UNIVERSITÀ DEGLI STUDI FIRENZE

DOTTORATO DI RICERCA TOSCANO IN NEUROSCIENZE

CICLO XXXV

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Pharmacological and functional characterization of adenosine "A_2" receptors as emerging targets in cerebral ischemia and oligodendrogliogenesis: an *in vitro* study

Settore Scientifico Disciplinare BIO/14

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Table of contents

INTRODUCTION

1 Purinergic signalling

The purinergic system consists of adenine nucleosides and nucleotides, such as adenosine and adenosine triphosphate (ATP), more commonly known as *purines,* and their receptors. Purines are ubiquitous molecules involved in various metabolic pathways and in various cellular homeostatic processes. In particular, adenosine is an endogenous neuromodulator that recently emerged as a most pervasive mechanism for intercellular communication in the nervous system. The first report of adenosine activity dates back to 1929, when Drury e Szent-Gyorgyi (Drury e Szent-Györgyi, 1929) described its vasodilating and bradycardising activities. It will be necessary to wait until 1963 to have further confirmation of the actions of adenosine, when Berne identified a physiological role for adenosine as a mediator of vasodilation and in the regulation of coronary blood flow (Berne, 1963). It was later demonstrated that in the Central Nervous System (CNS), adenosine raises cAMP levels in guinea pig cerebral cortex slices, an action that is suppressed by methylxanthines like caffeine and theophylline (Sattin and Rall, 1970).Later on, it was shown that adenosine inhibits excitatory synaptic potential amplitude in cortical and hippocampal slices as well as the firing of cortical and cerebellar neurons (Dunwiddie and Hoffer, 1980; Kostopoulos et al., 1975; Okasa and Ozawa, 1980; Phillis et al., 1979). Moreover, it was observed in different brain areas that adenosine inhibited the release of several important neurotransmitters such as acetylcholine, noradrenaline, excitatory amino acids and serotonin (Corradetti et al., 1984; Michaelis et al., 1979).

1.1 Adenosine

Adenosine is an important neuromodulator involved in many biochemical processes and signal transduction pathways. It carries out numerous actions at central level: it acts as an endogenous anticonvulsant, influences the control of motility, pain, learning and memory (Pedata et al., 2007). Moreover, adenosine has a further crucial role in the modulation of emotional states, conditioning social interactions and aggressive behaviors. In physiological conditions, extracellular adenosine exerts an inhibition on synaptic transmission and this makes it a highly protective neuromodulator.

The nucleoside adenosine is formed by an adenine and ribose molecule linked through an N9-glicosidic bond and it is continually formed both at intracellular and extracellular level (Fredholm et al., 2001). This metabolite is generated mainly by two enzymatic systems: intra- (cN-I) or extracellularly localized 5'-nucleotidases (e5'-NT) and cytoplasmic S-adenosylhomocysteine hydrolase (SAHH; hydrolysis of SAH is associated with transmethylation reactions) (Borowiec et al., 2006). In the first case, the ATP released in the synaptic terminal becomes a substrate of 5'-NT which sequentially degrades it into adenosine diphosphate (ADP), adenosine monophosphate (AMP) and finally into adenosine. ATP inhibits the 5'-NT that is an enzyme with an elevated affinity to AMP; for this reason, when the cell is exposed to an intense metabolic activity with increased ATP consumption and consequent elevated production of AMP, the enzyme has very high enzymatic activity (Pedