fragmentation. (ROS) Reactive oxygen species. (NO) Nitric oxide. (DNA) Deoxyribonucleic acid. Figure modified from (Nilton et al., 2014; Shalak and Perlman, 2004)

2. Animal models

The only approved medical treatment for acute ischemic stroke is intravenous thrombolysis with tPA (Adams et al., 2007). However, the therapeutic window of tPA treatment is up 4.5 hours after stroke, and consequently, tPA is applicable as treatment in only up to 5% of all patients (Fonarow et al., 2011). Thus, there is an urgent need for other, more widely applicable, treatment options.

Over the last four decades, a variety of animal stroke models have been developed, with the aim of identifying the mechanisms that underlie cerebral ischemia and developing new agents for stroke therapy. In fact, the success of preclinical stroke research in developing new therapeutics might rely on the selection of the appropriate animal stroke model to use (Fluri et al., 2015).

2.1 Stroke model selection

First, the majority of stroke experiments are carried out in small animals (mice, rats, rabbit), for their clear advantages including the lower cost. In particular, the rat is one of the most commonly used animals in stroke studies for its similarity with the human cerebral vasculature and physiology (Yamori et al., 1976).

Various stroke models have been developed to mimic different stroke subtypes or pathological mechanisms and can be generally classified into two categories: focal cerebral ischemia models and global cerebral ischemia models. Global ischemia models mimic the clinical conditions of brain ischemia following cardiac arrest while focal models represent the most common clinical ischemic stroke subtype (Liu et al., 2009). The most commonly used focal ischemia models are the intraluminal filament model (Koizumi et al., 1986) and the Tamura model (Tamura et al., 1981a). Some additional stroke models involve special modalities to induce artery occlusion, such as the thromboembolic, endothelin and photochemical models.

The selection of *in vivo* stroke models is influenced by the potential protection mechanism of the putative neuroprotective drugs. For example, if the predicted neuroprotection is associated with a particular brain cortex region, the phochemical model will be preferable because this model is able to produce ischemic injury at any location on the brain surface (see Table 1).

Stroke Model	Preferred application
Thromboembolic model	Clot-related protection mechanisms
Endothelin-1 model	Transient to semi-permanent focal cerebral ischemia that need outcome measurements of both histology and neurofunctions
Photochemical model	Neuroprotection is associated with a particular brain cortex region; Free radicals and endothelial injury play a major role in lesion development
Intraluminal suture model	Permanent and transient focal cerebral ischemia that need outcome measurements of both histology and neurofunctions
Tamura model	Permanent focal cerebral ischemia; Transient focal cerebral ischemia in which functional assessment is not needed.

Table 1: Summary of stroke model selection. Modified from (Liu et al., 2009).

2.2 The focal ischemia models

2.2.1 Thromboembolic model

The thromboembolic clot model is based on the application of spontaneously formed clots from autologous blood (Niessen et al., 2003), injecting thrombin directly into the intracranial segment of the internal carotid artery (ICA) (Zhang et al., 1997) or into middle cerebral artery (MCA) (Ansar et al., 2014). In particular, this model is reserved for clot-related protection mechanisms which other stroke models cannot address (Liu et al., 2009), but it is not suitable for validating neuroprotective effects because of its uncontrollable reperfusion and unacceptable variation of infarct area (see Table 2) (Zhang et al., 1997; Wang et al., 2001).

2.2.2 Endothelin-1 model

This model of focal stroke is based on the application of endothelin-1 (ET-1), that is a potent and long-acting vasoconstrictive peptide (Yanagisawa et al., 1988). It can be applied directly onto the exposed MCA (Robinson et al., 1990), as an intracerebral (stereotactic) injection (Hughes et al., 2003), or onto the cortical surface (Fuxe et al., 1997), which leads to a dose-dependent ischemic lesion with marginal ischemic edema (Fuxe et al., 1997; Hughes et al., 2003). After ET-1 administration, a rapid Cerebral Blood Flow reduction (70%-90%) is observed, followed by a reperfusion that occurs over several hours (Biernaskie et al., 2001). One of the advantages of this model is the possibility of inducing direct focal ischemia in deep and superficial brain regions, however one of the limits is that ET-1 receptors and ET-1 converting enzyme are also

expressed by neurons and astrocytes (Nakagomi et al., 2000), thus generating astrocytosis and axonal sprouting (see Table 2) (Uesugi et al., 1998), interfering with the interpretation of the results (Carmichael, 2005).

2.2.3 Photochemical model

This method consists in intravenous injection of a photosensitive dye (eg. Rose Bengal, erythrosine B) (Watson et al., 1985) that penetrates the BBB, and in irradiation within minutes by a light beam at specific wavelength (Watson et al., 1985) of the intact skull. The photochemical reaction generates singlet oxygen and free radicals, which cause endothelial injury and formation of microthromboses. This model is useful for neuroprotection associated with a particular cortex region that involves free radical scavenging as a protective mechanism (De Ryck et al., 2000; Chen et al., 2004).

2.2.4 Intraluminal suture Middle Cerebral Artery occlusion (MCAo) model

The MCA and its branches are the cerebral vessels that are most affected in human ischemic stroke, accounting for approximately 70% of infarcts (Bogousslavsky et al., 1988). Thus, techniques that occlude this artery are closest to human ischemic stroke (Longa et al., 1989; Tamura et al., 1981a). This technique involves temporarily occluding the common carotid artery (CCA), introducing a suture directly into the ICA, and advancing the suture until it interrupts the blood supply to the MCA. In a modified manner, the suture is inserted into the transected external carotid artery (ECA), using the ECA trunk as a path to advance a suture through the ICA. The ECA approach is a better choice for transient MCAo because it maintains the anatomic integrity required for reperfusion (Liu et al., 2009). This method enables permanent MCAo or transient ischemia with reperfusion and generates an infarct that involves the striatum and the overlying frontoparietal and temporal cortices (Garcia et al., 1995).

The MCAo model has several advantages (see Table 2): first of all, this model mimics human ischemic stroke, which often originates from an MCAo, and exhibits a *penumbra* that is similar to that of human stroke, moreover it is characterized by large infarct volumes and high reproducibility (Fluri et al., 2015).

	Advantages	Disadvantages
Thromboembolic model	Mimics most closely the pathogenesis of human stroke; Appropriate for studies of thrombolytic agents	Low reproducibility of infarcts; Spontaneous recanalization; High variability of lesion size
Endothelin-1 model	Low invasiveness; Induction of ischemic lesion in cortical and subcortical regions; Low mortality	Duration of the ischemia not controllable, Induction of astrocytosis and axonal sprouting, which may complicate the interpretation of the results; Low reproducibility of infarcts
Photochemical model	Enables well-defined localization of an ischemic lesion; Highly reproducibility, Low invasiveness	Causes early vasogenic edema that is uncharacteristic for human stroke, Not suitable for investigating neuroprotective agents
Intraluminal suture model	Mimics human ischemic stroke, Exhibits a *penumbra*; Highly reproducibility; Reperfusion highly controllable; No craniectomy	Hyper-/hypothermia, Increased haemorrhage with certain suture type; Not suitable for thrombolysis studies
Tamura model	High long-term survival rates; Visual confirmation of successful MCAo	High invasiveness and consecutive complications; Require a high degree of surgical skill

Table 2: Advantages and disadvantages of the most used rodent stroke models. Modified from (Fluri et al., 2015).

2.2.5 Tamura model (Craniectomy model)

In 1981, Tamura described a rat model of MCAo (Tamura et al., 1981a; 1981b) which can induce either permanent or temporary occlusion of the MCA. This method includes direct surgical MCAo requiring a craniectomy to expose MCA. In particular, the occlusion of MCA is performed by an electrocoagulation to induce a permanent occlusion, whereas for a temporary occlusion a microclip or an artery ligation are applicated (Tamura et al., 1981a; 1981b). Cerebral ischemia induced by this model compromises most of the frontal, parietal, temporal and rostral occipital cortices, and a marginal part of the striatum (Buchan et al., 1992). In comparison with the intraluminal suture model, Tamura method generates infarct variation (Chen et al., 2008) and a smaller infarct only on cortex which does not produce consistent functional deficit (Chen et al., 1986; Roof et al., 2001), therefore it has become an additional or alternative approach to validate neuroprotective efficacy in rodents.

Histaminergic System

Histamine [2-(4-imidazolyl)-ethylamine] (Figure 5) was isolated and characterized in the beginning of the 20th century by Sir Henry H. Dale (Barger and Dale, 1910; Dale and Laidlaw, 1910). One of the first described function was its ability to mimic anaphylaxis and has since demonstrated to play a major role in inflammatory processes. In 1927, histamine was isolated from liver and lung tissue, demostrating that it is a natural constituent of the body.

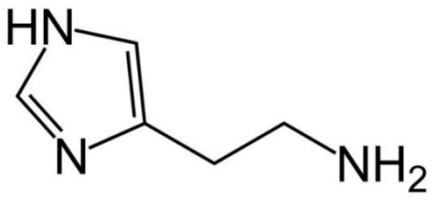

Figure 5 Histamine molecular structure.

Histamine is involved in the regulation of many physiological functions including cell proliferation and differentiation, haematopoiesis, embryonic development, regeneration and woud healing (Jutel et al., 2002; Schneider et al., 2002; Akdis and Blaser, 2003; MacGlashan, 2003; Dy and Schneider, 2004). For this wide spectrum of activities, including its potent mediator role in immediate hypersensitivity reactions (Daugherty, 2004), histamine has been one of the most studied substances in medicine for nearly a century.

3. Histamine: synthesis, regulation and metabolism

Histamine is synthesized in several cell types of peripheral and central tissue by decarboxylation of histidine by L-hystidine decarboxylase (HDC), which is dependent on the cofactor pyridoxal-5'-phosphate (Endo, 1982).

Histamine regulation is dependent on the gene of HDC enzyme, which is expressed in the cells throughout the body. The location of the HDC gene is found on chromosome 2 in mice and chromosome 15 in human, and their expression is controlled by lineage-specific transcription factor (Nakagawa et al., 1997). Moreover, the HDC activity is modulated, *in vitro*, by citokynes, such as IL-1, IL-3, IL-18 and TNF-α (Schneider et

al., 1987; Yoshimoto et al., 1999). *In vivo* study has demonstrated HDC activity in particular conditions, such as lipopolysaccharide (LPS) stimulation, infection, inflammation and graft rejection (Dy et al., 1981).

There are two major pathways of histamine metabolism: ring methylation and oxidative deamination by diamine oxidase (Schwartz et al., 1991). In the brain, histamine is metabolized by histamine-*N*-methyltransferase to form *tele*-methylhistamine. Most of the tele-methylhistamine formed is converted by monoamine oxidase B to *tele*-methylimidazoleacetic acid (Adachi, 2005) (Figure 6). The second pathway is specific for peripheral tissue, where histamine is mainly converted to imizoleacetic acid by diamino oxidase (Adachi, 2005).

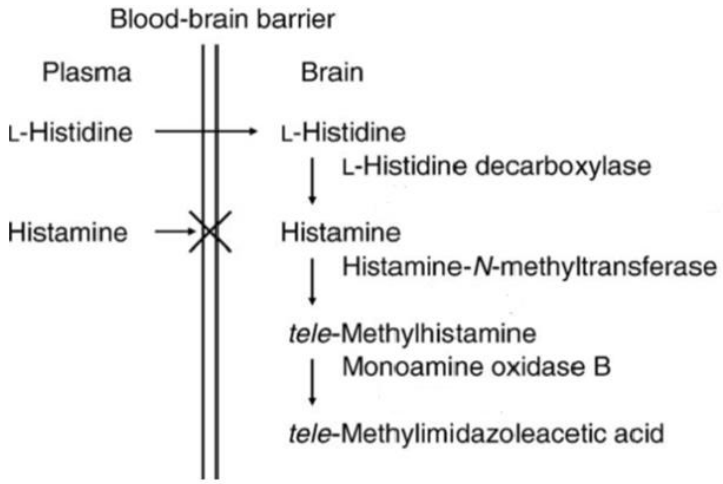

Figure 6 Biosynthesis and metabolism of brain histamine. Histamine in the brain is not transported from plasma but is formed from L-histidine by a specific enzyme, L-histidine decarboxylase (HDC). There are two major pathways of histamine metabolism; ring methylation and oxidative deamination by diamine oxidase. In the brain, most of histamine is catalized by histamine-*N*-methyltransferase to form *tele*-methylhistamine, which is converted by monoamine oxidase B to *tele*-methylimidazoleacetic acid. Figure modified from (Adachi, 2005).

3.1. The role of histamine in the periphery

The most important peripheral histamine sources are mast cells (Riley and West, 1952) and basophilis (Falcone et al., 2006), where histamine is associated in inactive form with the anion proteoglican and chondroitin-4-sulphate and stored in cytosolic granules. Histamine secreted from cells exerts its immunomodulatory role in response to various immunological and non-immunological stimuli (Kakavas et al., 2006; Krishnaswamy et al., 2006). The generation of HDC-deficient mice provided histamine-free system to

study the role of endogenous histamine in a broand range of normal and disease processes. These mice show decreased numbers of mast cells and significantly reduced granule content, suggesting a role of histamine in the synthesis of mast cell granule proteins (Ohtsu et al., 2001). Another peripheral source of histamine are the gastric enterochromaffin-like cells on the gastric mucosa, where it acts as a paracrine stimulant to control acid secretion in response to hormonal and neural stimuli (Prinz et al., 2003; Grandi et al., 2008).

In addiction, several myeloid and lymphoid cell types, such as dendritic cells, lymphocytes (Zwadlo-Klarwasser et al., 1994) and platelets (Masini et al., 1998). In particular, dendritic cells do not store histamine, show high HDC activity and are capable of production of high amount of histamine (Kubo and Nakano, 1999; Szeberényi et al., 2001). Histamine can influence numerous fuctions of the cells involved in the regulation of immune response and haematopoiesis. In fact, all these cells express histamine receptors and also secrete histamine, which can selectively recruit the major effector cells into the tissue sites and affect their maturation, activation, polarization and functions, leading to chronic inflammation (Jutel et al., 2009). Moreover, histamine can trigger inflammation acute symptoms due to its very rapid activity on vascular endothelium and bronchial and smooth muscle cells, leading to the development of such symptoms as acute rhinitis, bronchoconstriction, cramping, diarrhoea or cutaneous weal and flare responses. (Jutel et al., 2009). In particular, the release of histamine from mast cell induces not only acute allergic symptoms but also can be crucial for sustaining this response into a chronic phase (Jutel et al., 2002; Akdis and Blaser, 2003), enhancing the secretion of pro-inflammatory cytokines like IL-1α, IL-1β, IL-6 as well as chemokines (Merétey et al., 1991; Vannier and Dinarello, 1993; Jeannin et al., 1994; Bayram et al., 1999). Histamine is an important modulator of barrier function in endothelial and epithelial cells. *In vitro*, it can interact with endothelial cells (Rotrosen and Gallin, 1986; Ehringer et al., 1996) and epithelial cells (Takeuchi et al., 2001; Zabner et al., 2003), leading to increase in paracellular permeability while, *in vivo*, the effects on endothelial cells cause directly changes in vascular permeability, allowing for leukocytes infiltration and oedema formation (Jutel et al., 2002; Dy and Schneider, 2004).

3.2 The role of histamine in the CNS

The presence of histamine in the brain was first mentioned in 1943 (Kwiatkowski, 1943). In the mammalian CNS, histamine acts as an important neurotrasmitter or neuromodulator (Takagi et al., 1986), but it is not transported from the plasma since it cannot penetrate the BBB and so it is formed from L-histidine by a decarboxylase (see Figure 6) (Garbarg et al., 1983). The synthesized histamine is carried into vesicles by the vestibular monoamine-transporter VMAT-2. After release, it is methabolized in the cytoplasm by histamine-*N*-methyltransferase into the inactive tele-methilhistamine in the postsynapses or glia (Schwartz et al., 1991) (see Figure 6).

There are two important pools that contain histamine: neurons and mast cell (non-neuronal pool). The most important difference between neuronal and non-neuronal pools, is that the neuronal histamine pool turns over rapidly, with a half-life of less than 1 hour, while non-neuronal histamine turns over very slowly, with a half-life of several days (Martres et al., 1975; Garbarg et al., 1976).

Mast cells contain almost half of the histamine (Garbarg et al., 1976), and they have been identified in the brain parenchyma, mainly in the thalamus and hypothalamus (Ibrahim, 1974).

The soma of histaminergic neurons is located in the tuberomamillary nucleus in the hypothalamus, and their fibers are widely distributed throughout the brain (Wada et al., 1991). Studies aimed at assessing the role of the histaminergic system have utilized the intracerebroventricular (i.c.v.) administration of histamine and its antagonists, since histamine is not trasported to the brain across the BBB (Kalivas, 1982; de Almeida and Izquierdo, 1986). The histaminergic system regulates various brain functions, such as cognition, memory, cycle of sleeping and waking, energy and endocrine homeostasis (Higuchi et al., 2000; Haas and Panula, 2003). Moreover, the system regulates the release of non-histaminergic neurotrasmitters through histamine heteroreceptors located on the non-histaminergic nerve endings (Philippu et al., 1984; Prast et al., 1999). Thus, the histaminergic system forms a part of the neuronal network by modulating other neuronal system, such as catecholaminergic and cholinergic systems (Philippu et al., 1984; Prast et al., 1999).

3.3 Histamine receptors

The fundamental pleiotropic regulatory character of histamine in cellular events is attributed to its binding to four subtypes of G-protein-coupled receptors, designated H_1, H_2, H_3 and H_4 that are differentially expressed in various cell types (Dy and Schneider, 2004; Akdis and Simons, 2006; Chazot and Tiligada, 2008). In 1966, histamine receptors were first differentiated into H_1 and H_2 receptors (Ash and Schild, 1997). In 1987, a third histamine receptor subtype was cloned and termed as H_3 receptor (Lovenberg et al., 1999). Subsequentely, in 2000, the fourth histamine receptor was cloned and identified as H_4 receptor (Oda et al., 2000).

All these receptors are heptahelical transmembrane molecules that transduce the extracellular signal by using G-proteins and intracellular second messenger systems (Gantz et al., 1991; Le Coniat et al., 1994; Nakamura et al., 2000; Oda et al., 2000) (Figure 7).

Over the years, the study about different expressions, signal transduction or functions of histamine receptors improved the understanding of the role of histamine in physiology and disease.

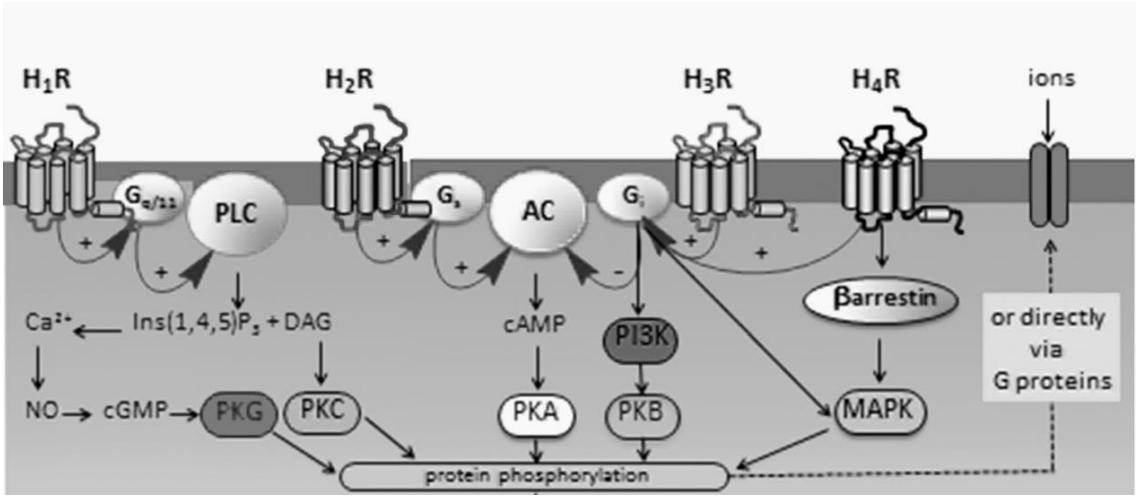

Figure 7 Schematic overview of the main signal transduction routes of the four different histamine receptors. Modified from (Panula et al., 2015).

3.3.1 H_1 receptor: distribution, peripheral and central functions

The histamine H_1 receptor (HR1) is coupled to G_q protein and its activation stimulates the inositol phospholipid signalling pathways, resulting in the formation of inositol-1,4,5-triphosphate and diacylglicerol and in an increase of intracellular Ca^{2+} (Bakker et al., 2001). Moreover, it also activates PLA2 and PLD (Jutel et al., 2009). The HR1 is expressed in numerous cells including airway and vascular smooth muscle cells, hepatocytes, endothelial cells, dendritic cells, monocytes, neutrophils, T and B cells (Leurs et al., 1995; Smit et al., 1999; Togias, 2003).

Activation of HR1 results in airway and vascular smooth muscle contraction, due to Ca^{2+} mobilization from intracellular stores (Leurs et al., 1995). Moreover, HR1 stimulation induces various cellular responses in vascular endothelial cells including changing in vascular permeability (Leurs et al., 2002; Akdis et al., 2004).

Histamine has a role in the functions and activity of dendritic cell precursors as well as their immature and mature forms, it is actively synthesized during cytokine-induced dendritic cell differentiation, acting in an autocrine and a paracrine fashion and modifies dendritic cell markers (Szeberényi et al., 2001). In fact, in the differentiation process of dendritic cell from monocytes, HR1 acts as positive stimulant that increases antigen-presentation capacity and pro-inflammatory cytokines production (Szeberenyi et al., 2001).

Histamine has been shown to intervene in the Th1, Th2 and Treg cell balance and consequently antibody formation. In particular, Th1 and Th2 present a differential pattern of histamine receptor, determining different responses to histamine stimulation. Th1 cells show predominant, but not exclusive expression of HR1 (Jutel et al., 2001), in mice, delection of HR1 results in the suppression of IFN-γ and dominant secretion of Th2 cytokines (IL-4 and IL-13) (Jutel et al., 2001).

Histamine increases adhesion molecule expression such as ICAM-1, VCAM-1 and P-selectin through HR1 activation in endothelial cells (Lo and Fan, 1987; Kubes and Kanwar, 1994; Yamaki et al., 1998).

Histamine, acting on granulocytes, regulates eosinophil migration in a dose-dependent manner. Whereas high doses inhibit eosinophil chemotaxis via histamine H_2 receptor, low doses enhance eosinophil chemotaxis via HR1 (Clark et al., 1977).

In human brain, higher densities of HR1 are found postsynaptically in neocortex, hippocampus, nucleus accumbens, thalamus, and posterior hypothalamus (Chang et al., 1979; Kanba and Richelson, 1984; Martinez-Mir et al., 1990; Villemagne et al., 1991;

Yanai et al., 1992). HR1 activation causes inhibition of firing and hyperpolarization in hippocampal neurons (Haas, 1981). However, many other notably hypothalamic supraoptic (Haas and Bucher, 1975), thalamic (Mccormick and Williamson, 1991), and human cortical neurons (Reiner and Kamondi, 1994) are excited by HR1 activation through a block of a potassium conductance. Targeted distruption of the HR1 gene in mice results in the impairment of neurologic fuctions such as memory, learning, locomotion and nociperception and in aggressive behaviour (Yanai et al., 1998).

Pharmacology

Histamine contributes to the progression of allergic-inflammatory responses by enhancement of the secretion of pro-inflammatory cytokines and chemokines both in several cell types and local tissue (Merétey et al., 1991; Vannier and Dinarello, 1993; Jeannin et al., 1994; Bayram et al., 1999). In particular, through HR1, it induces CC chemokines, monocyte chemotactic protein 1 and 3 in explant cultures of human nasal mucosa (Fujikura, 2001). So, HR1 antagonists, commonly referred to as antihistamines, are widely used in the treatment of allergy (Hill et al., 1997), they potently inhibit allergen-induced accumulation of eosinophils in the skin, nose and airways (Fadel et al., 1987), may be through the dowregulation of NF-kB, which acts as a potent transcription factor in initiating inflammation (Bakker et al., 2001). Their potential efficacy has been investigated in asthma (Malick and Grant, 1997), indeed it was demonstrated that inhalation, intravenous or oral administration of clemastine ((+)-2-[2-[(p-chloro-a-methyl-a-phenylbenzyl)oxy]ethyl]-1-ethylpyrrolidine) or chlorpheniramine induced significant bronchodilatation (Malick and Grant, 1997).

The first generation of antihistamines have been shown to penetrate the BBB and often have shown strong sedative effects, and also anticholinergic activities. The second generation of antihistamines has displayed long-term action having weak sedative and anticholinergic effects with relatively poor capacity for BBB penetration (Yanai et al., 1995a, 1995b). The sedative effects of antihistamines were initially recognized as side effects for the treatment of allergic rhinitis, but such action is now responsible for the use of the induction of sleep (Hu and Chen, 2017). Moreover, HR1 was found to be occupied by other drugs, such as antidepressant and antipsycotic from positron emissiontomography study in humans (Sato et al., 2015).

3.3.2 H_2 receptor: distribution, peripheral and central functions

The histamine H_2 receptor (HR2) is coupled with Gs protein determining adenylyl cyclase activity and conseguent cyclic AMP (cAMP) accumulation. HR2 is expressed in numerous cells and tissue such as gastric parietal cells, airway and vascular smooth muscle, T cells, neutrophils, dendritic cells and cardiac tissue. HR2 has a potent effect on gastric acid secretion, and the inhibition of this secretory process by HR2 antagonists has provided evidence for an important physiological role of histamine in the regulation of gastric secretion (Black et al., 1972; Black and Shankley, 1985; Soll and Berglindh, 1987). High concentrations of histamine are also present in cardiac tissues and can mediate positive chronotropic and inotropic effects on atrial or ventricular tissues via HR2 stimulation (Black et al., 1972; Hattori et al., 1983; Hattori and Levi, 1984; Inui and Imamura, 1976; Levi et al., 1982; Levi and Alloatti, 1988). Moreover, HR2 mediates smooth muscle relaxation in airway and vascular smooth muscle (Black et al., 1972; Edvinsson et al., 1983; Eyre and Chand, 1982; Foreman et al., 1985; Gross et al., 1981; Ottosson et al., 1989). Another effect of HR2 is the inhibition of a variety of functions within the immune system. In monocytes, histamine inhibits the production of pro-inflammatory IL-1-like activity, TNF-α, IL-12 and IL-18, but enhances IL-10 secretion through HR2 stimulation (Morichika et al., 2003; Takahashi et al., 2004). The inhibitory effect of histamine via HR2 is probably due to the regulation of ICAM-1 expression, leading to the reduction of the innate immune response stimulated by LPS (Morichika et al., 2003). Moreover, it has been shown that HR2 negatively regulates the release of histamine on basophils and mast cells (Lichtenstein and Gillespie, 1975), inhibits antibody synthesis, T cells proliferation and cytokines production (Jutel et al., 2002).

In human brain, HR2 is widely distributed postsynaptically with highest densities in the basal ganglia, hippocampus, amygdala, and cerebral cortex (Traiffort et al., 1992). Activation of cerebral HR2 can inhibit nerve cells (Haas and Bucher, 1975; Haas and Wolf, 1977), but the most intriguing action is a block of the long-lasting after-hyperpolarization and the accommodation of firing, an effect with a remarkably long duration leading to potentiation of excitation in rodents (Haas and Konnerth, 1983; Haas and Greene, 1986) and human brain (Haas et al., 1988).

Pharmacology

HR2 antagonists are widely used for the treatment of gastrointestinal disorders, in fact cimetidine (Tagamet^{TM}) became the first billion-dollar drug in the 1980s for the treatment of peptic ulcer disease (Hu and Chen, 2017). Despite the wide-spread use of the HR2 antagonist for the control of gastric acid release, they are rarely considered CNS drugs. There are some relevant preclinical research studies highlighting their potential to enhance the effects of opiate analgesics (Nalwalk et al., 1995) and for the treatment of psycotic disorders (Kaminsky et al., 1990; Mehta and Ram, 2014).

3.3.3 H_3 receptor: distribution and central functions

The histamine H_3 receptor (HR3) is coupled with Gi protein leading to inhibition of cAMP and accumulation of Ca^{2+} and activation of MAPK pathway. It has initially been identified in the central and peripheral nervous system as pre-synaptic autoreceptor controlling the release of histamine (Leurs et al., 2005) and as heteroreceptor on non-histaminergic fibers regulating the release of other neurotrasmitters (dopamine, serotonine, noradrenaline, GABA and acetylcoline) (Philippu et al., 1984; Prast et al., 1999) (Figure 8).

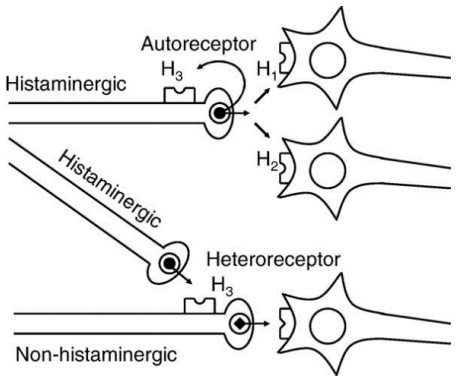

Figure 8 Autoreceptors and heteroreceptors. In the brain, HR3 receptors are located on histaminergic fibers as presynaptic autoreceptors and regulate release and synthesis of histamine. HR3 receptors also play a role as heteroreceptors located on non-histaminergic fibers and regulate release of non-histaminergic neurotransmitters by receiving histaminergic innervation (Adachi, 2005).

In particular, HR3 control of histamine release is a negative feedback mechanism, so reducing central histaminergic activity (Leurs et al., 2005).

It is mainly involved in brain functions, as cognition, sleep-awake status, energy homeostasis and inflammation. HR3 activation inhibits the firing of the histamine-neurons in the posterior hypothalamus through a mechanism different from autoreceptor functions found on other aminergic nuclei, presumably a block of Ca^{2+}-current (Haas, 1992). Also, the peripheral effect of histamine on mast cell via HR3, which mainly involved the nervous system, might be connected to a local neuron-mast cell interaction (Dimitriadou et al., 1994). The interaction of histamine on mast cell through HR3 involves neuropeptide-containing nerves and might be related to a local neuron-mast cell feedback loop controlling neurogenic inflammation (Dimitriadou et al., 1994). Dysregulation of this feedback loop may lead to excessive inflammatory responses and it may suggest a novel therapeutic approach by using HR3 agonists.

Pharmacology

HR3 has been viewed as a potential pharmacological target for the treatment of cerebral disorders, because it is primaly expressed in the brain (Nuutinen and Panula, 2010). HR3 antagonist/inverse agonists have been reported to have potential value in the treatment of wake-sleep disorders, cognitive impairment in Alzheimer's Disease, and schizophrenia. In March 2016, pitolisant (WakixR), an HR3 inverse agonist, was approved by the European Commission for the treatment of narcolepsy (Hu and Chen, 2017).

3.3.4 H_4 receptor: distribution, peripheral and central functions

The histamine H_4 receptor (HR4) has a 58% homology to HR3 (Jutel et al., 2009), and it is coupled with Gi protein inhibiting adenylyl cyclase and cAMP formation, activating MAPK and PLC with Ca^{2+} mobilization. HR4 appears to have higher affinity for histamine compared with HR1 and is highly expressed on the cells of immune system, such as neutrophils, eosinophils, mast cells, dendritic cells and T cells; on peripheral tissue, including spleen, thymus, colon and bone marrow (Nakamura et al., 2000; Zhu et al., 2001). Its expression is induced or altered in response to inflammatory stimuli (Oda et al., 2000; Lippert et al., 2004; Gutzmer et al., 2005; Damaj et al., 2007; Dijkstra et al., 2008), indeed its most clear activity consists in the recruitment and activation of cells

involved in inflammatory responses such as eosinophils, mast cells, neutrophils, T cells and dendritic cells (De Esch et al., 2005; Dunford et al., 2006; Gutzmer et al., 2005; Ling et al., 2004).

Histamine, more than 40 years ago, was first described as a selective chemoattractant for eosinophils (Clark et al., 1975), in fact it can induce actin polymerization, mobilization of intracellular Ca^{2+}, alteration in cell shape and up-regulation of adhesion molecule expression (Clark et al., 1975). The reported histamine effects on eosinophils chemotaxis can now be attributed to the HR4 (O'Reilly et al., 2002). Concrete evidence that HR4 controls leukocytes trafficking and pro-inflammatory responses is derived from the HR4-mediated histamine-induced activation of eosinophils: increased expression of adhesion molecules like Mac1 and ICAM-1 and rearrangement of the actin cytoskeleton leading to eosinophil migration into the bloodstream to the sites of inflammation (O'Reilly et al., 2002; Buckland et al., 2003; Ling et al., 2004; Barnard et al., 2008).

Human mast cells constitutively express HR4 that governs autocrine and paracrine histamine-induced processes (Lippert et al., 2004). Stimulation of HR4 also induces chemotaxis of mast cells (Hofstra et al., 2003). Experiment in mice showed that mast cells from wild-type and HR3-deleted mice migrated in response to histamine, while mast cells from HR4-deleted mice did not. These experiments demostrate that the chemotaxis of mast cells via histamine is triggered mainly through the HR4 (Hofstra et al., 2003). Selective recruitment of these effector cells into the tissue amplifies the histamine mediated reaction, leading to chronic allergic inflammation (Hofstra et al., 2003).

In general, histamine can enhance Th1 responses through HR1 activation and negatively regulate both Th1 and Th2 responses acting on HR2 (Jutel et al., 2001). HR4 modulates cytokine secretion during the integration of Th1/Th2 differentiation (Jutel et al., 2001), via the signal transduction and activators of trascription (STAT). Activation of STAT6 causing a shift towards the Th2 response, is implicated in allergic state development, while activation of STAT1 and STAT4 play a role in pathogenesis of asthma (Zampeli and Tiligada, 2009).

Moreover, HR4 activation induces Ca^{2+} mobilization and down-regulation of synthesis and release of Th2-linked chemokine CCL2 from monocytes. This negative feedback mechanism would avoid the Th2 enviroment in case of high histamine levels in allergic

inflammation and would contribute to the shift to Th1 that is observed in the transition from acute to chronic allergic inflammation (Dijkstra et al., 2007).

At central level, the HR4 mRNA was detected in human dorsal root ganglia (DRG), spinal cord and brain regions including hippocampus, cortex, thalamus and amygdala. In the rat HR4 was found in DRG, spinal cord, cortex, cerebellum, amygdala, thalamus and striatum (Strakhova et al., 2009). Moreover, HR4 was immunohistochemically indentified in the DRG and spinal cord (Strakhova et al., 2009; Lethbridge and Chazot, 2010) as well as in several regions of human and mouse brain, such as thalamus, hippocampus and cerebral cortex (Connelly et al., 2009).

Sanna and colleagues (2017) have characterized the behavioural phenotype of HR4-deficent mice. They have observed that the HR4-deficent mice showed an increased of locomotor activity, an axiogenic like phenotype and an increase of food intake (Sanna et al., 2017) demostrating an involvement of neuronal HR4 in locomotor activity, anxiety and food intake. In particular, they have shown that a prominent role of neuronal HR4 appears to be the modulation of pain perception (Sanna et al., 2017). In fact, behavioral experiments performed after i.c.v. administration of HR4 agonist, VUF 8430, in mice revealed pain-reducing effects in acute thermal nociception (Galeotti et al., 2013) and neuropathic pain (Smith et al., 2007; Sanna et al., 2015). While HR4-deficient mice showed a significantly increased hypersensitivity after spared nerve injury, a surgical procedure to produce peripheral neuropathy (Sanna et al., 2017). These results are in accordance with the data emerged after subcutaneously (s.c.) administration of HR4 antagonist, JNJ7777120, in a rat model of carrageenan-induced acute inflammation and thermal hyperalgesia. The antagonist significantly reduced paw edema and hyperalgesia provoked by subplantar injection of carrageenan (Coruzzi et al., 2007). These results highlight an opposite involvement of peripheral and neuronal HR4 on pain processes, with peripheral HR4 involved in promoting inflammatory pain, and neuronal HR4 involved in the relief from neuropathic pain (Sanna et al., 2017).

Besides neurons HR4 is expressed on microglial cells. The presence of HR4 mRNA was demostrated on primary rat cortex microglia cells and on murine N9 microglial cell line (Ferreira et al., 2012). The presence of HR4 protein was also confirmed by Western blot analysis and immunocytochemistry (Ferreira et al., 2012).

Moreover, in primary rat microglial cells histamine stimulates dose-dependently microglia activation and subsequently production of pro-inflammatory factors TNF-α and IL-6 (Dong et al., 2014) and ROS (Zhu et al., 2014) through the activation of HR4,

and these effects were abolished by HR4 antagonist (Dong et al., 2014; Zhu et al., 2014).

Pharmacology

The discovery of HR4 has allowed a better understanding of the role of histamine in pulmonary allergic responses (Jutel et al., 2009). In fact, HR4 deficient mice and mice treated with HR4 antagonists exhibited decreased allergic lung inflammation, with decreases in lung infiltrating eosinophils and lymphocytes and decreases in Th2 responses (Dunford et al., 2006). The evidence for a role of HR4 in many cells associated with asthma could explain the general lack of clinical efficacy of HR1 ligands in the disease, even if no clinical trials with HR4 antagonists have been reported (Jutel et al., 2009). Only a study about the effect of the dual HR4/HR2 agonist, 4-methylhistamine, on a mouse model of asthma that reduces airway hyperreactivity and inflammation when given by an inhaled route (Morgan et al., 2007).

The indole carboxamide compound, JNJ7777120, is the first selective and widely used HR4 antagonist, displaying high selectivity over other histamine receptors (Ling et al., 2004). Moreover, a 2-aminopyrimidine compound, ZPL-3893787, has completed phase I studies and is under development as an oral treatment for atopic dermatitis (Liu, 2014). However, HR4 ligands are not currently under evaluation to treat CNS disorders due to the enigmatic role of this receptor in the brain (Hu and Chen, 2017).

Histamine and Cerebral Ischemia

In literature, numerous studies are present about the role of histamine and its receptors in cerebral ischemia by acting on multiple targets (Hu and Chen, 2012, 2017).

In the experimental model of focal cerebral ischemia induced by MCAo, the levels of histamine and tele-methylhistamine in the striatum and surrounding cerebral cortex gradually increase and these changes become statistically significant 6-12 hours after ischemia (Adachi et al., 1991), reaching levels three- and two-fold higher, respectively, than those of the controlateral side (Adachi et al., 1991) (Figure 9).

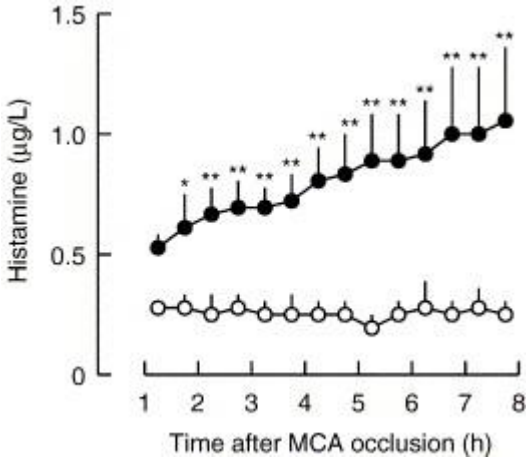

Figure 9 Concentration of histamine in micro dialysates was determined in conscious freely moving rats. The histamine increase is gradual and long lasting in the occluded group. Sham-operated animals (○), occluded animals (•). Each value represents the mean±SD of five animals. *p<0.05, **p<0.01, as compared with each corresponding value in the sham-operated group. Modified from (Adachi et al., 1991).

Moreover, the histamine release is reversed by preischemic administration of α-fluoromethylhistidine (α-FMH), an irrevirsible inhibitor of HDC (Adachi et al., 1992). In particular, α-FMH deplets histaminergic neurons of histamine within the first few hours after treatment. Since the turnover rate of neuronal histamine is more rapid than that of mast cell histamine (Martres et al., 1975; Garbarg et al., 1976; Kollonitsch et al., 1978), Adachi and collaborators (1992) proposed that the histamine release after ischemia was due to activation of histaminergic neurons. However, an increase of the number of mast cell undergoing degranulation has been shown after global cerebral ischemia (Hu et al., 2004). Mast cells accumulate in the ipsilateral hemisphere after

focal cerebral ischemia, therefore mast cell degranulation might account for increment of histamine content in thalamus and striatum regions (McKittrick et al., 2015).

4. Protective effects of histamine in cerebral ischemia

Considerable evidence shows that histamine has a protective effect on neurological injury after cerebral ischemia (Hu and Chen, 2012).

Intraperitoneal (i.p.) administration of the precursor of histamine, histidine, immediately and 6 hours after reperfusion, alleviates ischemic infarction after MCAo (Adachi et al., 2005). Moreover, histamine administrated by i.c.v. improves the delayed ischemic damage in hippocampal CA1 pyramidal cells, induced by transient forebrain ischemia (Fujitani et al., 1996). In addiction, histamine from mast cells protects against neuronal death induced by oxigen-glucose deprivation (OGD), an *in vitro* model of ischemia (Hu et al., 2007). In accordance, rats pretreated with α-FMH show significantly more necrotic hippocampal CA1 pyramidal cells than controls, after cerebral ischemia induced by 4-vessel occlusion (Adachi et al., 1993).

The expression and the binding density of histamine receptors also change after cerebral ischemia (Lozada et al., 2005). After 48 hours from transient global ischemia, HR1 mRNA expression increases while HR2 binding densities decrease in the caudate-putamen. HR3 mRNA expression increases in the caudate putamen but it decreases in the globus pallidus and the thalamus; in binding studies HR3 binding densities increases in the cortex, caudate-putamen, globus pallidus and hippocampus (Lozada et al., 2005).

4.1 Histamine and the regulation of excitotoxicity in the acute period after ischemia

Histamine alleviates the neuronal excitotoxicity induced by NMDA via HR2 and cAMP/PKA pathway (Dai et al., 2006). Although H1R is less studied in the context of ischemia, blockage of HR1 enhances the excitotoxic response of NMDA in cerebellar neurons (Díaz-Trelles et al., 2000).

After cerebral ischemia, the expression of astrocytic glutamate tansporter (GLT-1) and of glutamine synthetase (GS) declines, aggravating the accumulation of glutammate in the extracellular space and the excitotoxicity (Oliver et al., 1990; Chen et al., 2005). It

was demostrated that histamine upregulated the GLT-1 mediated current and the expression of GS via HR1, providing protection against cerebral ischemic injury in MCAo models (Fang et al., 2014). In particular, this effect was abrogated by HR1 antagonist, pyrilamine, in cultured astrocytes exposed to OGD (Wang et al., 2013) (Figure 10).

4.2 Histamine and the regulation of inflammation in the subacute period after ischemia

In the subacute period after ischemia, inflammation is initiated by microglia activation and by the release of proinflammatory cytokines, followed by the infiltration of neutrophils, macrophages, and monocytes (Hu and Chen, 2017).

Histamine is known to augment inflammatory reactions via HR1 in the periphery while stimulation of the HR2 shows an inhibitory effect on inflammation through downregulation of proinflammatory cytokines combined with the chemotactic responsiveness of leukocytes (Azuma et al., 2001; Takahashi et al., 2002; Akdis and Simons, 2006). After MCAo reperfusion, facilitation of histaminergic activity suppresses inflammatory cell infiltration via the HR2 (Hiraga et al., 2007). Moreover, administration of L-histidine, immediately and 6 hours after MCAo reperfusion, reduces the number of neutrophils to 52%, while the HR2 antagonist, ranitidine, reverses the L-histidine effects (Hiraga et al., 2007). These results suggest that the histamine suppression of inflammatory cell recruitment after ischemia may be due to HR2 (Hiraga et al., 2007) (Figure 10).

Simultaneous administration of thioperamide with L-histidine completely prevents oedema formation after MCAo (Irisawa et al., 2008). On the other hand, the HR2 antagonist ranitidine attenuates water accumulation and the maldistribution of ions in the brain in a bilateral common carotid artery occlusion model (Tósaki et al., 1994).

4.3 Histamine and the regulation of glial scar formation in the late period after ischemia

In the late period after focal cerebral ischemia, the reactive astrocytes and microglia contribute to glial scar formation, which impedes axonal regeneration and neurological recovery (Silver and Miller, 2004). Recently, it was demonstrated that treatment with histidine yields remarkable long-term neuroprotection at the late period after cerebral ischemia (Liao et al., 2015). Interestingly, this protection results from a facilitation of astrocyte migration towards the infarct *core*, inhibiting glial scar formation through the HR2 (Figure 10).

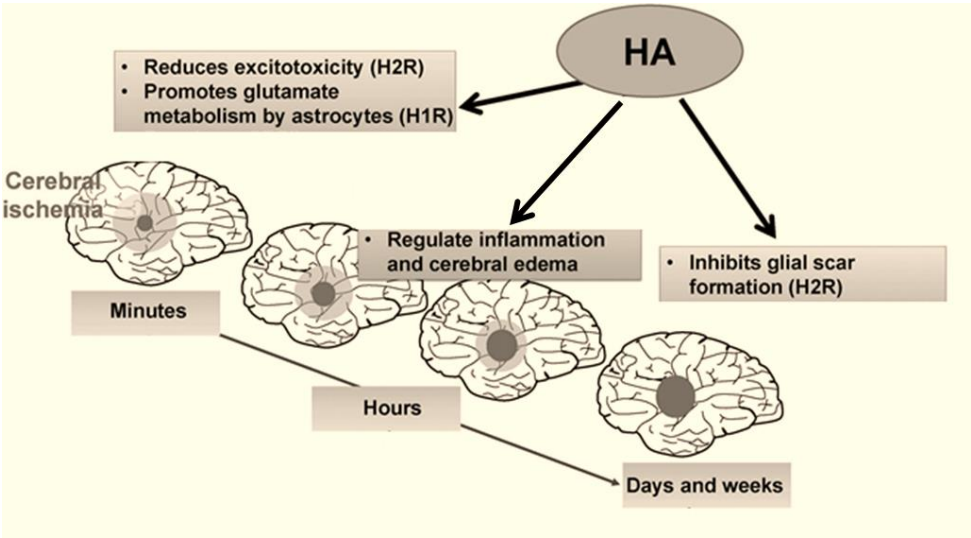

Figure 10. Schematic representation of the multitarget-directed protective activity of histamine and its receptors at different time-points following cerebral ischemia. Histamine reduces excitotoxicity via the HR2, promotes glutamate metabolism in astrocytes via the HR1 at the acute period after cerebral ischemia. Meanwhile, during the subacute period, histamine may be involved in neuroinflammation and cerebral edema. During the late period of cerebral ischemia, histamine still has a beneficial effect through suppression of glial scar formation. HA, histamine. Modified from (Hu and Chen, 2017).

4.4 Role of histamine H_3 receptor in cerebral ischemia

Due to the exclusive CNS distribution of HR3, agents targeting this receptor have peculiar neuroprotection potential, and their functions are the focus of much attention (Hu and Chen, 2012). Since, it is a presynaptic negative modulator of histamine release, it is thought that an HR3 antagonist would enhance histamine release and protection after ischemia events (Hu and Chen, 2012). Mice treated with HR3 antagonists, thioperamide or clobenpropit, and HR3-knockout mice show less impairment of

neurological function and a reduced infarct area after MCAo (Hu and Chen, 2012). While, HR3 agonist, α-methilhistamine, aggravates delayed neuronal death following ischemia induced by 4-vessel occlusion in rats (Adachi et al., 1993).

4.5 Role of histamine H_4 receptor in cerebral ischemia

To date there are no data in the scientific literature concerning the role of HR4 in cerebral ischemia.

Results exist about the HR4 role in ischemia/reperfusion liver injury in rats. It was demonstrated that the s.c. injection of HR4 agonist, clozapine, mimicked the protective effects of histamine, alleviating liver damage (Adachi et al., 2006).

In our studies, object of the present thesis, we investigated the effect of a chronic treatment (i.p., twice/day for 7 days), with the selective HR4 antagonist, JNJ7777120, in a rat model of transient (1h) ischemia induced by MCAo.

The Adenosinergic System

The nucleoside adenosine (Figure 11) is formed by a molecule of the purinergic nucleobase adenine bound to a ribose. Adenosine is a neuromodulator/neurotransmitter in the CNS, usually with a cytoprotective function (Fredholm et al., 2001). This nucleoside 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.

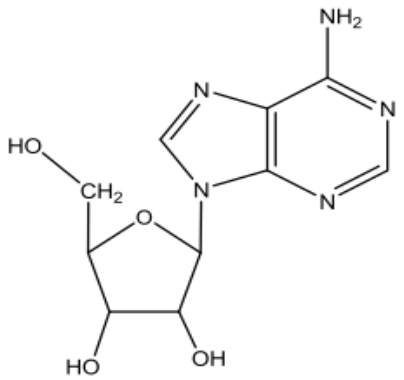

Figure 11 Adenosine molecular structure.

Many of the actions mediated by adenosine, are responsible of tissue protection during situations of stress, 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 Nervous System, 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. 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. 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 (Kuroda et al., 1976; Dunwiddie and Hoffer, 1980; Okasa and 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.

5. Adenosine formation

In the CNS, adenosine originates from neurons and from non-neuronal cells such as glial cells (Halassa et al., 2009). Its formation occurs by dephosphorylation of AMP operated by 5'-nucleotidases. These enzymes are present both intracellularly, (5'-endonucleotidase), and extracellularly, (5'-ectonucleotidase) (Zimmermann, 1992). Intracellularly adenosine is generated from AMP and extracellularly by enzimatic conversion of released nucleotides through coupled ectonucleotidases: CD39 that converts ATP/ADP to AMP and CD73 that hydrolizes AMP to adenosine. A further enzyme able to generate adenosine by sequential hydrolysis of ATP, is the alkaline phosphatase (Zimmermann, 2000).

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).

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 signalling is linked to adenylate cyclase, by favouring the accumulation of cAMP, may regulate the adenosine levels and thus the inhibitory effects in the CNS (Latini and Pedata, 2001).

Adenosine can also derive from S-adenosylhomocysteine (SAH) hydrolysis by the enzyme SAH-hydrolase (Figure 12). This reaction occurs mainly at cardiac level, where SAH represents an important source of intracellular adenosine but only in physiological and not under hypoxic conditions (Latini et al., 1996a). At neuronal level, this synthesis pathway does not significantly contribute to the adenosine production either in normoxic or in hypoxic conditions (Latini et al., 1996a; Pak et al., 1994).

Under normal physiological conditions, extracellular adenosine levels are between 20 and 300 nM, rising to a low micromolar range under extreme physiological situations (intensive exercises or low atmospheric oxygen levels) and high micromolar levels (30 µM) in pathological conditions such as ischemia (Newby, 1984).

5.1 5'-endonucleotidases

Six 5'-endonucleotidases with different subcellular localization have been cloned in mammals (Table 3).

5'-Nucleotidase (abbreviation)		**Aliases**	**Natural substrates**
cytosolic 5'-nucleotidase I (cN-I)	cN-IA	AMP-specific;	AMP, dTMP, dCMP, dGMP, dAMP, dIMP, (probable substrate = dUMP)
	cN-IB	cN-IA homologue; AIRP	AMP
cytosolic 5'-nucleotidase II (cN-II)		high *Km* 5'-NT; purine 5'-NT; GMP, IMP-specific 5'-NT	Nucleotidase reaction: IMP, dIMP, GMP, dGMP, XMP. Phosphotransfer reaction: inosine and Deoxyinosine
cytosolic 5'-nucleotidase III (cN-III)		PN-I; P5'N-1; UMPH	Nucleotidase reaction: CMP, UMP, dUMP, dCMP, dTMP. Phosphotransfer reaction: Uridine, Cytidine, Deoxycytidine
cytosolic 5'(3')-nucleotidase (cdN)		dNT-1; PN-II	3'-dUMP, 3'-dTMP, 3'-UMP, 2'-UMP, 5'-dIMP, 5'-dUMP, 5'-dCMP,5'-dTMP, 5'-dAMP
mitochondrial 5'(3')-nucleotidase (mdN)		dNT-2	5'-dUMP, 5'-dTMP, 3'-dTMP, 5'-, 3'-, 2'-dUMP

Table 3 Classification of 5'-endonucleotidases. Modified from (Bianchi and Spychala, 2003).

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 hydrolyse several substrates (see Table 3) 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 and 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 resynthesis, with a consequent production of adenosine (Dunwiddie and Fredholm, 1997). 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).

5.2 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 hydrolyzes 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 and Lowenstein, 1981) 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 and Barron, 1978; Zimmermann, 1992; Schoen et al., 1993; Resta et al., 1998; Spychala, 2000). Pathological conditions such as ischemia and hypoxia are major inducers of CD73 expression (Kitakaze et al., 1996; Braun et al., 1997; 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 signalling 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 and Richardson, 1993).

6. Adenosine release

The mode of adenosine release may differ depending on experimental conditions. After electrical stimulation *in vitro,* adenosine seems to originate directly from cells, while in ischemic experimental models *in vitro,* adenosine might have also an extracellular origin due to the degradation of the adenosine nucleotides released following changes in membrane permeability (Pedata et al., 1993).

Adenosine concentrations are regulated by a bidirectional flow mediated by transporters. These transporters are divided into two categories: those capable of a bidirectional transport across the plasma membrane of both purines and pyrimidines, according their concentration gradient (equilibrative transporters); and transporters that mediate the nucleotide influx thank to the coupling with sodium transporters (concentrative). Both transporters have been cloned and classified as: ENT1 and ENT2 equilibrative transporters; CNT1 and CNT2, concentrative transporters (Williams and Jarvis, 1991; Anderson et al., 1996; Baldwin et al., 1999). The equilibrative transporters work bidirectionally in order to maintain the intracellular and extracellular concentrations of adenosine in a range of 30-300 nM (Dunwiddie and Fredholm, 1997). 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 (Pazzagli et al., 1993; Melani et al., 2012). Conversely, under conditions of energetic imbalance, dypiridamole decreases extracellular adenosine indicating that the equilibrative carrier transports adenosine out of the cells (Latini and 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 (Pazzagli et al., 1993; Dobolyi et al., 2000).

On the all 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).

7. Adenosine catabolism

Adenosine metabolism is regulated by two enzymes: adenosine deaminase (ADA) and adenosine kinase (AK) (Figure 12). ADA degradates adenosine to inosine having a Km in the range of 17–45 μM (Phillips and Newsholme, 1979) and it is present both at intracellular and at extracellular sites where is anchored to the plasma membrane (Franco et al., 1986).

AK is an enzyme that converts adenosine to AMP, it is characterized by high specificity and a Km for adenosine of 2 μM (Phillips and Newsholme, 1979).

Since the 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 ADA is important only for the significant increases in adenosine concentration, such as those that occur during ischemia (Latini and Pedata, 2001).

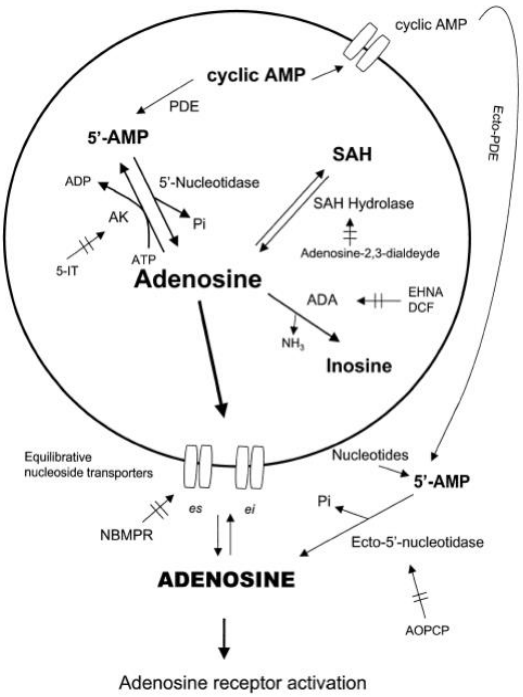

Figure 12 Adenosine synthesis, catabolism and transport. Adenosine is produced intracellularly by AMP dephosphorylation or by SAH hydrolysis and then released from the cell through specific membrane transporters, or extracellularly by ATP hydrolysis. Adenosine is deaminated by ADA and converted to inosine. ADA, adenosine deaminase; AK, adenosine kinase; AOPCP, α,β-methyleneADP; DCF, deoxyicoformicin; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenosine; es, sensitive transporter ei, insensitive transporter; 5-IT, 5-iodotubercidin; NBMPR, nitrobenzylthioinosine; PDE, cAMP-phosphodiesterase; SAH, S-adenosyl-homocysteine. From (Latini and Pedata, 2001).

8. 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 (van 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).

All adenosine receptors have been cloned from rat, mouse and human tissues (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 receptors are metabotropic heteromeric G-protein coupled receptor (GPCR). 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 (ECL1-3) and three intracellular loops (ICL1-3) (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) (Figure 13). 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).

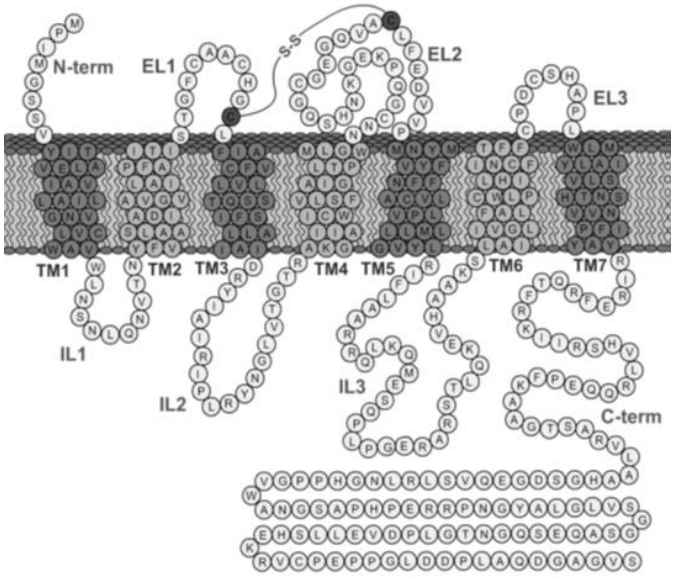

Figure 13 Schematic representation of the molecular structure of the A_{2A} receptor. From (Cristalli et al.,2008).

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 is the latest cloned and pharmacologically characterized (Linden et al., 1993), and 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 transduct signals via G proteins. Hence, A_1 and A_3 subtypes are associated with Gi activation, adenylyl cyclase inhibition and decrease of intracellular cAMP levels, while A_{2A} and A_{2B} receptors are linked to Gs 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 cyclase (and contrary to adenosine A_{2A} receptors) adenosine A_1, A_{2B} and A_3 receptors are also characterized by their stimulatory effect on PLC (Abbracchio et al., 1995; Feoktistov and Biaggioni, 1997) while A_1 and A_3 receptors can also activate 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 14).

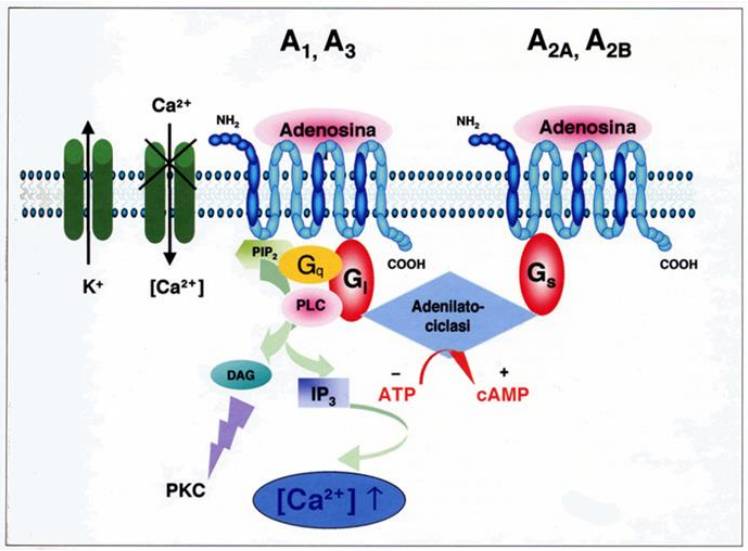

Figure 14 Adenosine receptors and signal transduction mechanisms. Adenosine receptors are G protein-coupled receptors. The A_1 and A_3 subtypes inhibit adenylate cyclase via Gi protein, while the A_{2A} and A_{2B} receptors mediate stimulation of the enzyme via Gs protein. In addition, A_1, A_3 and A_{2B} receptors can modulate, by Go protein or Gq, phospholipase activity, inducing the production of diacylglycerol (DAG) and inositol-triphosphate (IP3) and an increase in Ca^{2+} release from intracellular deposits. From (Gessi and Borea, 2011).

Another key feature for adenosine receptor subtypes classification is the affinity for their endogenous ligand. A_1, A_{2A} and A_3 receptors have high affinity for the endogenous ligand, being activated by nanomolar concentrations of adenosine (Fredholm et al., 2011). On the contrary, the affinity values of A_{2B} receptors for adenosine evaluated in binding and functional experiments are higher than 1 µM (Fredholm et al., 2011). Under physiological conditions, extracellular adenosine concentrations are estimated in the range of 30 to 200 nM (see: Latini and Pedata, 2001). These levels are sufficient to activate A_1, A_{2A} and A_3 receptors subtypes, but not A_{2B}, which require higher concentrations (micromolar range) of adenosine to be activated. Such higher adenosine concentrations are only reached under pathological conditions, such as during hypoxia or ischemia *in vivo* (Pedata et al., 2001) and *in vitro* (Latini et al., 1999).

8.1 A_1 receptor: molecular structure, localization 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. It is coupled with the $G_{i/0}$ protein at the TM3 loop. A_1 receptor mediates: inhibition of

adenylate cyclase, activation of several types of K^+ channels (probably via β,γ-subunits), inactivation of N, P and Q-type Ca^{2+} channels, stimulation of phospholipase C (Burnstock et al., 2011), and stimulation of phospholipase D (Fredholm et al., 2001) (Figure 15).

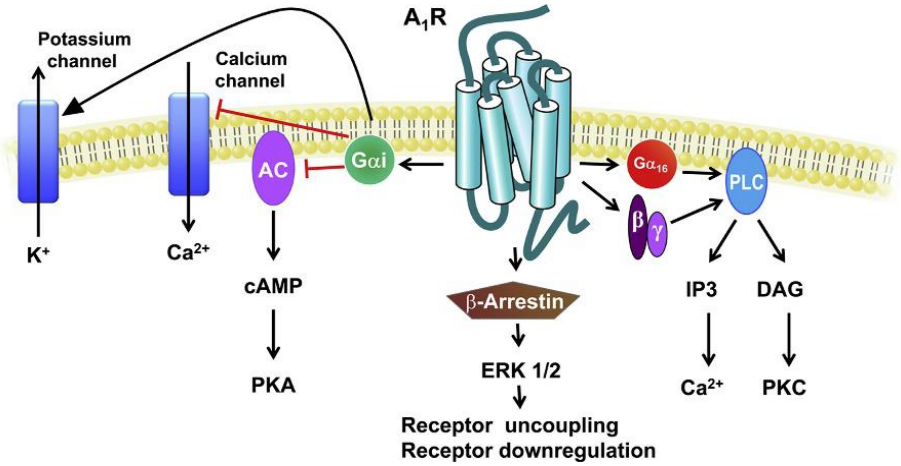

Figure 15 Major signalling pathway of A_1 adenosine receptor (A_1R). From (Chen et al., 2014b).

The highest levels of A_1 receptor expression are found in the CNS (Table 2), suggesting a particularly important role of adenosine in brain functions. In rat the highest expression of A_1 receptors has been found in the cortex, hippocampus, cerebellum and dorsal horn of the spinal cord; intermediate levels in basal ganglia structures including the striatum (Dixon et al., 1996). mRNA encoding A_1 receptors is present in large striatal cholinergic interneurones (Dixon et al., 1996) and in neurons of the globus pallidus, subthalamic nucleus and thalamus (Fredholm et al., 2001). 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). Neuronal A_1 receptors are localized both pre- and post-synaptically (Deckert and Jorgensen, 1988). In the hippocampus subcellular analysis of nerve terminals revealed that A_1 receptor immunoreactivity is strategically located in the active zone of presynaptic terminals, as expected on the basis of the ability of A_1 receptor agonists to depress neurotransmitter release. It has also been demonstrated that A_1 receptor immunoreactivity is evident at postsynaptic sites together with NMDA receptor subunits NR1, NR2A and NR2B and with N-and P/Q-type Ca^{2+} channel immunoreactivity, emphasizing the importance of A_1

receptors in the control of dendritic integration (Rebola et al., 2003). A_1 receptors can be found also extrasynaptically on dendrites (Rivkees et al., 1995) and on the axonal fibres of the hippocampus (Swanson et al., 1995). Activation of A_1 receptors along the axon may be a powerful extrasynaptic mechanism by which adenosine alters axonal electric transmission to inhibit neurotransmitter release (Swanson et al., 1995).

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 (Von Lubitz, 1999; Latini and Pedata, 2001). The inhibitory effect of adenosine A_1 receptor stimulation has a pre- and post-synaptic component. At pre-synaptic level, the activation of A_1 receptor reduces Ca^{2+} influx through the preferential inhibition of N-type and, probably, Q-type channels (Yawo and Chuhma, 1993; Wu and Saggau, 1994), with a consequent drecrease in trasmitters release (Prince and Stevens, 1992). In fact, adenosine, by stimulation of A_1 receptor, has been found to inhibit the release of all classic neurotrasmitters: glutamate, acetylcoline, dopamine, noradrenaline and serotonin (see Fredholm and Dunwiddie, 1988). In particular, a powerful suppression of glutamate release from presynaptic terminals has been described in the hippocampus (Corradetti et al., 1984; Burke and Nadler, 1988).

At post-sypatic level, A_1 receptors mediate a direct hyperpolarization of neurones via activation of G-protein-coupled inwardly rectifying potassium (GIRK) channels (K_{ir} 3.2 and 3.4 channels) (Takigawa and Alzheimer, 1999, 2002) and by a direct increase of Cl^- conductance (Greene and Haas, 1991) thus, stabilizing the membrane potential.

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

-Agonist. 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 (Bruns et al., 1987).

-Antagonist. Methylxanthines are non-selective antagonist of adenosine receptors (Sattin and 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(1*H*)-yl)-3,7-dihydro-1,3-dipropyl-1*H*-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).

8.2 A_{2A} receptor: molecular structure, localization and function

A_{2A} receptor is a glycoprotein of approximately 45 kDa with an amino acid sequence highly conserved 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 Gs protein at TM2 and TM3 loop (Moreau and Huber, 1999), it stimulates adenylate cyclase and increases intracellular cAMP concentrations (Moreau and Huber, 1999) and mobilizes the intracellular Ca^{2+} (Mirabet et al., 1997) (Figure 16).

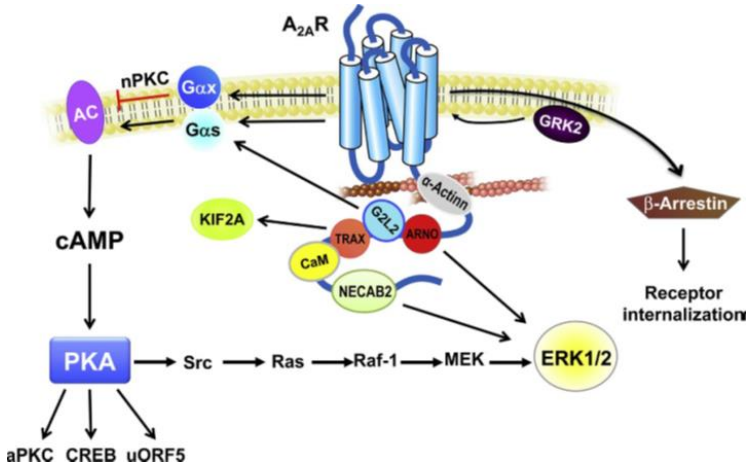

Figure 16 Major signalling pathway of A_{2A} adenosine receptor ($A_{2A}R$). From (Chen et al., 2014b)

A_{2A} receptors are expressed throughout all the CNS (Dixon et al., 1996). They are principally located 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 neurones (where it co-localises with dopamine D_2 receptors, but not on GABAergic-dynorphin striatal neurones (Fredholm and 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). In the CNS 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) (see Figure 17).

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. Various lines of evidence indicate that such an interaction occurs postsynaptically in the striatum between A_{2A} adenosine and D_2 dopamine receptors, this heterodimerization inhibits D_2 receptor functions (Ferre et al., 1991). While 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).

In the nervous system, A_{2A} receptor activation mediates excitatory actions, contrary to the A_1 receptor that exerts synaptic inhibition (Pedata et al., 1984; Spignoli et al., 1984; Latini et al., 1996b; Sebastião and Ribeiro, 1996). Electrophysiological investigations about the role of A_{2A} receptors under physiological conditions have shown that they increase synaptic neurotransmission. In fact, the A_{2A} receptor G_s-mediated signalling 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 Gs protein, which can promote the phosphorylation, mediated by PKC, of Ca^{2+} channels of N-type (Gubitz et al., 1996; Gonçalves et al., 1997). In fact, in the hippocampus *in vitro*, A_{2A} receptor stimulation results in a Ca^{2+}-dependent release of acetylcholine (Spignoli et al., 1984; Cunha et al., 1995). Moreover, the selective stimulation of adenosine A_{2A} receptors augments the amount of glutamate released in hippocampus and striatum of young rats (Corsi et al., 1999, 2000; Popoli et al., 1995), supporting the theory about that A_{2A} receptors increase excitatory amino acid release.

A_{2A} receptor also impacts in long-term potentiation (LTP), a form of synaptic plasticity associated with memory, central for learning process, that is increased by A_{2A} receptor activation (Almeida et al., 2003). In fact, a decreased LTP in the nucleus accumbens was found in knock-out (KO) mice for the A_{2A} receptor (d'Alcantara et al., 2001).

It is worth noticing that the role of A_{2A} receptors in the striatum is recently gaining interest in light of their heterodimerization with D_2 dopamine receptors. 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} antagonists not only provide symptomatic relief but also decelerate dopaminergic neuron degeneration in patients (Xu et al., 2005).

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 (Haskó et al., 2008; Antonioli et al., 2014). Lower levels are also found in the heart, lung and blood vessels (Fredholm et al., 2001).

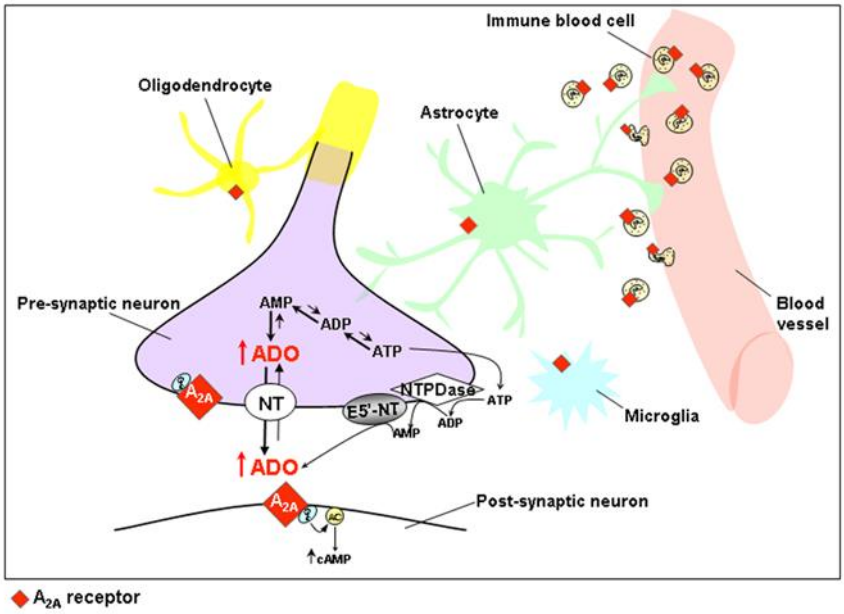

Figure 17 Schematic drawing of adenosine A_{2A} receptor localization on different cell types. Adenosine A_{2A} receptors are expressed at central level on pre-synaptic and post-synaptic neurons, on astrocytes, on microglia and on oligodendrocytes. A_{2A} receptors are present also at peripheral level on leukocytes and vasculature. Abbreviations: AC: adenylate cyclase; ADO: adenosine; ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; E5'-NT: ecto-5'-nucleotidase; NT: nucleoside transporter; Gs: stimulatory G-protein; NTDPase: ecto-nucleoside triphosphate diphosphohydrolases. The proportions of the various components of the nervous tissue have not been kept. From (Pedata et al., 2014).

Pharmacology

-Agonist. 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 (2-[p-(2-carboxyethyl)-phenethylamino]-5'-Nethylcarboxamidoadenosine) have been synthetized. NECA was long considered to be a selective A_2 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 (Fredholm et al., 2001). However, based on evidence that 2-substitution of NECA molecule increased selectivity, CGS21680 was developed as an A_{2A} selective agonist (Hutchison et al., 1989). This compound is less potent and selective in humans than in rats (Kull et al., 1999), but it has been replaced by another recently developed A_{2A} 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 (Rieger et al., 2001). By substitution in position N6, the compound DPMA has been obtained (Bridges et al., 1988).

-Antagonist. 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 and 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} 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]-7*H*-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) (Poucher et al., 1995). 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). Recently, the A_{2A} antagonist istradefylline (KW-6002: 8-[(*E*)-2-(3,4-dimethoxyphenyl)vinyl]-1,3-diethyl-7-methyl-3,7-dihydro-1*H*-purine-2,6-dione)) is approved (2014) for treatment of Parkinson's disease (Borea et al., 2018).

8.3 A_{2B} receptor: molecular structure, localization and function

The A_{2B} receptor is a glycoprotein of approximately 36-37 kDa, coupled to Gs protein, that stimulates adenylate cyclase, intracellular Ca^{2+} mobilization (Mirabet et al., 1997) and activates PKC and PLC (Abbracchio et al., 1995; Feoktistov and Biaggioni, 1997) (Figure 18).

A_{2B} receptors are expressed both in the CNS and peripheral nervous system (PNS) (Dixon et al., 1996). A_{2B} receptors are shown to be ubiquitously distributed in the brain and their mRNA has been detected in all rat cerebral areas studied (Dixon et al., 1996; Von Lubitz, 1999) with a prevalent presence on hippocampal neurons (Mogul et al., 1993; Kessey et al., 1997) and on glial cells (Peakman and Hill, 1994; Fiebich et al., 1996).

A_{2B} receptors appear to 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). The signalling pathways generated by A_{2B} receptors stimulation are strongly influenced by the signalling of other receptors that affect the PLC-Ca^{2+}-PKC pathway. In brain slices, 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).

At peripheral level, A_{2B} subtype is particularly abundant in the gastrointestinal tract, mainly in caecum, colon, urinary bladder, lung, blood vessels and adipose tissue (Fredholm et al., 2001). Increasing evidences indicate a role for this receptor in the modulation of inflammation and immune responses in selected pathologies like cancer, diabetes, as well renal, lung and vascular diseases (Borea et al., 2018).

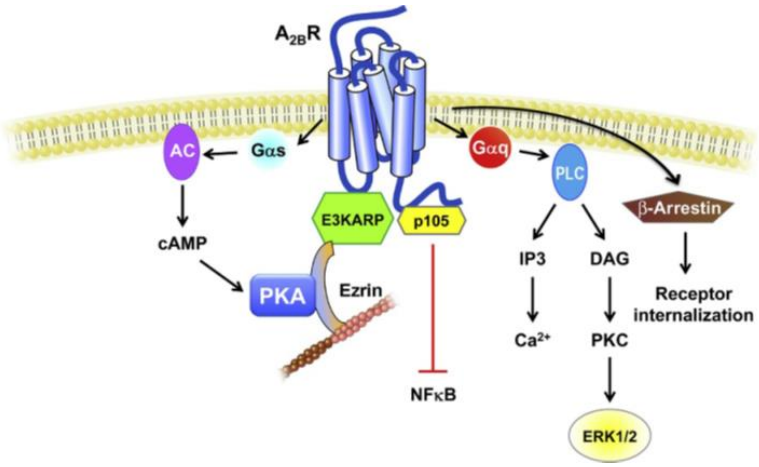

Figure 18 Major signalling pathway of A_{2B} adenosine receptor (A_{2B}R). From (Chen et al., 2014b)

Pharmacology

-Agonist. A class of non-adenosine A_{2B} 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) 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.

-Antagonist. Several potent and relatively selective antagonists have been found among anilide derivatives of xanthines with K_i values in the low nanomolar range, such as MRS 1754 (*N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-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). 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) (Kim et al., 2001; Stewart et al., 2004; Gessi et al., 2005).

8.4 A_3 receptor: molecular structure, localization 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., 2004, 2005) and interacts with MAPKs (Schulte and Fredholm, 2003) (Figure 19).

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) (Table 2) where they are mainly expressed at the presynaptic level (Lopes et al., 2003).

In literature discrepancies about the role of adenosine A_3 receptors in the CNS are present. An excitatory role of A_3 receptor has been supported by evidence indicating that, in the rat hippocampus, its activation attenuates long-term depolarization (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 and Fredholm, 1997). 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 (2001), 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.

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 and Fredholm, 1997; 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 (Linden et al., 1993; Salvatore et al., 1993). Interestingly, A_3 receptor is overexpressed in several cancer cells and tissues and is therefore likely to have an important antitumoral role (Borea et al., 2014).

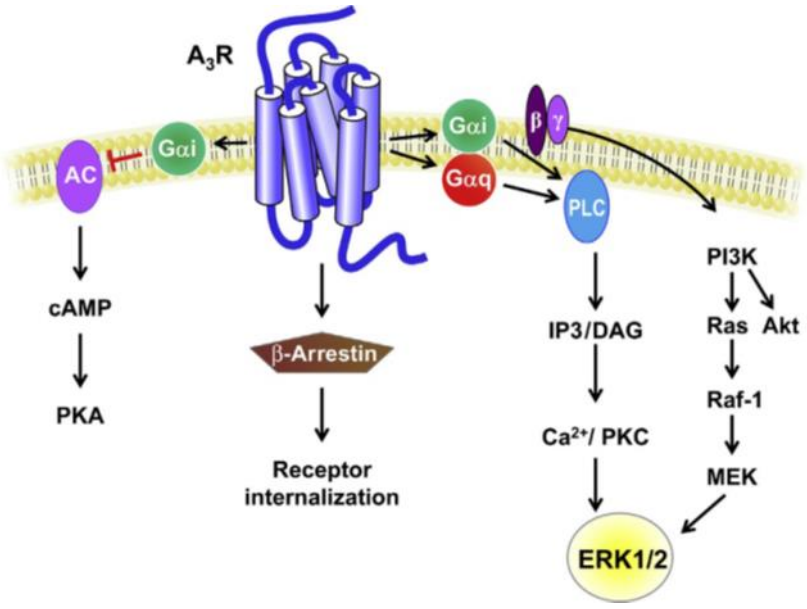

Figure 19 Major signalling pathway of A_3 adenosine receptor (A_3R). From (Chen et al., 2014b).

Pharmacology

-Agonist. The most widely used A_3 agonist is Cl-IB-MECA (2-Chloro-N^6-(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 (Jeong et al., 2003). Cl-IB-MECA affinity does not vary beyond an order of magnitude among mammal species, while the A_3 agonist MRS 1523 (*N*-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide) is the unique rat-selective. Particularly potent agonists can be obtained through the 4'-thio modification of adenosine derivatives, such as in LJ 568 (Jeong et al., 2003).

-Antagonist. 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 (Fredholm, 1995). Hence, A_3 antagonists are dihydropyridines, pyridines and flavonoids (Baraldi et al., 2000). 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 K_i value of 4 nM versus human A_3 receptors but not versus the rat isoform (van Muijlwijk-Koezen et al., 2000). 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 on human than rat receptors, as described for MRS 1220 (*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).

Role of Adenosine A_{2A} and A_{2B} Receptors in Cerebral Ischemia

9. Adenosine in cerebral ischemia

The increase of extracellular adenosine concentration during *in vivo* ischemia (Dux et al., 1990; Hagberg et al., 1987; Matsumoto et al., 1992; Melani et al., 1999; Phillis et al., 1994, 1996; Sciotti et al., 1992) is attributable to different reasons. Early after ischemia, the increase of adenosine is mainly due to extracellular released ATP (Melani et al., 2012) that is hydrolysed by ectonucleotidases (NTPDases 1, 2 and 3 that convert ATP o ADP and AMP) and ecto-5'-nucleotidase that converts AMP to adenosine (Zimmermann, 2000; Fausther et al., 2012). Thereafter adenosine *per se* is mainly released from cells likely by the ENT2 (Melani et al., 2012). Inhibition of adenosine-uptake processes due to down-regulation of CNT2 and 3 and of the ENT1 also contributes to the extracellular adenosine increase after stroke (Medina-Pulido et al., 2013).

The extracellular adenosine concentrations reached after ischemia allow the stimulation of all adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3). In particular, their wide distribution on neuronal, glial and inflammatory cells (Fiebich et al., 1996; Brodie et al., 1998; Svenningsson et al., 1999; Hettinger et al., 2001; Yu et al., 2004), suggests that the role of adenosine in ischemia is the consequence of an interplay among different receptor activation, which changes depending on the time-related development of the pathological condition (Pedata et al., 2014).

During ischemia, adenosine has long been identified as a neuroprotectant endogenous agent (Cunha, 2001; Pedata et al., 2007; Fredholm et al., 2003; Ongini et al., 1997; Ribeiro et al., 2002; Schwarzschild et al., 2002). 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). Protective effects are greatly attributed to A_1 receptor activation due to reduced Ca^{2+} influx, thus lowering presynaptic release of excitatory neurotransmitters (Andiné; Corradetti et al., 1984; Dunwiddie, 1984; Zetterström and Fillenz, 1990; Pedata et al., 1993; Kitagawa et al., 2002) and in particular of glutamate which exerts an excitotoxic effect during ischemia mainly by overstimulation of NMDA receptors (Choi, 1990). In

addition, by directly increasing the K^+ and Cl^- ion conductances, adenosine stabilises the neuronal membrane potentials, thus reducing neuronal excitability (Choi, 1990). Nevertheless, the use of selective A_1 agonists is hampered by undesirable effects such as sedation, bradycardia, hypotension (White et al., 1996; Fredholm et al., 2005).
More recently adenosine A_{2A} receptors emerged as an interesting target in ischemia.

9.1 Adenosine A_{2A} receptor antagonists protect against primary ischemic injury

9.1.1 Adenosine A_{2A} receptor antagonists are protective against ischemic damage

Gao and Phillis, (1994) demonstrated for the first time that the non-selective A_{2A} antagonist CGS15943 (9-chloro-2-(furan-2-yl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine), reduced cerebral ischemic injury in a model of global forebrain ischemia in the gerbil. Thereafter many reports have confirmed the neuroprotective role of A_{2A} receptor antagonists in different models of ischemia. For example: CSC (8-(3-chlorostyryl)caffeine), as well as less selective antagonists, such as CGS15943 and CP66713 protected against hippocampal cell injury in different models of ischemia in gerbil and rat (Phillis, 1995; Von Lubitz et al., 1995).
In all the mentioned studies, adenosine A_{2A} receptor antagonists were administered preischemia. However, postischemia administration is more relevant to a possible clinical use of drugs in stroke. The selective A_{2A} receptor antagonist, SCH58261, acutely (Monopoli et al., 1998; Melani et al., 2003) and subchronically administered was protective from brain damage, neurological deficit (Melani et al., 2006, 2009; Pedata et al., 2005) and disorganization of myelin (Melani et al., 2009) 24 hours after focal cerebral ischemia in the adult rat. Moreover, a noxious role of A_{2A} receptors during ischemia is also supported by the observation that A_{2A} receptor KO mice show significantly decreased infarct volume up to 48 hours after transient MCAo when compared with their wild-type littermates (Chen et al., 1999; Gui et al., 2009).
In particular, SCH58261 behaves as a significant protective agent at the dose (0.01 mg/kg) that does not have cardiovascular effect. This low dose does not affect motor activity in naive animals but decreases contralateral turning behaviour after ischemia induced by monofilament technique (Melani et al., 2003, 2006).

Most recently, the question has been rised if A_{2A} receptor continuous blockade over an extended time-window after ischemia is protective. CSC continuously administered over 72 hours, after permanent MCAo in hypertensive rats, did not decrease brain infarct volume 3 days after the beginning of ischemia (Fronz et al., 2014). Authors attributed the lack of protection to high hepatic metabolism and elimination of CSC (Fronz et al., 2014).

In our studies, object of the present thesis, we investigated if the selective adenosine A_{2A} receptor antagonist, SCH58261, chronically administered (i.p., twice/day for 7 days) maintained a protective effect 7 days after transient (1h) MCAo in the rat.

9.1.2 A_{2A} receptor antagonists protect from the increase of glutammatergic excitatory transmission

The ability of adenosine A_{2A} receptor antagonists in protecting against ischemic damage is largely attributed to the control of excessive glutamatergic transmission and of the ensuing acute excitotoxicity after ischemia (Pedata et al., 2016).

A_{2A} receptors are expressed on neurons at high levels in the striatum (Fink et al., 1992) and at lower levels in all other brain regions as detected by autoradiography (Johansson and Fredholm, 1995) and real time PCR (Dixon et al., 1996). A_{2A} receptors in the striatum are located presinaptically (Hettinger et al., 2001; Rebola et al., 2005a; Rosin et al., 2003) on glutamatergic terminals (Rebola et al., 2005b) where they can directly regulate glutamate release under normoxix (Lopes et al., 2002; Rodrigues et al., 2005) and ischemic conditions *in vivo* (Marcoli et al., 2003, 2004). Consistently, A_{2A} receptors play an important modulation of synaptic transmission (Sebastião and Ribeiro, 1996; Lopes et al., 2011) as demonstrated in the hippocampus (Cunha et al., 1994; Lopes et al., 1999). In the CA1 area of the rat hippocampus, which is the most sensitive region to ischemia, the selective A_{2A} receptor antagonists, ZM241385 and SCH58261, in model *in vitro* of severe (7 min) OGD, delay the appearance of AD, a phenomenon strictly related to cell damage and death (Somjen, 2001), protect from the synaptic activity depression, and protect CA1 neuron and astrocytes from injury (Marcoli et al., 2003). Same effects of ZM241385 were observed after a severe (9 min) OGD period in the gyrus dentatus of the hippocampus (Maraula et al., 2013). The time-window of the protective effects of the A_{2A} receptor antagonists in the hippocampus overlaps with the delay obtained by treating the slices with glutamate receptor antagonists (Tanaka et al.,

1997; Yamamoto et al., 1997), indicating that their effects are attributable to reduced glutamate excitotoxicity.

Moreover, adenosine acting on A_{2A} receptor regulates AMPA (Dias et al., 2012) and NMDA receptor function (Rebola et al., 2008). In the hippocampus A_{2A} and mGlu5 receptors are co-located and A_{2A} receptors play a permissive role in mGlu5 receptor-mediated potentiation of NMDA effects (Tebano et al., 2005). By the use of SCH58261, it was demonstrated that A_{2A} receptors support the expression and recruitment of Ca^{2+}-permable AMPA receptors during LTP induced by OGD in rat hippocampal slices (Dias et al., 2013).

In vivo, a definite overexpression of A_{2A} receptors was found in neurons of the striatum and cortex 24 hours after focal ischemia (Trincavelli et al., 2007) and it was demonstrated that the low dose of SCH58261 that protects against tissue damage induced by MCAo or quinolinic acid excitotoxicity, also reduces glutamate extracellular concentrations (Popoli et al., 2002; Melani et al., 2003). This supports that protective effects of low doses of A_{2A} receptor antagonists administered early after brain ischemia are largely due to reduced excitotoxicity and to the ensuing excitotoxic cascade attributable to stimulation of NMDA receptors (Melani et al., 2003).

A further mechanism by which A_{2A} receptor antagonism is protective may be due to the capability of increasing GABA extracellular concentration during ischemia. The major part of excitatory glutamatergic innervation is modulated by inhibitory GABA releasing interneurons. Potentiation of GABAergic synaptic transmission has neuroprotective effects in several experimental models of cerebral ischemia (Schwartz-Bloom and Sah, 2001). GABA is strongly increased in the cortex and striatum during ischemia (O'Regan et al., 1992; Melani et al., 1999) and evidence shows that selective A_{2A} receptor stimulation decreases ischemia-evoked GABA outflow (O'Regan et al., 1992; Saransaari and Oja, 2005).

9.1.3 A_{2A} receptor antagonists protect from ischemia-induced activation of intracellular pathways

Adenosine A_{2A} receptor antagonists might exert protection also because regulate the activation of proteins involved in transcriptional or post-trascriptional mechanisms playing important roles in ischemia (Pedata et al., 2016).

Twenty-four hours after focal ischemia, a definite over expression of A_{2A} receptors (Trincavelli et al., 2007) and an activation of p38 and ERK1/2 MAPKs was found in neurons and in microglia of the ischemic tissue (Irving et al., 2000; Takagi et al., 2000; Piao et al., 2003; Melani et al., 2006). Subchronic administration of the A_{2A} receptor antagonist, SCH58261, has reduced phospho-p38 in microglia while it did not affect ERK1/2 MAPK activation 24 hours after ischemia (Melani et al., 2006). A control of p38 activation by A_{2A} receptor antagonism (Melani et al., 2006) might account for neuroprotection after ischemia as demonstrated in brain slices after OGD (Barone et al., 2001). These effects might be directly due to A_{2A} receptors located on microglia, whose stimulation is known to be the cause of microglial activation (Orr et al., 2009), and the block with antagonists suppresses microglia activation in murine N9 microglial cells exposed to an inflammatory stimulus such as LPS (Gomes et al., 2013).

Twenty-four hours after MCAo, A_{2A} receptor antagonist, SCH58261, subchronically administered, also reduces phospho-JNK MAPK, expressed in mature oligodendrocytes, in oligodendrocytes progenitors (OPC) and in few neurons (Melani et al., 2009). Phospho-JNK MAPK is a factor involved in oligodendrocyte death (Bonetti et al., 1999; Howe et al., 2004; Jurewicz et al., 2006). This is also proved by the fact that JNK2/3 MAPK KO mice are protected from damage following cerebral ischemia (Lee et al., 2003; Gelderblom et al., 2004). Thus, JNK MAPK activation in oligodendrocytes and neurons represents a noxious event after ischemia that can damage oligodendrocytes bringing to myelin damage and disorganization (Melani et al., 2009).

Besides a direct effect of the A_{2A} receptor antagonists on receptors located on oligodendrocytes or microglia, we should consider that the reduced MAPK activation by SCH58261, in the initial hours after *in vivo* ischemia is secondary, to overall reduction of the excitotoxic cascade that in turn primes microglial activation (Bruce-Keller, 1999) and MAPK activation (Melani et al., 2003). In fact, oligodendroglia are extremely sensitive to glutamate receptor overactivation and ensuing oxidative stress (Matute et al., 1997; Mcdonald et al., 1998) as well as to cytokines (Back, 2006) and p38 MAPK activation is definitely induced by NMDA receptor stimulation in cerebellar

granule cells (Kawasaki et al., 1997) and in spinal cord cultures (Tikka and Koistinaho, 2001).

9.2 Adenosine A_{2A} receptor agonists protect against secondary injury

9.2.1 Adenosine A_{2A} receptor agonists are protective against ischemic damage

While many data support that A_{2A} receptor antagonists protect against central excitotoxicity, also A_{2A} receptor agonists were found protective but the protection appears attributable to different mechanism. The A_{2A} receptor antagonist ZM241385 administered repeatedly (1 mg/kg i.p.) in the 12 hours after traumatic brain injury was protective 15 min after trauma when cerebro spinal fluid (CSF) glutamate concentration rose; conversely, the A_{2A} receptor agonist, CGS21680, administered repeatedly (0.1 mg/kg i.p.) in the 12 hours after trauma was protective 3 hours after trauma when CSF glutamate concentrations were down (Dai et al., 2010). The adenosine A_{2A} receptor agonist 2-[(2-aminoethylamino)-carbonylethylphenylethylamino]- 50-N-ethylcarboxoamidoadenosine (APEC), administered systemically and chronically, before a global 10 min ischemia in the adult gerbil, ameliorated animal and neuron survival (Von Lubitz et al., 1995). A high dose of the selective A_{2A} receptor agonist, CGS21680 (10 mg/kg i.p.), administered immediately after 5 min of global ischemia in gerbil, exhibited highly significant protection against neuronal loss (Sheardown and Knutsen, 1996). Melani et al., (2014) have demonstrated that the A_{2A} receptor agonist, CGS21680, chronically administered at a low dose of 0.01 and 0.1 mg/kg, starting 4 hours after transient (1 hour) MCAo, induced protection from neurological deficit, weight loss, cortical infarct volume, myelin disorganization and glial activation evaluated 7 days after ischemia.

In considering translation to clinic, a main problem of A_{2A} receptor agonists consists in their cardiovascular effect because adenosine A_{2A} receptors located on vascular smooth muscle and endothelial cells exert a vasodilatory effect (Phillis, 2004). CGS21680 administered at the dose of 0.5 mg/kg i.p in conscious rats induces a decrease of blood pressure and an increase of heart frequency (Schindler et al., 2004, 2005). Relevantly

Melani et al. (2014) have demonstrated that the low dose of CGS21680 of 0.01 mg/kg did not modify either mean blood pressure or heart frequency.

Since CGS21680 crosses the BBB (Sihver et al., 2009), to some extent protection after ischemia can be attributed to central effects. In rat model of intracerebral hemorrhage, CGS21680 administered directly into the striatum reduces parenchymal neutrophil infiltration and tissue damage: an effect that was related to the inhibition of TNF-α expression (Mayne et al., 2001). Moreover activation of central A_{2A} receptors is known to increase expression and release of neurotrophic factors (Sebastião and Ribeiro, 1996) such as nerve growth factor (NGF) 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); glial cell line-derived neurotrophic factor (GDNF) 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.

9.2.2 A_{2A} receptors and neuroinflammation

Minutes to hours after onset of cerebral ischemia, a cascade of inflammatory events is initiated through activation of resident cells (Macrez et al., 2011). While some protection by A_{2A} agonists could be attributed to central effects, 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} 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 antinflammatory effect. (Haskò et al., 2000; Lappas et al., 2006; Sitkovsky et al., 2004; Varani et al., 1998).

In the tMCAo model in the rat, selective immunostaining for granulocytes, by anti-HIS-48 antibody, shows numerous infiltrated cells in ischemic striatal and cortical *core* two days after tMCAo (Melani et al., 2014). This is in agreement with observation that after tMCAo, a peak of neutrophil infiltration occurs at 6 and 48 hours thereafter (Zhang et al., 1994). Seven days thereafter, infiltrated blood cells were anymore observed (Melani et al., 2014). Two days after MCAo, chronic treatment with the A_{2A} adenosine receptor

agonist, CGS21680 at the dose of 0.01 mg/kg, has definitely reduced the number of infiltrated blood cells in the ischemic areas (Melani et al., 2014).

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, neutrophils) (Haskó et al., 2008). In support that A_{2A} receptors on blood cells are greatly responsible of the protective effects of A_{2A} agonists, the protection of motor deficits by A_{2A} receptor agonists, systemically administered after spinal trauma, is lost in mice lacking A_{2A} receptors on bone marrow derived cells (BMDCs) but is restored in A_{2A} receptor KO mice reconstituted with A_{2A} receptors on BMDCs (Li et al., 2006). Moreover, in the spinal cord trauma model in the mouse, CGS21680 protected from damage when injected systemically but not when centrally injected into the injured spinal cord (Paterniti et al., 2011).

Consistent with its anti-inflammatory and immunosuppressive role, the protective effect of adenosine A_{2A} receptor stimulation has been observed in different pathologies where inflammatory process has an important role in tissue damage (Howe et al., 2004; Odashima et al., 2005; Choukèr et al., 2008; Genovese et al., 2010) such as ischemia/reperfusion liver injury (Day et al., 2004), spinal cord trauma (Paterniti et al., 2011), rheumatoid arthritis (RA) (Mazzon et al., 2011), acute lung inflammation (Impellizzeri et al., 2011), intestine ischemia/reperfusion injury (Di Paola et al., 2009), and experimental autoimmune encephalomyelitis (Xu et al., 2013).

All the therapies under study in ischemia, if translated into clinic would need to be associated with thrombolytic drugs, since thrombolytic drugs have potential hemorrhagic effects, it appears crucial to verify if the adenosine A_{2A} agonists exert antiplatelet effects.

In agreement one of the aims of the experiments reported in the present thesis was to assess if the adenosine A_{2A} receptor agonist, CGS21680, chronically and systemically administered (twice/day for 7 days) at the dose of 0.01 mg/kg, did not exert antiplatelet effects in the rat.

9.3 Adenosine A_{2B} receptors in brain ischemia

Adenosine A_{2B} receptor is expressed at low levels uniformly throughout the CNS (Puffinbarger et al., 1995; Dixon et al., 1996; Fredholm et al., 2000) and has a low affinity for adenosine. Their 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 (Li et al., 2017). Thus, during conditions of hypoxia or ischemia when the extracellular adenosine levels rise, A_{2B} receptors might be well activated (Xu et al., 2013). Because of paucity of A_{2B} selective agonists and antagonists (Müller and Jacobson, 2011) few studies are till now present on the role of A_{2B} receptors in brain ischemia.

Recently, the role of A_{2B} receptors 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, N-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) phenoxy]–acetamide (MRS1754) and 8-[4-[4-(4-Chlorophenzyl) piperazide-1-sulfonyl) phenyl]] -1-propylxanthine) (PSB603) 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 h after the end of OGD and prevented astrocyte modification (Fusco et al., 2018).

The selective A_{2B} 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 defence 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, reduced the ceramide

production in astrocytes and attenuated inflammatory responses and neuronal damage after 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 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 (Gessi et al., 2005; Eckle et al., 2008) where in most cases they are coexpressed with A_{2A} receptors. They are also expressed at low levels on platelets, where they are upregulated following injury and systemic inflammation *in vivo* 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 hypoxia inducible factor (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., 2007, 2008). Attenuation of hypoxia-associated increases in tissue neutrophil numbers appeared to depend largely on hematopoietic cell A_{2B} receptor signalling (Yang et al., 2006; Eckle et al., 2007).

Recent introduction of new pharmacological tools (Hinz et al., 2014) led to understand a role of A_{2B} receptors in ischemia. Intravenous treatment with the selective A_{2B} receptor agonist BAY 60-6583 (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 tPA (administered after ischemic stroke to dissolve intravascular clots), BAY 60-6583 also mitigated sensorimotor deficits and reduced tPA induced hemorrhages at 24 hours after ischemia (Li et al. 2017). The neurovascular protection afforded by BAY 60-6583 appears to derive from stimulation of the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) production, inhibition of tPA-induced matrix metalloprotease (MMP) activation, and prevention of tight junction protein degradation. In fact, over-activation 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 tPA and could be a promising strategy for decreasing the risk of hemorrhages during treatment for ischemic stroke (Li et al. 2017).
All together these studies point toward a role of central A_{2B} receptors, in synergy with A_{2A} receptors in promoting brain excitotoxicity, while A_{2B} receptors located on vascular endothelial cells would play a pivotal role in attenuating hypoxia-induced increases in vascular leak. Blood immune cells would be implicated in dampening vascular adhesion signals and hypoxia-induced inflammation (Koeppen et al., 2011).
To date there are no evidences in literature on the protective effects of A_{2B} receptor agonists at more distant times from ischemia when a defined neuroinflammation develops. In our studies, object of the present thesis, we explored the protective effect of A_{2B} receptor agonist, BAY 60-6583, in a rat model of transient (1h) ischemia induced by MCAo, with a chronic treatment for 7 days, when a clear inflammatory response has developed.

Aims of the Research.

To study the protective effects of the selective histamine H_4 receptor antagonist, JNJ7777120, in a rat model of transient cerebral ischemia

Consistent results characterize the H_4R as the main immune system histamine receptor with a pro-inflammatory role. H_4R is mainly expressed on hematopoietic cells of the immune system, including mast cells, leukocytes, dendritic cells, and T lymphocytes. However, its role in brain immune responses was scarcely studied. To date there is no information on the role of H_4R in brain ischemia.

The aim of our study was to assess the putative neuroprotective effects of the potent and selective H_4 receptor antagonist, JNJ7777120, chronically administered (1 mg/kg, i.p., twice/day for 7 days) on damage parameters in a model of focal ischemia induced in the rat by the transient (1 hour) occlusion of the middle cerebral artery (tMCAo) by the monofilament technique.

To study the time-course of protection by the selective A_{2A} receptor antagonist, SCH58261, after transient focal cerebral ischemia

Evidences indicate that adenosine A_{2A} receptor subtype is of critical importance in stroke. An overexpression of A_{2A} receptors was found in neurons and microglia of the striatum and cortex 24 hours after focal ischemia induced by permanent MCAo. Protective effect of A_{2A} receptor antagonists administered early after brain ischemia is attributable largely to reduced excitotoxicity and ensuing excitotoxic cascade due to stimulation of NMDA receptors. Most recently, the question has been raised if A_{2A} receptor continuous blockade over an extended time-window after ischemia is protective.

Purpose of our studies was to investigate if the selective adenosine A_{2A} receptor antagonist, SCH58261, chronically administered (0.01 mg/kg i.p., twice/day for 7 days) maintained a protective effect 7 days after transient (1h) MCAo in the rat.

To study the putative protection by the adenosine A_{2B} receptor agonist, BAY 60-6583, from behavioral and histological damage after focal transient brain ischemia in the rat

Adenosine A_{2B} receptors is expressed at low levels uniformly throughout the CNS and has a low affinity for adenosine. An overexpression of A_{2B} receptors occurred on endothelial cells, neurons, and astrocytes 24 hours after tMCAo in the rat. Thus, during conditions of hypoxia or ischemia when the extracellular adenosine levels rise, A_{2B} receptors might be well activated.

Besides brain cells, A_{2B} receptors are present on blood immune cells i.e neutrophils, lymphocytes. Thus, A_{2B} receptors might play a crucial role in the inflammatory phenomena that occur hours and days after ischemia. To date there are no evidences in literature on the protective effects of A_{2B} receptor agonists at more distant times from ischemia when a defined neuroinflammation develops.

In our study, we explored the putative protective effect of A_{2B} receptor agonist, BAY 60-6583, in a rat model of transient (1h) ischemia induced by MCAo, with a chronic treatment for 7 days, when a clear inflammatory response has developed.

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 and authorized by the Italian Ministry of Health (Authorization n. 118/2016-PR). 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. 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 et al., (1999) (Figure 20).

One hour after occlusion, animals were re-anesthetized with isofluorane and reperfused by withdrawing the filament. The sham operation was conducted by inserting the filament into the internal carotid artery and immediately withdrawing it.

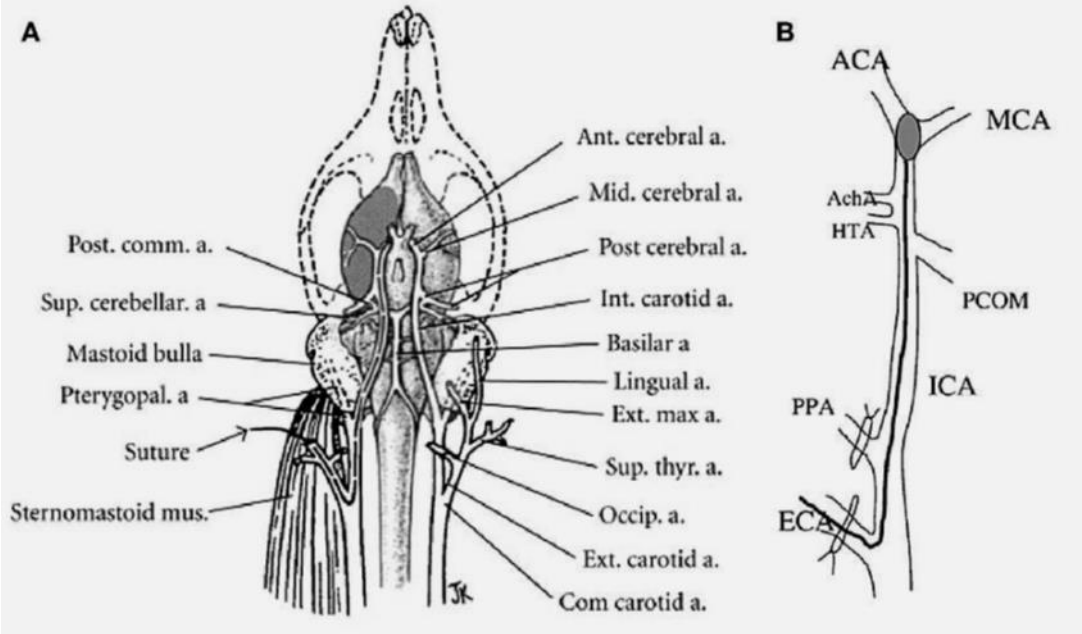

Figure 20 Intraluminal middle cerebral artery occlusion. Abbreviations: ACA: anterior cerebral artery; ECA: external carotid artery; ICA: internal carotid artery; MCA: middle cerebral artery; PCOM: posterior communicating artery; PPA: pterygopalatine artery. From (Canazza et al., 2014).

3. Treatments and Experimental Groups

3.1 Chronic treatment with histamine H_4 receptor antagonist, JNJ7777120

3.1.1 Drug administration

Histamine H_4 receptor antagonist 1-[(5-chloro-1H-indol-2yl)carbonyl]-4methylpiperazine, (JNJ7777120) (Johnson & Johnson, San Diego, CA, USA) (Figure 21) was dissolved in saline with 1.1% dimethyl sulfoxide (DMSO). JNJ7777120 was chronically administered at the dose of 1 mg/kg, intraperitoneally (i.p.).

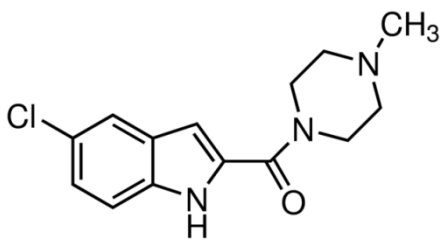

Figure 21 JNJ7777120

3.1.2 Experimental groups

Animals subjected to tMCAo were sacrificed 7 days after ischemia. Rats were randomly allocated in the following groups: (1) sham-operated rats (n=6): did not receive any treatment; (2) tMCAo+vehicle group (n=13): saline with DMSO (1.1%) administered (i.p.) twice/day for 7 days, starting 4 hours after tMCAo; (3) tMCAo+JNJ7777120 group (n=10): JNJ7777120 administered (i.p.) twice/day for 7 days, starting 4 hours after tMCAo.

A group of animals subjected to tMCAo was sacrificed 2 days after ischemia. Rats were randomly allocated in the following groups: (1) Sham-operated rats (n=3): did not receive any treatment; (2) tMCAo+vehicle group (n=4): saline with DMSO (1.1%) administered (i.p.) twice/day for 2 days, starting 4 hours after tMCAo; (3) tMCAo+ JNJ7777120 group (n=4): JNJ7777120 administered (i.p.) twice/day for 2 days starting 4 hours after tMCAo.

3.2 Chronic treatment with adenosine A_{2A} receptor antagonist, SCH58261

3.2.1 Drug administration

Adenosine A_{2A} receptor antagonist 7-(2-feniletil)-5-ammino-2-(2-furil)-pirazolo-[4,3-e]-1,2,4,triazolo[1,5-c]pirimidina (SCH58261) (Sigma-Aldrich, St. Louis, Missouri, USA) (Figure 22) was dissolved by sonication in saline with 1% Tween 80. The dose of SCH58261 administered (0.01 mg/kg, i.p.) and the protocol of administration were chosen on the basis of those found protective against brain ischemia in previous *in vivo* studies (Melani et al., 2003, 2006, 2009; Monopoli et al., 1998).

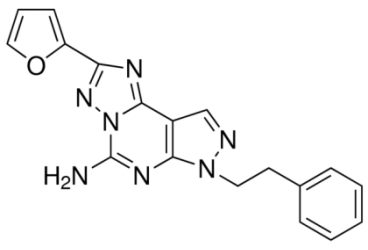

Figure 22 SCH58261

3.2.2 Experimental groups

Animals subjected to tMCAo were sacrificed 7 days after ischemia. Rats were randomly allocated in the following groups: (1) sham-operated rats (n=10): did not receive any treatment; (2) tMCAo+vehicle group (n=9): saline with Tween 80 (1%) administered (i.p.) twice/day for 7 days, starting 5 min after tMCAo; (3) tMCAo+SCH58261 group (n=5): SCH58261 administered (i.p.) twice/day for 7 days, starting 5 min after tMCAo.
A group of animals subjected to tMCAo was sacrificed 2 days after ischemia. Rats were randomly allocated in the following groups: (1) Sham-operated rats (n=3): did not receive any treatment; (2) tMCAo+vehicle group (n=3): saline with Tween 80 (1%) administered (i.p.) twice/day for 2 days, starting 5 min after tMCAo; (3) tMCAo+ SCH58261 group (n=6): SCH58261 administered (i.p.) twice/day for 2 days starting 5 min after tMCAo.

3.3 Chronic treatment with adenosine A_{2A} receptor agonist, CGS21680

3.3.1 Drug administration

Adenosine A_{2A} receptor agonist 2-[p-(2-carbox- yethyl)-phenethylamino]-5'-Nethylcarboxamidoadenosine (CGS21680) (Tocris bioscience, Bristol, UK) (Figure 23), was dissolved in saline with 0.1% DMSO. CGS21680 was chronically administered at the dose of 0.01 mg/kg, i.p., dose that does not modify blood pressure and heart frequency (Melani et al., 2014).

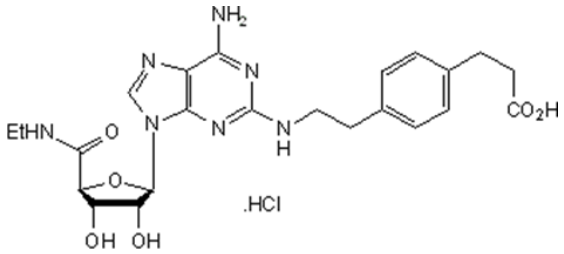

Figure 23 CGS21680

3.3.2 Experimental groups

Animals for platelet aggregation assay were randomly allocated in the following groups: (1) Control group (n=3): did not receive any treatment; (2) CGS21680 group (n=3): CGS21680 administered (i.p.) twice/day for 7 days.

3.4 Chronic treatment with adenosine A_{2B} receptor agonist, BAY 60-6583

3.4.1 Drug administration

Adenosine A_{2B} receptor agonist 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide (BAY 60-6583) (Tocris, Bristol, United Kingdom, UK) (Figure 24), was dissolved in DMSO 0.5%. BAY 60-6583 was chronically administered at the dose of 0.1 mg/kg, i.p.

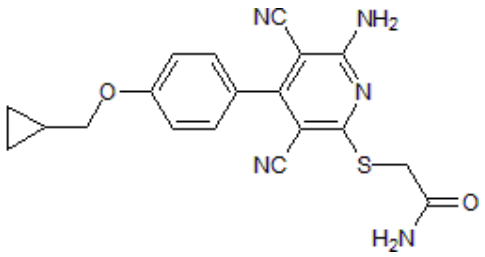

Figure 24 BAY 60-6583

3.4.2 Experimental groups

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=4): DMSO (0.1%) administered (i.p.) twice/day for 7 days, starting 4 hours after tMCAo; (3) tMCAo+ BAY 60-6583 group (n=6): BAY 60-6583 administered (i.p.) twice/day for 7 days, starting 4 hours after tMCAo.

4. 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 tMCAo and 1, 5 and 7 days after tMCAo.

The mNSS test evaluates the sensorimotor deficit: it is composed of motor, sensory, reflex and beam balance tests (see Figure 25). 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.

TABLE 1. 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 paw against table edge to stimulate limb muscles)	1
Beam balance tests (normal=0; maximum=6)	6
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall 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 cornea 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 25 Example of modified Neurological Severity Severity (mNSS) score points scale for sensorimotor deficit evaluation.

5. 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.

6. Ischemic brain damage

Rats were anesthetized with Zoletil 50/50 (100 mg/kg i.p., Virbac, Carros, Francia) 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 (20-30 µm) were collected at 200-450 µm intervals at 8-12 different levels through the striatum (König and Klippel, 1967). Seven days after tMCAo, 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, 8-12 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 8-12 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.

7. Blood cell infiltration and gliosis

Coronal sections (20-30 µm), stored at –20°C in antifreeze solution until assay, were mounted on gelatin-coated slides and washed with PBS-TX. Then they were incubated for 15 min in PBS-TX containing 0.75% H_2O_2, rinsed in PBS-TX and incubated at room temperature (RT) in Blocking Buffer for 60 min. After washing in PBS-TX, the sections were incubated overnight at 4°C with mouse monoclonal antibody, anti-HIS48 (specific for granulocytes) (1:50, Santa Cruz Biotechnology), mouse monoclonal antibody, anti-GFAP (specific for astrocytes) (1:300, BD Transduction Laboratories) and rabbit polyclonal antiboby, anti-IBA1 (specific for microglia) (1:300, Wako Chemicals), dissolved in Blocking Buffer.

The day after, sections were rinsed three times in PBS-TX and then were incubated for 1 hour at RT with the specific biotinylated secondary antibodies, anti-mouse-IgG or anti-rabbit-IgG (1:333) (Vector Laboratories, Burlingame, CA, USA) respectively, dissolved in Blocking Buffer. After washing in PBS-TX, sections were incubated for 1 hour in avidin-biotin-peroxidase complex (ABC kit Elite standard, Vectastain, Vector Laboratories, Burlingame, CA, USA). Finally, all sections were stained using diaminobenzidine (DAB-peroxidase, Vectastain, Vector) in the presence of NiCl.

All sections were examined using an Olympus BX40 microscope (Olympus, Milan, Italy) and photographed using a digital camera (Olympus DP50). To evaluate the number of granulocytes, astrocytes and microglia, cells were counted within an optical field at 40X magnification in ischemic cortex and striatum. HIS48-positive cells were counted at seven different levels per animal (+2.0 mm to -1.0 mm from the Bregma). GFAP- and IBA1-positive cells were counted at Bregma level. Data were then averaged and expressed as the mean±SEM of number cells per optical field of "n" animals.

8. MAG staining

Coronal sections (30 µm), stored at –20°C in antifreeze solution (30% ethylene glycol, 30% glycerol in phosphate buffer) until assay, were mounted on gelatin-coated slides and washed with phosphate buffer saline-0.3% Triton X-100 (PBS-TX) (for 3 times, 5 min each), blocked with blocking buffer (5 mg/ml of Bovine Serum Albumin/PBS-TX) for 1 hour at room temperature (RT). Sections were then incubated overnight at RT with the primary mouse monoclonal antibody, anti-myelin associated glycoprotein (MAG, 1:250; Millipore, Temecula, CA, USA) dissolved in blocking buffer, used to visualize myelinated processes of oligodendrocytes.

The day after, the primary antibody was removed. Slices were washed several times with PBS-TX solution and incubated, in the dark, for 2 hour at RT with secondary fluorescein-conjugated goat anti-mouse IgG antibody (1:400; Vector Laboratories, Burlingame, CA, USA), dissolved in blocking buffer. After a washing in PBS-TX, sections were mounted on gelatin-coated slides using Vectashield (Vectastain, Vector Laboratories, Burlingame, CA, USA) as a mounting medium. Sections were observed under an epifluorescent Olympus BX40 microscope (Olympus, Hamburg, Germany) with excitation wavelength at 488 and 568 nm and photographed using a digital camera

(Olympus DP50). Images were assembled into montages using Adobe Photoshop 6.1 (Adobe Systems, Mountain view, CA, USA).

9. Determination of TNF-α, IL-1β and IL-10

The levels of TNF-α, IL-1β, pro-inflammatory cytokines, and of IL-10, a regulatory cytokine, were measured on aliquots (200µl) of plasma using commercial ELISA kits (ELISA Ready-Set-Go, eBioscience, San Diego, CA, USA), following the protocol provided by the manufacturer (Lucarini et al., 2016). Results are expressed as pg of protein/ml of plasma.

10. Platelet aggregation assay

Female Wistar rats (Envigo, Italy) were used, in the body weight ranging from 220 to 240 g. Rats were anesthetized with isoflurane (Baxter, International) and blood was collected by cardiac puncture into vacuum tubes which contained 3.8% sodium citrate (1:9, v/v). Platelet aggregation was assessed in platelet-rich plasma (PRP), which was obtained by centrifugation of citrated whole blood at room temperature for 10 min at 1000 rpm. The platelet-poor plasma (PPP) was obtained by centrifugation of PRP at room temperature for 15 min at 4000 rpm, which was used to set zero (for details see Ma et al., 2016; Yang et al., 2015). The aggregation rate was measured by lumi-aggregometer after stimulation with ADP (10 µM). Data were expressed as percentage of aggregation.

11. 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 GraphPad Prism7.

Results.

The protective effects of the selective histamine H_4 receptor antagonist, JNJ7777120, in a rat model of transient cerebral ischemia

1. Effect of treatment with the histamine H_4 receptor antagonist on neurological deficit after tMCAo

The mNSS was performed according to Chen et al. (2001) before tMCAo and 1, 5 and 7 days after tMCAo. The test showed that sham-operated rats had a neurological score of 0.2-0.7 in the period from 1 to 7 days after tMCAo (Figure 26 A). Twenty-four hours after tMCAo, vehicle-treated rats showed a clear neurological deficit with a neurological score of 8.6±0.5 (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 6.8±0.4, seven days after tMCAo, the neurological score was further reduced to 5.8±0.4 that represents a mild injury. Chronic treatment with the H_4R antagonist, JNJ7777120, at the dose of 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 ($F_{2,26}$=82.35; $p<0.0001$), time factor ($F_{2,52}$=12.29; $p<0.0001$) and interaction between treatment and time ($F_{4,52}$=2.66; $p<0.042$) were statistically significant. The Tukey post hoc test indicated that sham-operated rats had a neurological score significantly different from vehicle-treated and JNJ7777120-treated rats at each time point (1, 5, 7 days after tMCAo, $p<0.001$). The chronic treatment with JNJ7777120 significantly reduced the neurological deficit with respect to vehicle-treated rats at 1, 5 and 7 days ($p<0.001$-0.0001) after tMCAo.

2. Effect of treatment with the histamine H_4 receptor antagonist on body weight loss after tMCAo

Male Wistar rats used in this study had a body weight in the range of 270-290 g. Twenty-four hours after operation, sham-operated rats lost 3.7±0.7 g weight, then they increased in body weight as evaluated up to 7 days after tMCAo. Vehicle-treated rats

lost 23.8±2.5 g 1 day after tMCAo, 41.9±9.0 g 5 days and 30.6±10.8 g 7 days after tMCAo (Figure 26 B). Chronic treatment with JNJ7777120 significantly reduced the body weight loss at 5 and 7 days after tMCAo with respect to vehicle-treated rats. Repeated Measures two-way ANOVA, calculated for the two factors: treatment and time from tMCAo, showed that treatment factor ($F_{2,26}$=11.87; p<0.0002), time factor ($F_{2,52}$=7.95; p<0.001) and interaction between treatment and time ($F_{4,52}$=6.08; p<0.0004), 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 JNJ7777120-treated rats at 5 days after tMCAo (p<0.025). The chronic treatment with JNJ7777120 significantly decreased body weight loss at 5 days (of 67.5%, p<0.01) and at 7 days (of 99.0%; p<0.004) after tMCAo, with respect to vehicle-treated rats.

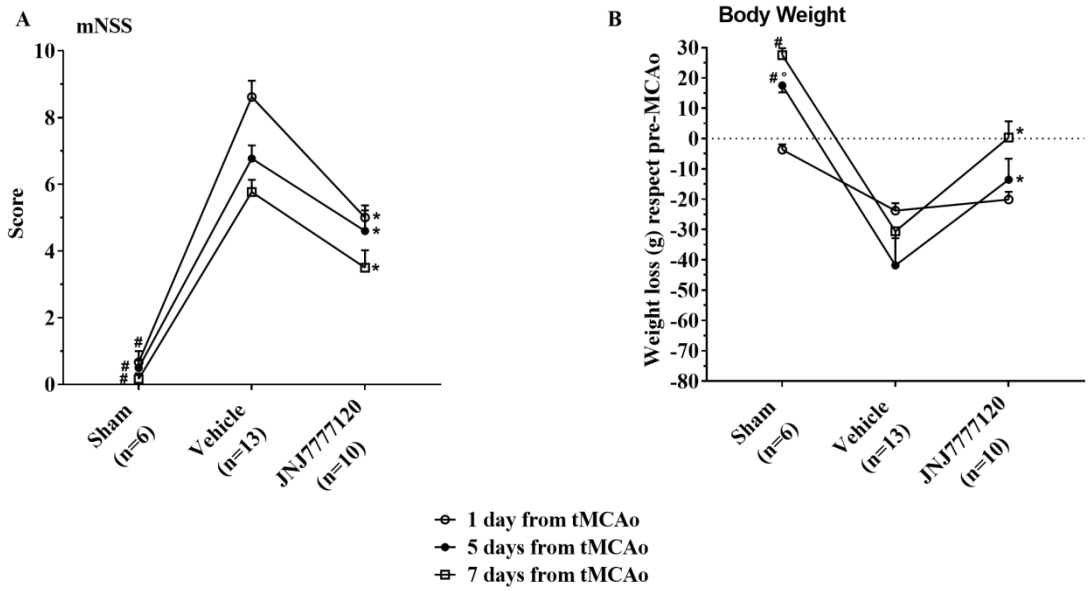

Figure 26 Effect of chronic treatment with JNJ7777120 (1 mg/kg i.p.) on neurological deficit (A) and body weight loss (B). Data are expressed as mean±SEM of "n" rats. A) mNSS test: the score is evaluated before and after 1, 5 and 7 days from tMCAo in each rat group. Repeated Measures two-way ANOVA followed by Tukey post hoc test: $^{\#}$p<0.0001 sham-operated versus chronic JNJ7777120- and vehicle-treated rats; *p<0.0001-0.001 chronic JNJ7777120-treated versus vehicle-treated rats. B) Body weight loss: sham-operated rats increase their body weight in the period after the operation. The body weight loss of tMCAo rats was calculated as the mean±SEM of the difference between body weight at each time point and pre-operation body weight. Repeated Measures two-way ANOVA followed by Tukey post hoc test: $^{\#}$p<0.0001 sham-operated versus vehicle-treated rats; $^{\circ}$p<0.025 sham-operated versus chronic JNJ7777120-treated rats; *p<0.004-0.01 chronic JNJ7777120-treated versus vehicle-treated rats.

3. Effect of treatment with the histamine H_4 receptor antagonist on brain ischemic damage after tMCAo

Figure 27 shows the extent of ischemic damage evaluated as infarct area (Figure 27 A, C) and infarct volume (Figure 27 B, D) in ischemic striatum and cortex of vehicle- and JNJ7777120-treated rats 7 days after tMCAo. Chronic treatment with JNJ7777120 significantly reduced the infarct in both areas. The striatal and cortical infarct volumes were reduced by 58.4% and 42.2% respectively. (Unpaired Student's t-test: p<0.0001-0.0005). Sham-operated rats did not show any damage.

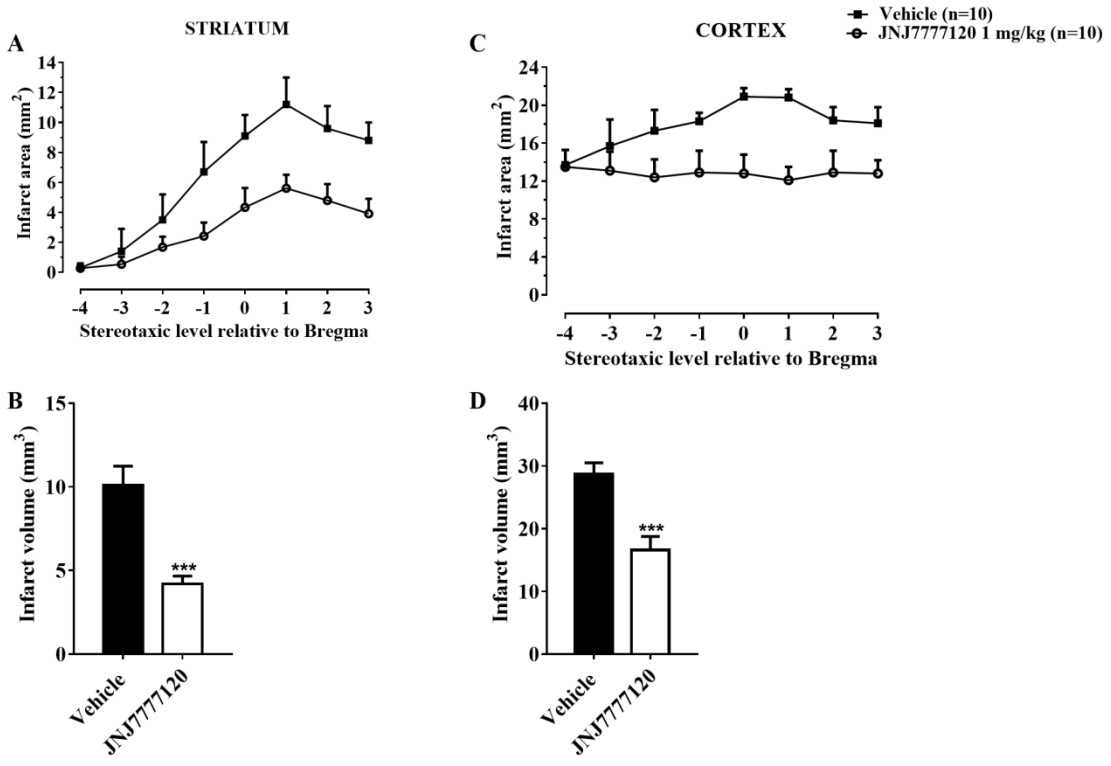

Figure 27 Effect of chronic treatment with JNJ7777120 (1 mg/kg i.p.) on infarct area (A, C) and infarct volume (B, D) in the striatum and cortex 7 days after tMCAo. Data are the mean±SEM of infarct area measured at 8 predetermined coronal levels through the brain of "n" rats. Bregma=0 (König and Klippel, 1967). Bar graphs show the infarct volume calculated as mean±SEM in the striatum and cortex. Unpaired Student's t-test: ***p<0.0001-0.0005 vs vehicle-treated rats.

To characterize the cytoarchitecture of the ischemic cortex and striatum 7 days after tMCAo, ischemic tissue was stained by H&E (Figure 28). Seven days after transient ischemia, H&E staining showed a decrease in staining intensity in vehicle-treated rats (Figure 28 C, D) compared to sham-operated rats (Figure 28 A, B). Parenchyma showed numerous small and heterochromatic nuclei both in ischemic cortex and striatum. The typical cytoarchitecture of these two regions (for a description see Danner and Pfister, 1981) was lost. In the striatum, the white matter *fascicula* (f) were no more recognizable; in the fronto-parietal cortex the columnar organization was not appreciable. Figure 28 E, F showed that chronic administration of JNJ7777120 was associated with a recovery of the staining intensity and with a reduction of heterochromatic small nuclei staining in both brain regions. The cytoarchitecture of the white matter *fascicula* was recognizable in the dorsal corpus striatum (Figure 28 E) and the columnar organization was appreciable in the fronto-parietal cortex (Figure 28 F). Quantitative analysis showed that chronic JNJ7777120 treatment significantly reduced heterochromatic nuclei number in the ischemic striatum ($p<0.02$; Figure 28 G) and in the ischemic cortex ($p<0.0005$; Figure 28 H).

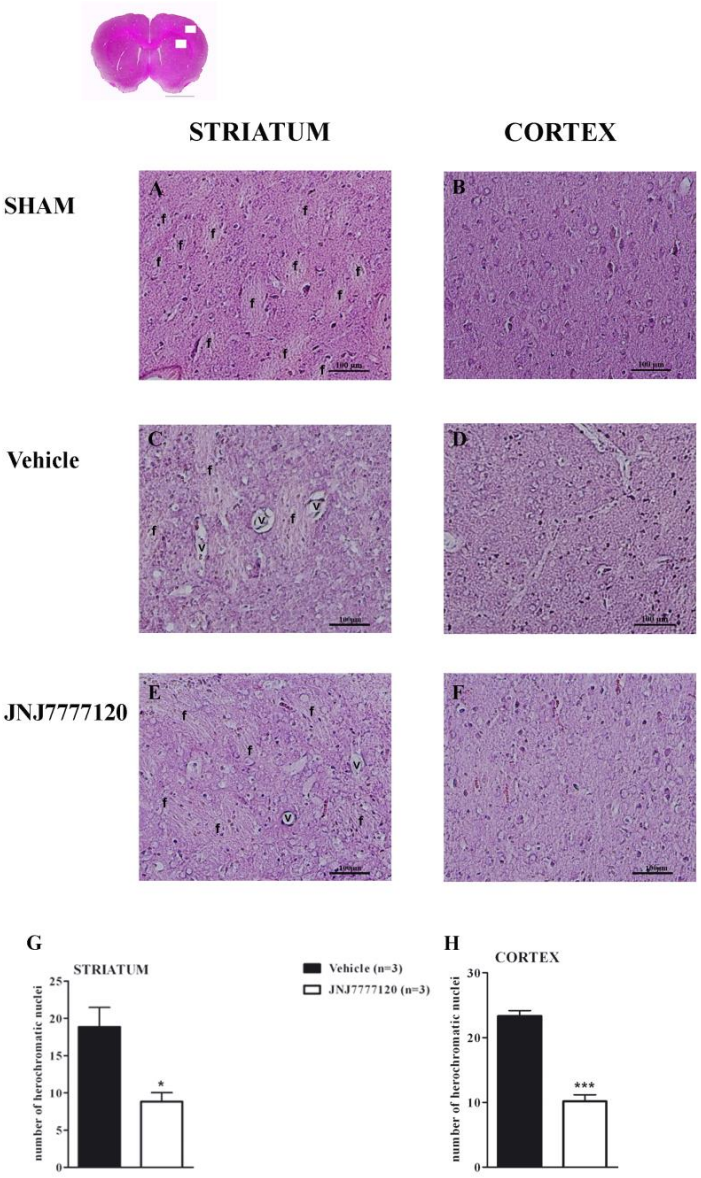

Figure 28 Effect of chronic treatment with JNJ7777120 (1 mg/kg i.p.) on cytoarchitecture of the ischemic striatum and cortex after tMCAo. Upper part: representative photomicrograph of a histological section of control rat (at Bregma +1.5 mm) (König and Klippel, 1967). The two white boxes indicate the ischemic striatal and cortical H&E stained area. Scale bar=2 mm. Sham-operated rats: (A) in the dorsal striatum, the typical caudate-putamen cytoarchitecture is appreciable, numerous transversally sectioned white matter *fascicula* (*f*) are surrounded by gray matter containing diverse type of neurons distinct on the basis of their size and shape (Danner and Pfister, 1981); (B) in the fronto-parietal cortex the typical columnar organization is appreciable. Vehicle-treated ischemic rats: (C) in most part of the ischemic dorsal striatum the cytoarchitecture is lost, numerous and dilated vessels (v) and small and heterochromatic nuclei are present; (D) in the ischemic fronto-parietal cortex the columnar organization is not appreciable, the interstitial spaces are enlarged, vessels are numerous and dilated and numerous heterochromatic small nuclei are present. JNJ7777120-treated ischemic rats: note the recovery of the staining intensity and the decrease of heterochromatic small nuclei; (E) in the ischemic dorsal striatum the cytoarchitecture of the white matter *fascicula* is again recognizable; (F) in the ischemic fronto-parietal cortex the columnar organization is appreciable. Scale bar=100µm. Bar graphs represent the mean±SEM of the number of heterochromatic nuclei per optical field (40X) present at Bregma=0 coronal level of 3 rats, in ischemic striatum (G) and in ischemic cortex (H). Unpaired Student's t-test: *$p<0.02$; ***$p<0.0005$ vs vehicle rats.

4. Effect of treatment with the histamine H_4 receptor antagonist on gliosis after tMCAo

Seven days after surgery, microglia and astrocytes were characterized by IBA-1 and GFAP immunostaining, respectively. In sham-operated rats, resting microglia (Figure 29 A, B, G, H) was diffusely distributed throughout the cortex and striatum. Microglia had morphological features typical of resting cells with a small cell body and faintly stained thin processes. Seven days after tMCAo, a definite increase of IBA-1 immunostaining was observed both in striatum and cortex. In the cortical and striatal *core*, microglia assumed a round macrophage-like morphology, typical of activated cells (Figure 29 C, I); in the boundary zone, microglial cells showed a large cell body with thick and short processes (Figure 29 D, L), an intermediate morphology between resting and activated microglia defined reactive microglia (Melani et al., 2006; Cerbai et al., 2012). Chronic treatment with JNJ7777120 reduced IBA-1 immunostaining intensity and hypertrophic features in the *core* and in the boundary zone of both cortex (Figure 29 E, F) and striatum (Figure 29 M, N). Quantitative analysis (Figure 29 P, Q) showed that JNJ7777120 treatment significantly reduced IBA-1^+ cell number in the cortical ischemic *core* ($p<0.005$) and in the boundary zone ($p<0.02$; Figure 29 P) and in the striatal ischemic *core* ($p<0.01$; Figure 29 Q) and in the boundary zone ($p<0.01$; Figure 29 Q).

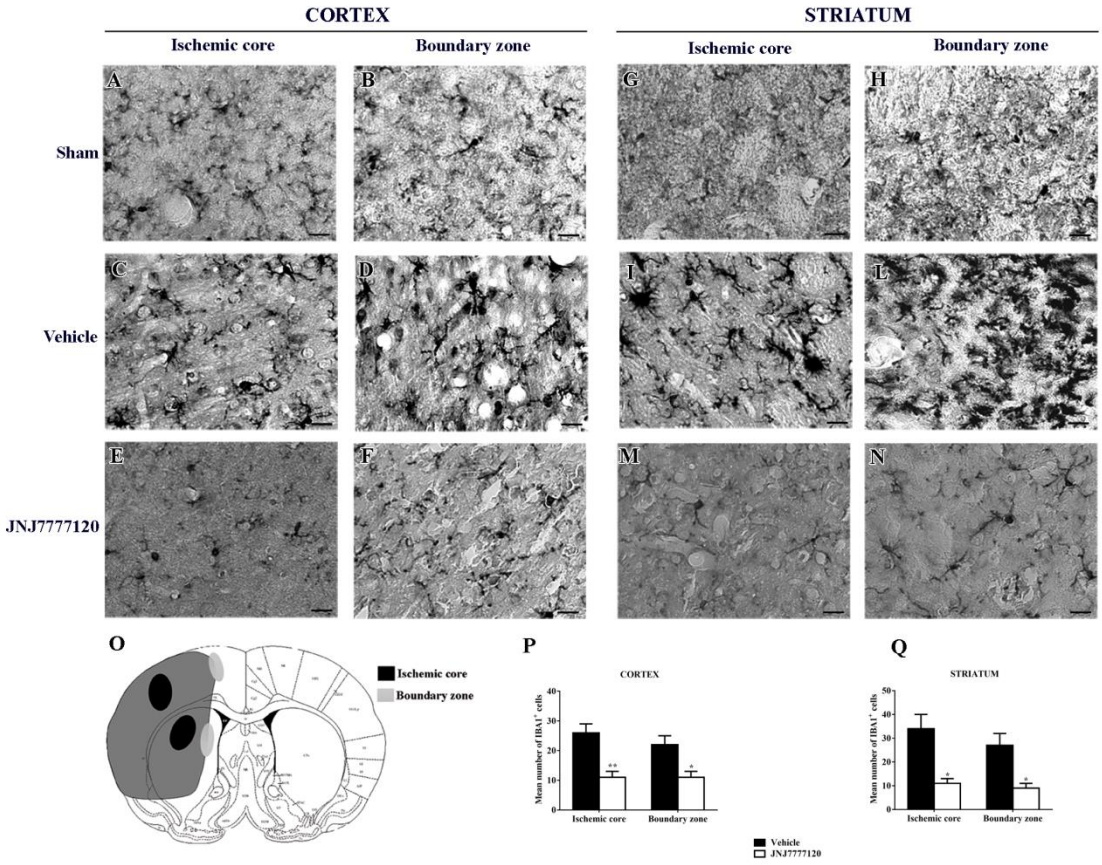

Figure 29 Effect of chronic treatment with JNJ7777120 (1 mg/kg i.p.) on microgliosis in the ischemic *core* and boundary zone 7 days after tMCAo. Schematic brain picture (O) indicates regions where photomicrographs of ischemic *core* and boundary zone were captured. In the sham-operated rats, microglia (black cells) has morphological features of resting cells with small cell body and faintly stained thin processes (A, B-G, H). Seven days after ischemia, an increase of IBA-1 immunostaining intensity is observed both in ischemic cortex and striatum. In the cortical and striatal ischemic *core*, microglia have a round macrophage-like morphology, typical of activated cells (C, I). In the cortical and striatal boundary zones, IBA-1 positive cells have a round cell body with short and thick processes, a morphology defined reactive microglia (D, L) (Cerbai et al., 2012). Treatment with JNJ7777120 reduces IBA-1 immunostaining intensity and hypertrophic features in the *core* and in the boundary zone of both cortex (E, F) and striatum (M, N). Scale bar=20µm. Bar graphs represent the mean±SEM of the number of $IBA1^+$ cells, per optical field (40X), present at Bregma=0 coronal level of 4 rats, in the ischemic *core* and boundary zone of both cortex (P) and striatum (Q). Unpaired Student's t-test: *p<0.01-0.02; **p<0.005 vs vehicle rats.

In sham-operated rats, resting astrocytes (Figure 30 A, B, G, H) were diffusely distributed throughout the cortex and striatum. Astrocytes had morphological features typical of resting cells with a small cell body and faintly stained thin processes. Seven days after tMCAo, a definite increase of GFAP immunostaining was observed both in striatum and cortex. In the ischemic *core*, astrocytes showed a smaller cell body and shorter processes (Figure 30 C, I) while in the boundary zone, they showed a highly hypertrophic cell body and long and thick processes (Figure 30 D, L). Chronic treatment

with JNJ7777120 reduced the GFAP immunostaining intensity both in *core* and boundary zone and astrocytes appeared less hypertrophic (Figure 30 E, F, M, N). Quantitative analysis (Figure 30 O, P) showed that JNJ7777120 treatment significantly reduced $GFAP^+$ cell number in cortical ischemic *core* ($p<0.02$; Figure 30 O) and boundary zone ($p<0.02$; Figure 30 O) and in the striatal ischemic *core* ($p<0.01$) and boundary zone ($p<0.001$; Figure 30 P).

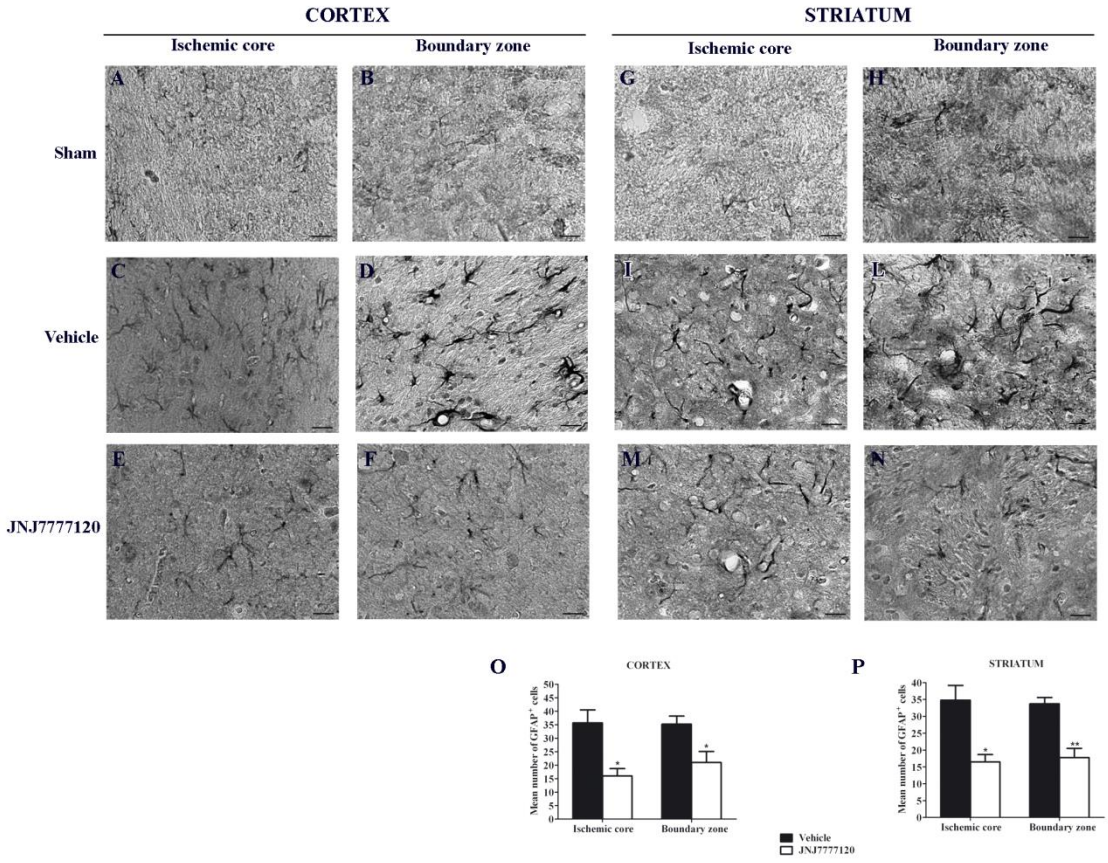

Figure 30 Effect of chronic treatment with JNJ7777120 (1 mg/kg i.p.) on astrogliosis in the ischemic *core* and boundary zone 7 days after tMCAo. In the sham-operated rats, astrocytes (black cells) have morphological features of resting cells with small cell body and thin ramified processes both in cortex (A, B) and in the striatum (G, H). Seven days after ischemia, an increase of GFAP immunostaining intensity is observed both in ischemic cortex and striatum. In the cortical and striatal ischemic *core*, astrocytes have morphological features of suffering cells (C, I). In the cortical and striatal boundary zones, GFAP-positive cells show a highly hypertrophic cell body and long and thick processes (D, L). Treatment with JNJ7777120 reduces GFAP immunostaining intensity and hypertrophic features in the core and in the boundary zone of both cortex (E, F) and striatum (M, N). Scale bar=20μm. Bar graphs represent the mean±S.E.M. of the number of $GFAP^+$ cells per optical field (40X), present at Bregma=0 coronal level of 4 rats, in the ischemic *core* and boundary zone of both cortex (O) and striatum (P). Unpaired Student's t-test: *$p<0.01$-0.02; **$p<0.001$ vs vehicle rats.

5. Effect of treatment with the histamine H_4 receptor antagonist on cytokine plasma levels after tMCAo

Seven days after tMCAo, plasma levels of pro-inflammatory cytokines IL-1β and TNF-α were significantly increased while the plasma level of IL-10, a regulatory cytokine with an anti-inflammatory action was reduced in vehicle-treated rats as compared to sham-operated rats (Figure 31 A, B, C). Chronic treatment with JNJ7777120 significantly reduced IL-1β and TNF-α levels while restored the plasma level of IL-10 (One-way ANOVA: $p<0.0005$-0.02) (Figure 31 A, B, C).

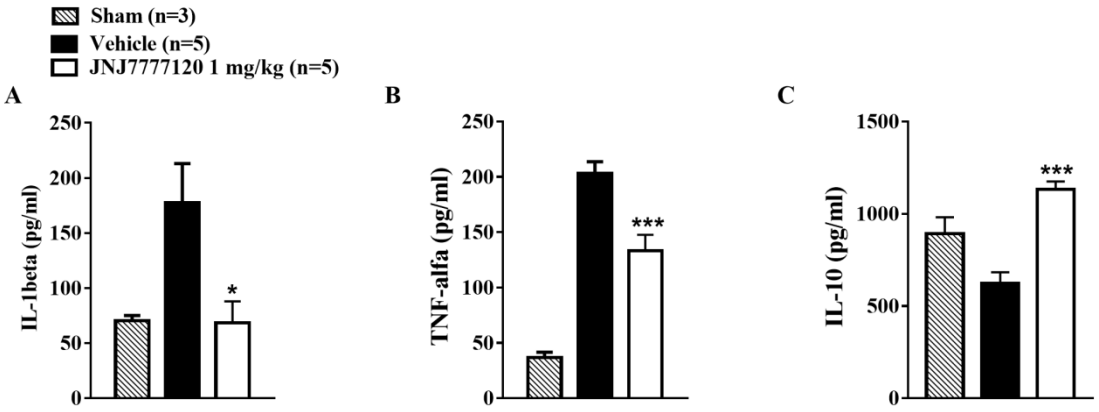

Figure 31 Effect of chronic treatment with JNJ7777120 (1 mg/kg i.p.) on IL-1β (A), TNF-α (B) and IL-10 (C) plasma levels. Results are expressed as pg of protein/ml of plasma and values are mean±SEM. One-way ANOVA: ***$p<0.0005$; *$p<0.02$ vs vehicle rats.

6. Effect of treatment with the histamine H_4 receptor antagonist on blood cell infiltration after tMCAo

Two days after tMCAo, HIS-48^+ cells (granulocytes) were detected in the cortical and striatal ischemic *core* (Figure 32 A). Seven days after tMCAo, granulocytes were anymore detectable in the ischemic tissue (data not shown).

Chronic treatment with JNJ7777120 significantly reduced the number of HIS-48^+ cells both in the cortical ischemic *core* (mean±SEM: 20.5±1.4 cells/optical field in vehicle- vs 7.2±0.5 cells/optical field in JNJ-treated rats; unpaired Student's t-test: $p<0.0002$) and in the striatal ischemic *core* (mean±S.E.M.: 24.3±1.7 cells/optical field in vehicle-

vs 6.9±0.4 cells/optical field in JNJ7777120-treated rats; unpaired Student's t-test: $p<0.0001$) (Figure 32 B).

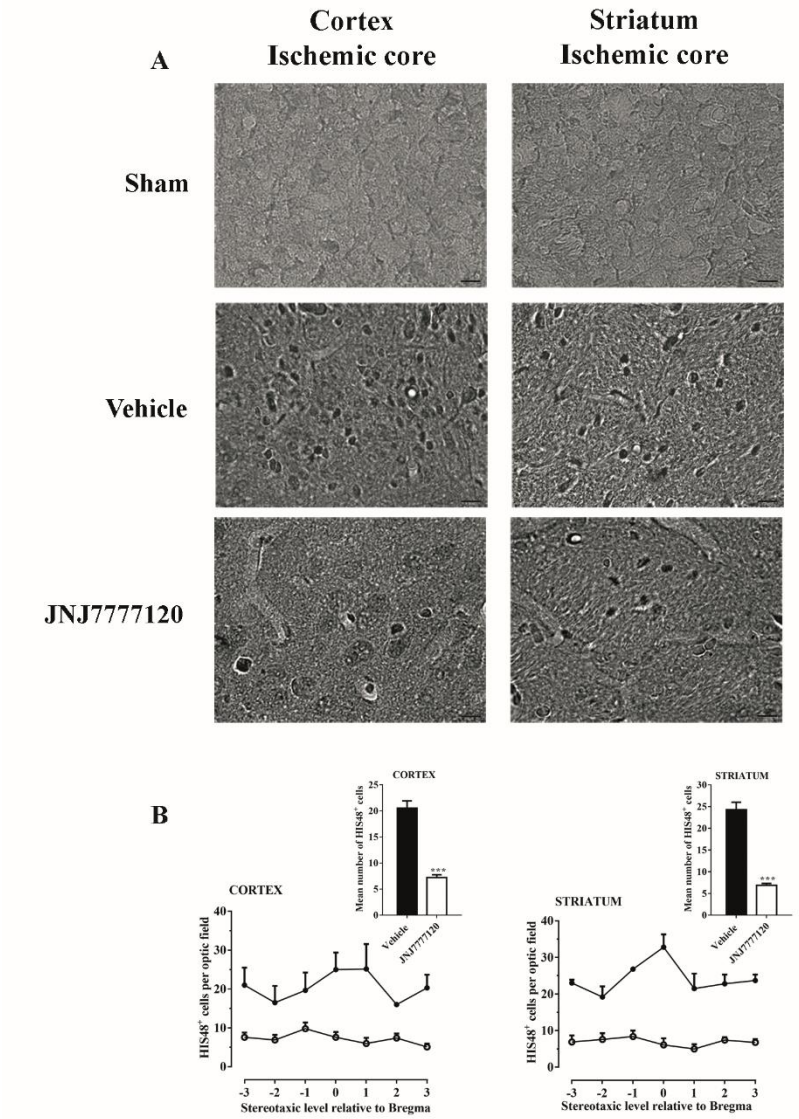

Figure 32 Effect of chronic treatment with JNJ7777120 (1 mg/kg i.p.) on blood cell infiltration in the ischemic *core* 2 days after tMCAo. (A) Representative micrographs of HIS-48-positive cells detected in cortical and striatal ischemic *core* obtained by optical microscopy at 60X of magnification. Scale bar=10 µm. JNJ7777120 reduces HIS-48-positive cells in cortex and striatum. (B) Data are mean±SEM of the number of HIS-48-positive cells per optical field (40X) counted in 7 coronal levels through the brain of "n" rats taking Bregma=0 (König and Klippel, 1967). Unpaired Student's t-test:***$p<0.0001$-0.0002.

Time-course of protection by the selective A_{2A} receptor antagonist, SCH58261, after transient focal cerebral ischemia

1. Effect of treatment with the adenosine A_{2A} receptor antagonist on neurological deficit after tMCAo

Figure 33 A shows that in the mNSS test, sham-operated rats had a neurological score of 0.15-0.70 in the period from 1 to 7 days after tMCAo showing any neurological deficit. Twenty-four hours after tMCAo, vehicle-treated rats had a neurological score of 12.6±0.5 (mean±S.E.M.) that defines a severe injury. Five days after tMCAo, the neurological score was reduced to 8.2±0.7 and 7 days after tMCAo, the neurological score was further reduced to 7.0±0.6 that represents a moderate injury. The chronic treatment with SCH58261 improved the neurological deficit only at 1 day after tMCAo. Two-way ANOVA, calculated for the two factors: treatment and time after tMCAo, showed that treatment factor ($F_{2,80}$=171.2; $p<0.0001$), time factor ($F_{3,80}$=106.6; $p<0.0001$) and interaction between treatment and time ($F_{6,80}$=24.66; $p<0.0001$) were statistically significant. The Bonferroni post hoc test indicated that sham-operated rats had a neurological score significantly different from vehicle- and SCH58261-treated rats at each time-point (1, 5, 7 days after tMCAo, $p<0.001$). Moreover, the Bonferroni post hoc test indicated that SCH58261, chronically administered, improved significantly the neurological deficit at 1 day ($p<0.01$) after tMCAo respect to vehicle-treated rats.

2. Effect of treatment with the adenosine A_{2A} receptor antagonist on body weight loss after tMCAo

Twenty-four hours after operation, 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 36.00±1.59 g 1 day after tMCAo, 62.37±7.89 g 5 days after tMCAo and 60.89±10.99 g 7 days after tMCAo (Figure 33 B). Treatment with the A_{2A} receptor antagonist, SCH58261, did not modify the body weight loss at any time point after

tMCAo respect to vehicle-treated rats. Two-way ANOVA, calculated for the two factors: treatment and time from tMCAo, showed that treatment factor ($F_{2,60}$=103.5; p<0.0001), but not time factor ($F_{2,60}$=2.29; p<0.1), was statistically significant. The interaction between treatment and time ($F_{4,60}$=5.88; p<0.0005) was also statistically significant. The Bonferroni post hoc test indicated that in sham-operated rats, body weight was different respect to vehicle- and SCH58261-treated rats at each time point (p<0.001) after tMCAo.

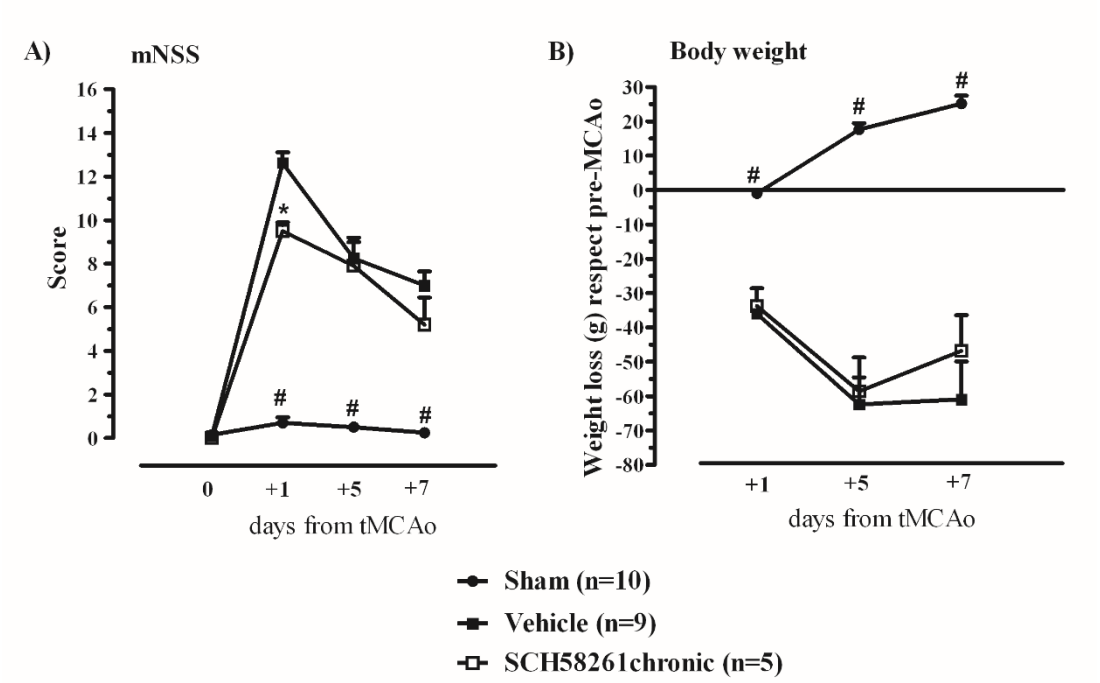

Figure 33 Effect of treatment with SCH58261 on neurological deficit and on body weight loss. Data are expressed as mean ± S.E.M. of "n" rats. A) mNSS test. The score is evaluated before (0 day) and after 1, 5 and 7 days from tMCAo in each rat group. Two-way ANOVA followed by Bonferroni post hoc test: $^{\#}$p<0.001 sham-operated versus chronic SCH58261- and vehicle-treated rats; *p<0.01 chronic SCH58261-treated versus vehicle-treated rats. B) Sham-operated rats increased their body weight in the period after the operation. The body weight loss of tMCAo rats was calculated as the mean ± S.E.M. of difference between body weight at each time-point and pre-operation body weight. Two-way ANOVA followed Bonferroni post hoc test: $^{\#}$p<0.001 sham-operated versus chronic SCH58261- and vehicle-treated rats.

3. Effect of treatment with the adenosine A_{2A} receptor antagonist on brain ischemic damage after tMCAo

Seven days after tMCAo, the volume of damage evaluated by cresyl violet in the striatum of vehicle-treated rats was 28.43±2.21 mm^3 and 75.15±5.13 mm^3 in the cortex (Figure 34). Treatment with SCH58261 did not modify brain infarct volume either in striatum or cortex. In sham-operated rats no ischemic damage was found.

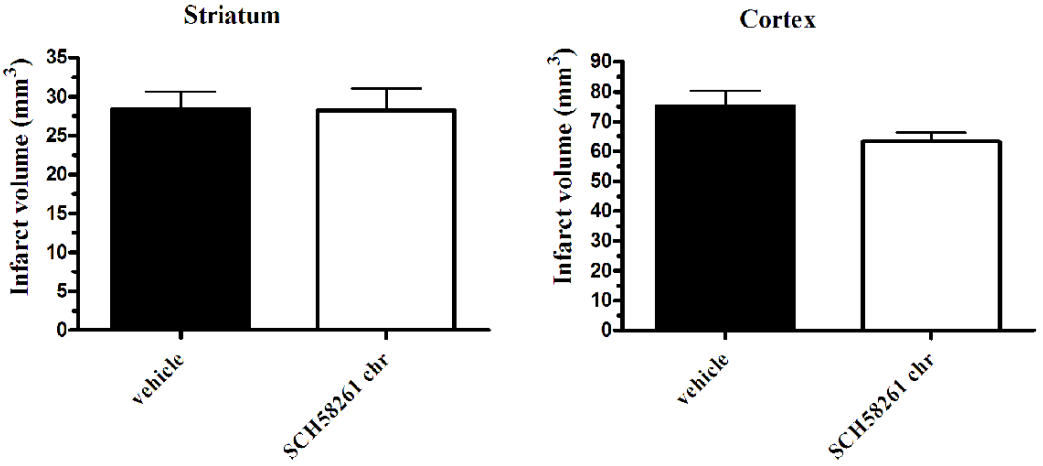

Figure 34 Effect of treatment with SCH58261 on brain ischemic damage 7 days after tMCAo. The infarct volume (mm^3) in striatum and in cortex is expressed as mean ±S.E.M. of "n" rats. Black column: vehicle-treated rats (n=9); white column: chronic SCH58261-treated rats (n=5).

Seven days after transient ischemia, the cytoarchitecture characterized by H&E staining showed a remarkable decrease in staining intensity in both cortex and striatum of vehicle-treated rats (Figure 35 C, D) compared to the sham operated rats (Figure 35 A, B). The paleness was due to enlargement of the interstitial spaces consequent to edema; in the parenchyma of ischemic cortex and striatum numerous small and heterochromatic nuclei belonging to astrocytes and microglia (Melani et al., 2014) were present. The typical cytoarchitecture of these two regions (for a description see Danner and Pfister, 1981) was lost. In the striatum, the white matter *fascicula* (*f*) were no more recognizable; in the fronto-parietal cortex the columnar organization was not appreciable. Figures 35 E and F show that chronic administration of SCH58261 did not substantially improve the cortical or striatal cytoarchitecture.

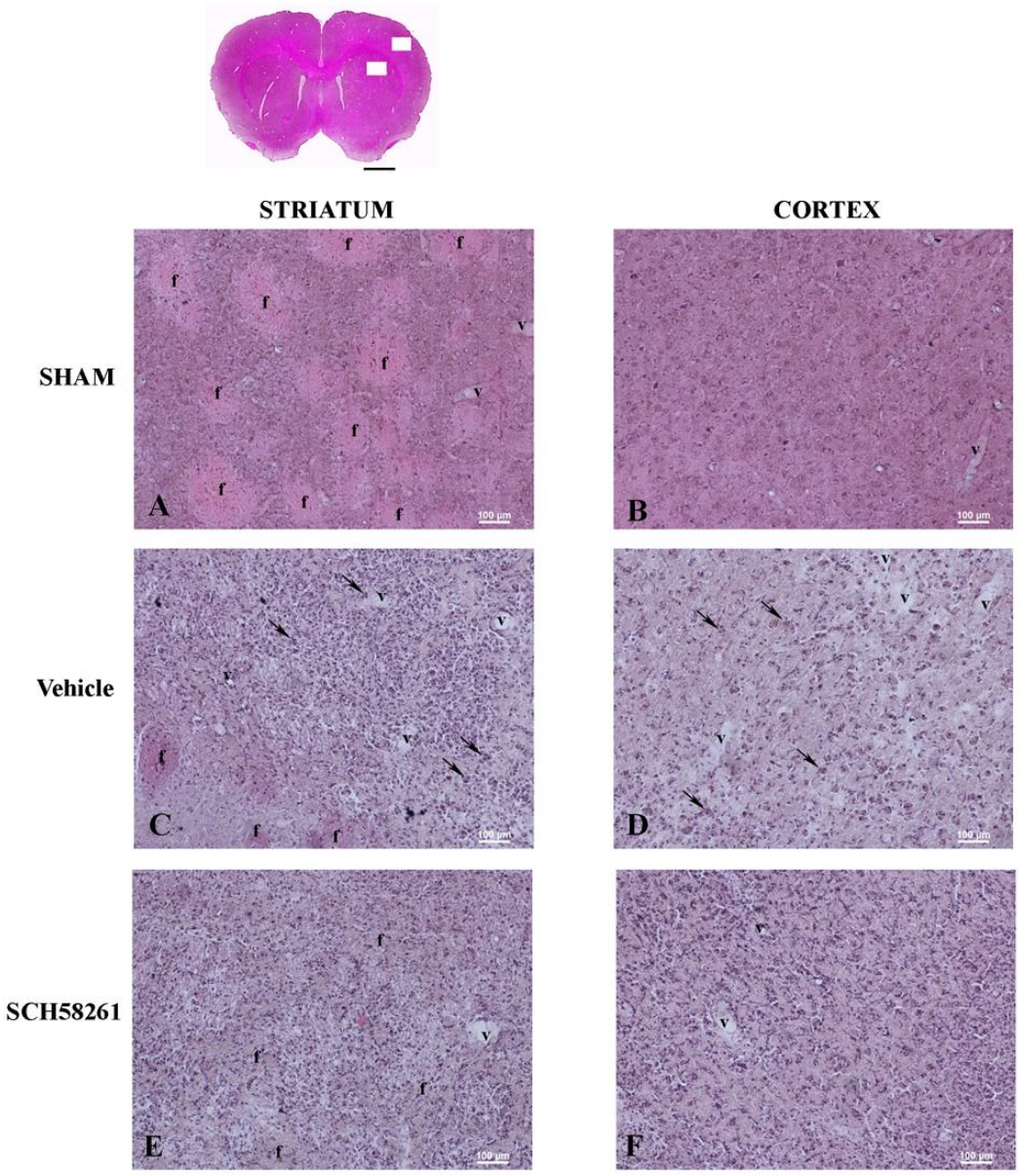

Figure 35 Effect of treatment with SCH58261 on histological damage 7 days after tMCAo. Upper part: representative photomicrograph of a histological section of a control rat (at Bregma +1.5 mm, Konig and Klippel, 1967). The two white boxes indicate the ischemic striatal and cortical area reported in the enlargements. H&E staining. Scale bar= 2 mm.
A-B: Sham-operated rats. **A**: Dorsal striatum. The typical caudate-putamen cytoarchitecture is appreciable: numerous transversally sectioned white matter *fascicula* (f) are surrounded by grey matter. **B**: fronto-parietal cortex. The typical columnar organization of the cortex is appreciable. **C-D**: Vehicle-treated ischemic rats. Note the paleness of the stained tissue due to the excess of interstitial fluid. **C**: Ischemic dorsal striatum. The cytoarchitecture is completely lost. The distinction between white and grey matter is no more appreciable; there are numerous and dilated vessels (v) and a consistent increase of the interstitial spaces. **D:** Ischemic fronto-parietal cortex. The columnar organization of the cortex is hardly visible, the interstitial spaces are enlarged, and vessels are numerous and dilated. In both striatum and cortex arrows indicate heterochromatic small nuclei. **E-F**: SCH58261-treated ischemic rats. Note the paleness of the stained tissue due to the excess of interstitial fluid. **E**: Ischemic dorsal striatum. Few fascicles (f) are hardly identifiable. **F**: Ischemic fronto-parietal cortex**.** The columnar organization is hardly detectable. Scale bar=100 µm.

Myelin distribution studied by an antibody against MAG showed to be well organized within the white matter *fascicula* of the caudate-putamen of sham-operated rats (Figure 36 A). The same distribution of MAG labeling appeared in the contralateral non-ischemic hemisphere (data not shown). In the ischemic striatum of vehicle-treated rats, MAG labeling was not characterized by the typical profile of the white matter *fascicula* but appeared irregularly distributed into *fascicula* (Figure 36 B). The same irregular distribution of MAG is present in the ischemic striatum of rats treated with SCH58261 (Figure 36 C).

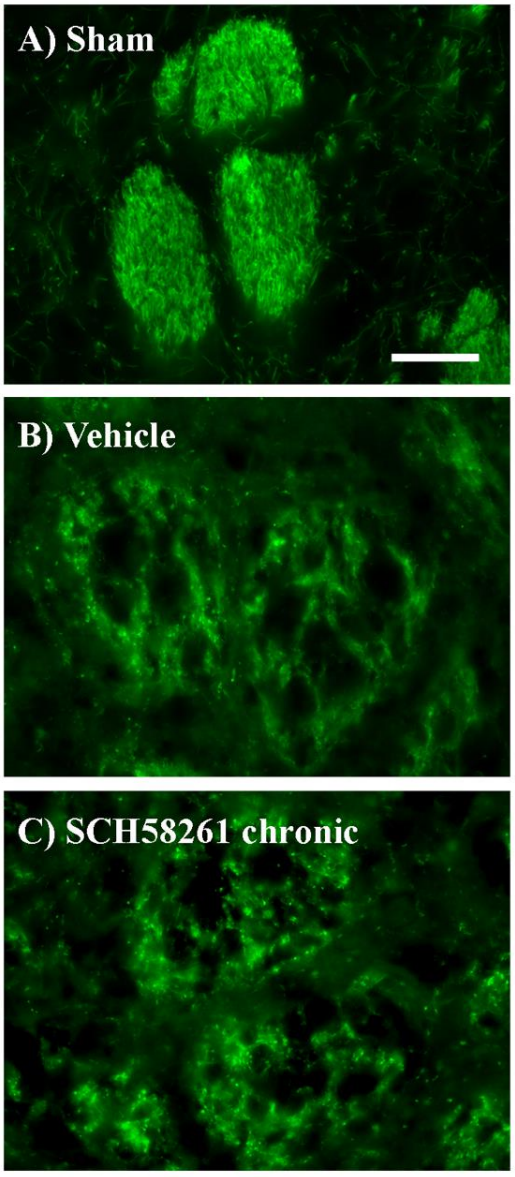

Figure 36 Effect of treatment with SCH58261 on myelin organization in the striatum 7 days after tMCAo. A) In sham-operated rats, myelin is well organized within the white matter *fascicula* of the caudate-putamen. B) In vehicle-treated rats, myelin organization is loss. C) In SCH58261-treated rats, myelin organization is lost in a similar way to that of vehicle-treated rats. Scale bar=50 µm

4. Effect of treatment with the adenosine A_{2A} receptor antagonist on blood cell infiltration after tMCAo

Table 4 shows that HIS48$^+$ cells (granulocytes) were found in cortical and striatal ischemic *core* of vehicle rats two days after tMCAo. No HIS48$^+$ cells were detected into the cortical and striatal tissue of sham-operated rats. Treatment with SCH58261 did not modify the number of HIS48$^+$ cells in cortical and striatal ischemic *core*.

Treatment	**HIS-48$^+$ cells**	
	Ischemic Cortex	**Ischemic Striatum**
Vehicle (n=4)	30.7±3.63	29.4±3.61
SCH58261 (n=3)	25.0±1.16	25.0±1.30

Table 4 Effect of SCH58261 on granulocyte infiltration into *core* ischemic tissue after 2 days from tMCAo. Data are the mean±S.E.M. of the number of HIS-48$^+$ cells per optical field (40X) counted in 7 coronal levels through the brain of "n" rats.

Effects of chronic treatment with the adenosine A_{2A} receptor agonist, CGS21680, on platelet aggregation

1. Adenosine A_{2A} receptor agonist, CGS21680, does not modify platelet aggregation

To assess the effect of CGS21680 on platelet aggregation, classical light transmission aggregometry was performed after stimulation with ADP 10 µM. In the control group the percentage of aggregation was 50.9%±11.5 (mean±SEM), the chronic treatment with CGS21680 at the dose 0.01 mg/kg did not modify platelet aggregation induced by 10 µM ADP (49.4%±1.3) (Figure 37).

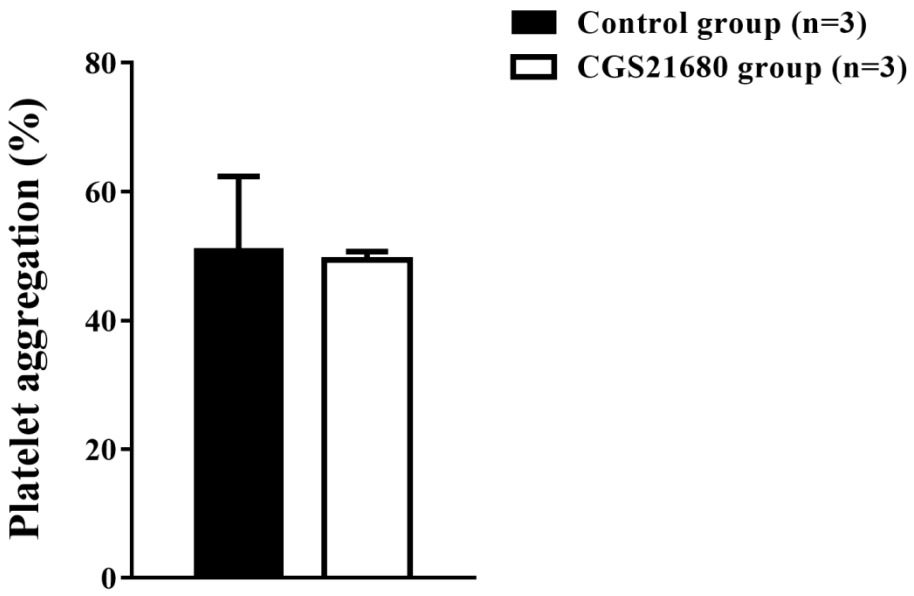

Figure 37 Effect of chronic treatment with CGS21680 (0.01 mg/kg i.p.) on platelet aggregation. The platelet aggregation activities was assessed using ADP (10 µM). Results are expressed as percentage of aggregation and values as mean±SEM.

Adenosine A_{2B} receptor agonist, BAY 60-6583, protects from behavioral and histological damage after focal transient brain ischemia in the rat

1. Effect of treatment with the adenosine A_{2B} receptor agonist on neurological deficit after tMCAo

Figure 38 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, seven days after tMCAo, the neurological score was further reduced to 6.8±0.9. Chronic treatment with the A_{2B} agonist, BAY 60-6583, 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 BAY 60-6583-treated rats at each time point (1, 5, 7 days after tMCAo, $p<0.0001$-0.03). The chronic treatment with BAY 60-6583 significantly reduced the neurological deficit with respect to vehicle-treated rats at 1, 5 and 7 days ($p<0.001$-0.02) after tMCAo.

2. Effect of treatment with the adenosine A_{2B} receptor agonist on body weight loss after tMCAo

Twenty-four hours after operation, 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 38 B). Treatment with the A_{2B} receptor agonist, BAY 60-6583, 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 BAY 60-6583-treated rats at each time point (p<0.0001-0.003) after tMCAo.

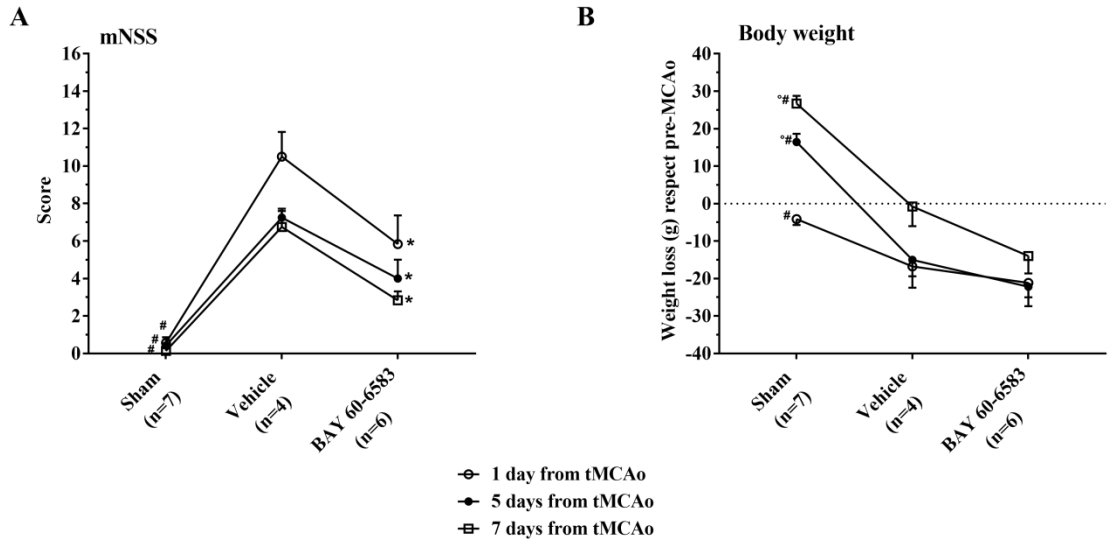

Figure 38 Effect of chronic treatment with BAY 60-6583 (0.1 mg/kg i.p.) on neurological deficit (A) and body weight loss (B). Data are expressed as mean±SEM of "n" rats. A) mNSS test: the score is evaluated before and after 1, 5 and 7 days from tMCAo in each rat group. Repeated Measures two-way ANOVA followed by Tukey post hoc test: $^{\#}$p<0.0001-0.04 sham-operated versus chronic BAY 60-6583- and vehicle-treated rats; *p<0.001-0.02 chronic BAY 60-6583-treated versus vehicle-treated rats. B) Body weight loss: sham-operated rats increase their body weight in the period after the operation. The body weight loss of tMCAo rats was calculated as the mean±SEM of the difference between body weight at each time point and pre-operation body weight. Repeated Measures two-way ANOVA followed by Tukey post hoc test: $^{\circ}$p<0.0001 sham-operated versus vehicle-treated rats $^{\#}$p<0.0001-0.003 sham-operated versus chronic BAY 60-6583-treated rats.

3. Effect of treatment with the adenosine A_{2B} receptor agonist on brain ischemic damage after tMCAo

Figure 39 shows the extent of ischemic damage evaluated as infarct area (Figure 39 A, C) and infarct volume (Figure 39 B, D) in ischemic striatum and cortex of vehicle- and BAY 60-6583-treated rats 7 days after tMCAo. Chronic treatment with BAY 60-6583 significantly reduced the infarct area within the cortex and showed an apparent trend towards a reduction for ischemic striatal infarct (Figure 39 B). The cortical infarct volume was reduced by 40% (Unpaired Student's t-test: $p<0.04$; Figure 39 D). Sham-operated rats did not show any damage.

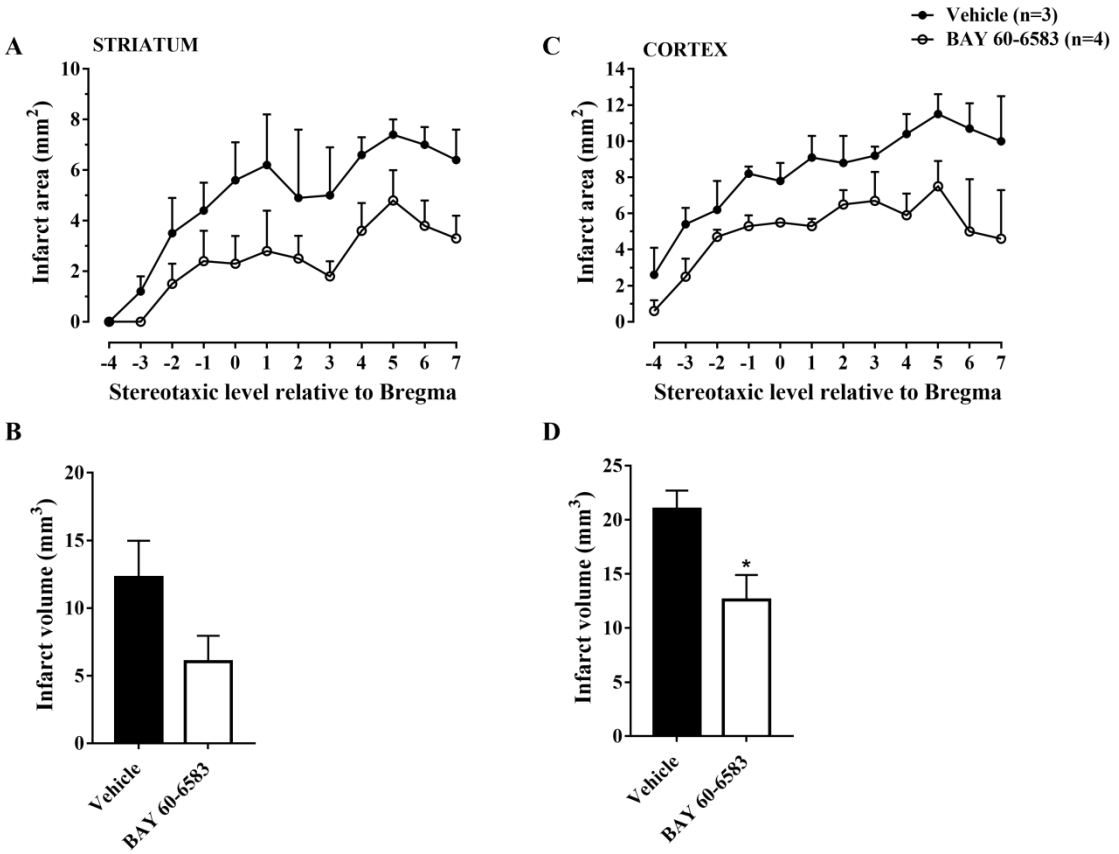

Figure 39 Effect of chronic treatment with BAY 60-6583 (0.1 mg/kg i.p.) on infarct area (A, C) and infarct volume (B, D) in the striatum and cortex 7 days after tMCAo. Data are the mean±SEM of infarct area measured at 12 predetermined coronal levels through the brain of "n" rats. Bregma=0 (König and Klippel, 1967). Bar graphs show the infarct volume calculated as mean±SEM in the striatum and cortex. Unpaired Student's t-test: *$p<0.04$ vs vehicle-treated rats.

To characterize the cytoarchitecture of the ischemic cortex and striatum 7 days after tMCAo, ischemic tissue was stained by H&E (Figure 40 A-F). Seven days after transient ischemia, H&E staining showed a decrease in staining intensity in vehicle-treated rats (Figure 40 C, D) compared to the sham operated rats (Figure 40 A, B). The typical cytoarchitecture of these two regions (for a description see Danner and Pfister, 1981) was lost. In the striatum, the white matter *fascicula* (*f*) were no more recognizable; in the fronto-parietal cortex the columnar organization was not appreciable and very numerous heterochromatic small nuclei were present. Figure 40 E, F shows that chronic administration of BAY 60-6583 at the dose of 0.1 mg/kg was associated with a recovery of staining intensity and with a reduction of heterochromatic small nuclei staining in both brain regions. The cytoarchitecture of the white matter *fascicula* was recognizable in the dorsal corpus striatum (Figure 40 E) and the columnar organization was appreciable in the fronto-parietal cortex (Figure 40 F).

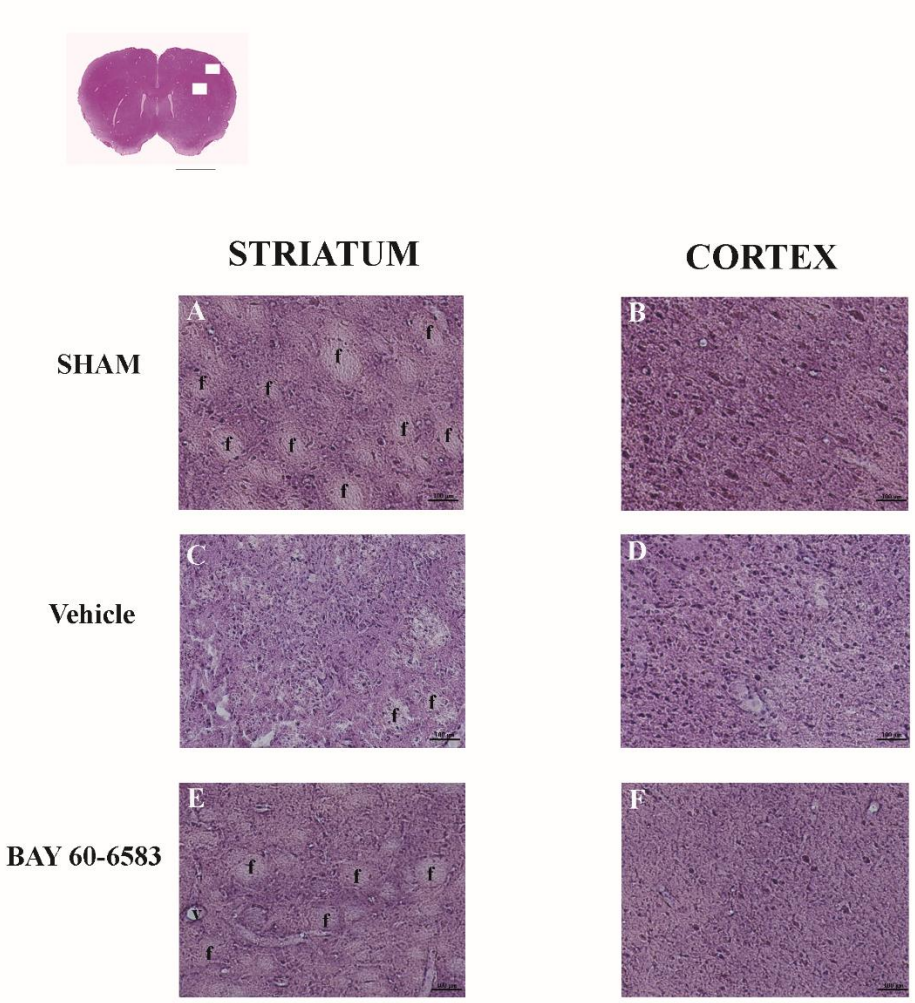

Figure 40 Effect of BAY 60-6583 (0.1 mg/kg i.p.) on cytoarchitecture of the ischemic striatum and cortex after tMCAo. Upper part: representative photomicrograph of a histological section of control rat (at Bregma +1.5 mm, König and Klippel, 1967). The two white boxes indicate the ischemic striatal and cortical area reported in the enlargements. Scale bar=2mm. Sham-operated rats: (A) in the dorsal striatum, the typical caudate-putamen cytoarchitecture is appreciable, numerous transversally sectioned white matter *fascicula* (*f*) are surrounded by gray matter containing diverse type of neurons distinct on the basis of their size and shape (Danner and Pfister, 1981); (B) in the fronto-parietal cortex the typical columnar organization is appreciable. Vehicle-treated ischemic rats: (C) in most part of the ischemic dorsal striatum the cytoarchitecture is completely lost. The distinction between white and gray matter is no more appreciable; a remarkable increase of the interstitial spaces and numerous heterochromatic small nuclei are present; (D) in the ischemic fronto-parietal cortex the columnar organization is hardly visible, the interstitial spaces are enlarged and dilated and numerous heterochromatic small nuclei are present. BAY 60-6583-treated ischemic rats: note the recovery of the staining intensity and the decrease of heterochromatic small nuclei; (E) in the ischemic dorsal striatum the cytoarchitecture of the white matter *fascicula* is again recognizable; (F) in the ischemic fronto-parietal cortex the columnar organization is appreciable. Scale bar=100µm.

Discussion.

The protective effects of the selective histamine H_4 receptor antagonist, JNJ7777120, in a rat model of transient cerebral ischemia

Results demonstrate that the selective antagonist of histamine H_4 receptor, JNJ7777120, systemically and chronically administered after ischemia, reduces the ischemic brain damage and improves the neurological deficit.

In the experimental model of focal cerebral ischemia induced by MCAo in rats, the levels of histamine and tele-methylhistamine evaluated by microdialysis in the striatum and cerebral cortex gradually increase reaching levels three- and 2-fold higher, respectively, than those of the contralateral side and become statistically significant 6–12 hours after induction of ischemia (Adachi et al., 1991). JNJ was described as a lipophilic drug that readily crosses BBB (Funke et al., 2013) and H_4 receptor recently identified in cells of CNS, can account for protective effects of the H_4 antagonist in MCAo rats. In the human brain, H_4 receptor mRNA was detected by RT-PCR in brain regions including hippocampus, cortex, thalamus and amygdala and in the rat brain in the cortex, cerebellum, brainstem, amygdala, thalamus and striatum (Strakhova et al., 2009). As to the cellular localization, expression of H_4 receptor mRNA was detected by immunohistochemistry in neurons of several regions of human and mouse brain, such as thalamus, hippocampus and cerebral cortex (Connelly et al., 2009). Although the most accurate qPCR technique has revealed only low levels of H_4 receptor mRNA in human brain and criticism was raised concerning the consistency of literature data that demonstrate the presence of H_4 receptor in the brain (Schneider and Seifert, 2016), the brain H_4 receptor is functional. Indeed, in the somatosensory cortex, the H_4 receptor agonist 4-methyl histamine directly hyperpolarizes neurons and promotes outwardly rectifying currents (Connelly et al., 2009). Acutely after ischemia, glutamate acting on "non-NMDA" and NMDA receptors acts as the main promoter of excitotoxicity that brings to glial reactivity and neuron death (Chamorro et al., 2016). However, the H_4 receptor hyperpolarization effect together with the observation that H_4R did not act as hetero-receptors on thalamocortical glutamatergic terminals (Connelly et al., 2009) do not support the notion that the protective effects of JNJ7777120 are attributable to reduced release of glutamate from neurons.

In addition to neurons, H_4 receptor has been localized by RT-PCR analysis, immunocytochemistry and western blotting on microglial cells (Ferreira et al., 2012). Here we report that JNJ7777120 chronically administered after ischemia, clearly hampers glial activation reducing both reactivity and number of microglia and astrocytes in the cortical and striatal ischemic core and boundary zones. Consistently, a selective histamine H_4 receptor antagonist inhibits the histamine induced TNF-α and IL-6 production by primary cultured microglia (Dong et al., 2014) supporting that protective effect of JNJ7777120 is attributable to direct antagonism of H_4 receptor located on rat microglial cells. So far, there is no evidence for the presence of H_4 receptor on astrocytes. After brain ischemia, the primary acute lethal mechanism of excitotoxicity induces the activation of resident immune cells especially microglia and production of inflammation mediators that triggers the so-called "secondary damage" characterized by endothelial expression of adhesion molecules and leukocyte infiltration that on their turn exacerbate neuroinflammation (Iadecola and Anrather, 2011; Pedata et al., 2016). Consistent part of the increase of brain histamine after ischemia (Adachi et al., 1991) might by accounted for by release from mast cells. In a model of transient cerebral ischemia induced by four-vessel occlusion, an increase of mast cells degranulation in association with an increase of histamine content was demonstrated seven days after ischemia in the adult rat (Hu et al., 2004). Moreover, mast cells were found involved in promoting BBB breakdown and neutrophil infiltration in a mouse model of focal ischemia (McKittrick et al., 2015). Observation that two days after tMCAo the H_4 receptor antagonist has significantly reduced granulocyte infiltration, supports that the histamine H_4 receptor is involved in promoting BBB breakdown and neutrophil infiltration.

Mast cells residing in the brain are located on or near the cerebrovasculature and, among other mediators, synthetize TNF-α and various eicosanoids as well as pro-inflammatory cytokines that concur to neurovascular inflammation (Hu and Chen, 2012).

Administration of the H_4 receptor antagonist, JNJ7777120, reduced TNF-α and IL-1β assayed in the peripheral plasma seven days after ischemia. Such reduction might reflect lower levels of pro-inflammatory cytokines produced in the brain and reaching peripheral blood. It might be also envisaged a peripheral mechanism of action of JNJ7777120. Stroke and inflammation are strictly interrelated. Single-photon emission computed tomography (SPECT) imaging in mice subjected to tMCAo, has revealed that

systemic inflammation preceding experimental stroke is associated with markedly augmented brain inflammation and impaired neurologic outcome, but conversely also that 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 protocols (Szigeti et al., 2015). Such peripheral inflammatory changes on their turn might contribute to a worse recovery after stroke. Precise cellular-molecular mechanisms underlying are unclear, but likely reflect a vicious circle responsible of inflammatory mediator production and BBB function. JNJ7777120 could reduce TNF-α and IL-1β and increase IL-10 production by antagonizing H_4 receptor on dendritic cells (Simon et al., 2011) that are activated by histamine that in turn is released by basophiles or perivascular mast cells secondarily to central inflammatory mediators released in the blood after ischemia.

Overall our results stress the key research questions of the predictive value of blood biomarkers in stroke and suggest that JNJ7777120 by controlling a secondary inflammatory damage after brain ischemia represents a new interesting target after brain ischemia.

Time-course of protection by the selective A_{2A} receptor antagonist, SCH58261, after transient focal cerebral ischemia

Our results demonstrate that the selective A_{2A} receptor antagonist, SCH58261, systemically and chronically administered (0.01 mg/kg i.p starting 5 min after tMCAO and twice/day for 7 days), protected from neurological deficit 1 day after tMCAo but not after 5 and 7 days (Melani et al., 2015). However, seven days after tMCAo, it has not protected from brain ischemic damage and from the body weight loss. Protection by the A_{2A} antagonist in the first 24 hours after ischemia confirms previous results (Melani et al., 2003, 2006, 2009; Monopoli et al., 1998) that demonstrated that the adenosine A_{2A} receptor antagonist SCH58261, administered at the same dose (0.01 mg/kg, i.p) 5 min, 6 h and 15 h after permanent ischemia, has protected from neurological and tissue damage 24 hours after permanent ischemia. Protective effect of SCH58261, in the first 24 hours, is attributed to antagonism of the early excessive glutamate outflow that occurs after ischemia (Pedata et al., 2014). However cerebral ischemia is a complex pathology that rapidly evolves in time. The early massive increase in extracellular glutamate after ischemia primes an excitotoxic cascade which in turn activates brain immune cells that produce mediators of inflammation (Dirnagl et al., 1999). After transient (1 hour) focal ischemia induced by MCAo, a definite microglial activation is present after 12 hours (Gelderblom et al., 2009). Activated immune cells produce proinflammatory cytokines that upregulate cell adhesion molecules (Stoll et al., 1998; Huang et al., 2006) and promote increased permeability of BBB. It is now accepted that a massive leukocyte infiltration into ischemic areas, by a disrupted BBB, amplifies the primary ischemic damage (Pantoni et al., 1998; Iadecola and Anrather, 2011). This is consistent with the view of a neurovascular unit where any cell type of the brain together with peripheral immune cells dynamically interact in the pathobiology of stroke (del Zoppo, 2009; Dirnagl, 2012). By anti-HIS-48 antibody, we showed numerous infiltrated granulocytes in ischemic striatal and cortical areas two days after tMCAo. This is in agreement with observation that after tMCAo, a peak of neutrophil infiltration occurs at 6 and 48 hours thereafter (Zhang et al., 1994). Three days after tMCAo the majority of immune cells were described to be neutrophils and at less extent

lymphocytes (Gelderblom et al., 2009). Two days after tMCAo, chronic treatment with SCH58261 did not reduce HIS-48 positive cell infiltration into ischemic tissue (Melani et al. 2015). Explanation of the lack of protection by the chronically administered A_{2A} receptor antagonist 7 days after ischemia, likely lies in the fact that the early protective effect is overwhelmed by subsequent damage brought about by massive cell infiltration and neuroinflammation (Gelderblom et al., 2009; Melani et al., 2014; Zhang et al., 1994).

Clarification of the time-limit in which A_{2A} receptor antagonism is protective after brain ischemia might let to devise a correct therapeutic strategy with A_{2A} receptor antagonists. Neuroinflammation is now recognized as a predominant mechanism of secondary progression of brain injury after ischemia. Since adenosine A_{2A} agonists are recognized as antinflammatory drugs acting on mostly peripheral adenosine A_{2A} receptors located on blood cells (Pedata et al., 2018), we further checked, the effect of adenosine A_{2A} agonists in the MCAo model of brain ischemia. While the A_{2A} receptor antagonist, SCH58261, does not maintain protection after 7 days from tMCAo, Melani and colleagues (2014) had reported that the A_{2A} receptor agonist, CGS21680, chronically administered at the dose of 0.01 mg/kg, twice/day for 7 days, starting 4 h after tMCAo, 7 days thereafter has protected from neurological deficit and ischemic damage and 2 days after tMCAo, it has protected from blood cell infiltration. CGS21680, administered i.p.at the dose of 0.01 mg/kg did not modify heart frequency or mean pressure from 15 min up to 1 hour after administration. In the present thesis we demonstrate that at this dose, chronically administered, twice/day for 7 days, it either does not modify platelet aggregation induced by 10 μM ADP in the rat.

Therapies under study in ischemia (i.e. neuroprotective drugs including hypothermia or antioxidant/antiinflammatory strategies) need to be associated with thrombolytic drugs. Indeed, restoration of oxygen and glucose by thrombolytic drugs, at the moment, is considered the best therapy to protect against cell death from stroke (Liu et al., 2018), although its efficacy may be limited by the potential hemorrhagic effects. Considering that tPA and/or antiplatelet drugs are also routinely used in prevention of the secondary stroke, administration of a further drug that has antiplatelet activity could potentiate a previous antiplatelet therapy increasing the hemorrhagic potential or on the contrary could be useful in maintaining an anti-platelet effect after the primary stroke (Pedata et al., 2018). Data demonstrate that adenosine A_{2A} receptor agonist exerts a protective

effects at doses lower than those necessary to inhibit platelet aggregation, suggesting the possible utility of a therapy with A_{2A} agonists in combination with thrombolytic drugs.

In conclusion, at the moment considering a possible therapy after ischemia with adenosine compounds active at adenosine A_{2A} receptor we cannot exclude that the protection from excitotoxicity by an early treatment with A_{2A} receptor antagonists can be enhanced by a subsequent treatment performed in a wider therapeutic time-window with A_{2A} agonists with the purpose of limiting the infiltration and ensuing inflammatory damage.

Adenosine A_{2B} receptor agonist, BAY 60-6583, protects from behavioral and histological damage after focal transient brain ischemia in the rat

Our preliminary results demonstrate that the selective adenosine A_{2B} receptor agonist, BAY 60-6583, chronically and systemically administered at the dose of 0.1 mg/kg, improves the neurological deficit and reduces the ischemic brain damage 7 days after tMCAo.

Adenosine A_{2B} receptor is characterized by a low affinity for adenosine (EC_{50} 5-20 µM), however it might be well activated in cerebral ischemia when extracellular adenosine levels increase and are estimated in the range of 30 µM (Latini and Pedata, 2001). Efficacy of adenosine on A_{2B} receptors might be also due to an increase of A_{2B} receptors that have been reported in ischemic cortex and striatum 24 hours after tMCAo by Li and colleagues (2017). The same authors have 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, reduced lesion volume and attenuated brain swelling and BBB disruption 24 hours after ischemia. In this work we report evidence that the adenosine A_{2B} receptor agonist BAY60-6583 administered systemically and chronically after ischemia is protective at more distant time from ischemia when a defined neuroinflammation occurs. BAY 60-6583 reduces significantly the volume of the ischemic infarct in the cortex and causes a trend towards a reduction of the ischemic striatal infarct 7 days after transient ischemia. Indeed, seven days after ischemia, H&E staining shows that chronic treatment with BAY 60-6583 reconstitutes the cortex and striatum cytoarchitecture and has decreased heterochromatic small nuclei in the ischemic area. Melani et al. (2014) have reported that the heterochromatic small nuclei belong to astrocytes and microglia that are activated seven days after ischemia. It is today well known that after an ischemic event over-activation of microglia is detrimental and these resident immune cells are involved in the neuroinflammation process (Schwartz, 2003; Kriz, 2006; Block et al., 2007). Adenosine A_{2B} receptor is expressed on microglia and their stimulation reduces TNF-α levels in primary microglia cultures (Merighi et al., 2015) and augmentes the release of IL-10 by murine microglial cells (Koscsó et al., 2012). In addiction, A_{2B} receptor is also

expressed on astrocytes where its levels increase 24 hours after transient ischemia (Li al., 2017). In a model *in vitro* of hypoxia/reoxygenation, A_{2B} receptor stimulates the increase of IL-6 from astrocytes (Maeda et al., 1994). It was demonstrated that IL-6 exerts neuroprotective actions in a model of permanent MCAo (Loddick et al., 1998). On the all these results support a central protective effect of BAY 60-6583 which is attributable to direct action on A_{2B} receptor on microglial cells and on astrocytes.

Besides microglia and astrocytes, A_{2B} receptor is present also on endothelial cells where their expression increases after transient ischemia (Li et al., 2017). After MCAo and subsequent reperfusion, there is an initial increase of BBB permeability (Sandoval and Witt, 2008) and a following biphasic increase at 5 and 72 hours (Kuroiwa et al., 1985). Stimulation of A_{2B} receptor decreases BBB permeability 24 hours after ischemia (Li et al., 2017) and also reduces vascular leak in a model of hypoxia in mice (Eckle et al., 2008). Changes in BBB permeability are responsible for cell infiltration, such as neutrophils that, expressing cytokines and chemotactic factors, promote expansion of the inflammatory response in the ischemic tissue (Haskó and Pacher, 2008). Correlations among neutrophils accumulation, severity of brain tissue damage and neurological outcome have been reported by Akopov et al., (1996). A_{2B} receptor KO mice, exposed to hypoxia, exhibit increased neutrophils infiltration suggesting that A_{2B} receptors exert an inhibitory role on neutrophils transmigration (Eckle et al., 2007, 2008). Thus, a direct protective action of BAY 60-6583 on endothelial cells of BBB may prevent from neutrophils infiltration in ischemic tissue. Moreover a possible direct action of BAY 60-6583 on A_{2B} receptor localized on blood immune cells, i.e. neutrophils and lymphocytes (Gessi et al., 2005; Eckle et al., 2008) where A_{2B} receptors exert anti-inflammatory actions, inhibiting vascular adhesion (Yang et al., 2006) and inflammatory cells migration (Wakai et al., 2001; Konrad et al., 2012) might contribute to protective effect of the A_{2B} agonist.

It worth discussing that the chronic treatment with BAY 60-6583 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 after ischemia. Adenosine is released from fat cells in adipose tissue (Schwabe et al., 1975; Capogrossi et al., 1986) and partecipates in the regulation of adipocyte function (Eisenstein and Ravid, 2014). In particular, stimulation of A_{2B} receptor inhibits adipogenesis (Gharibi et al., 2012). This effect might account for the lack of the chronic treatment with BAY 60-6583 in protecting from the body weight loss.

It is worth mentioning that in a model of ischemia *in vitro*, Fusco et al. (2018) have demonstrated the protective effects of selective A_{2B} receptor antagonists, MRS1754 and PSB603, that prevent the appearance of AD, a phenomenon caused by the sudden increase of extracellular K^+ and by the contemporary explosive rise in glutamate extracellular concentration (Somjen, 2001) that brings to an acute tissue damage. These results suggest that in the acute phase of brain ischemia when excitotoxicity plays a major role, A_{2B} receptor antagonism might represent a cell defence mechanism. Excitotoxicity plays a crucial role acutely after ischemia (Dirnagl et al., 1999). It has been demonstrated that striatal glutamate outflow reaches a peak between 60 and reaches a peak between 60 and 120 min after MCAo (Melani et al., 1999; Gui et al., 2009) and is maintained higher than basal up to 2 hours after reperfusion (Gui et al., 2009). However, after ischemia, the tissue damage is the result of a combination of the precocious acute excitotoxic damage and of a secondary damage due to neuroinflammation that develops hours and days after MCAo (Dirnagl et al., 1999).

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 both on central glial cells and on vascular endothelial and blood cells would play a pivotal role in attenuating hypoxia-induced increases in vascular leak infiltration and neuroinflammation that develops hours and days after ischemia. Importantly, A_{2B} receptor agonists might be adjuvant to the today accepted pharmacological strategy of tPA administration being a promising strategy for decreasing the risk of hemorrhages during treatment for ischemic stroke.

Further experiments will be performed to study the intimate brain cell transduction mechanisms involved in the A_{2B} protective effects after transient ischemia.

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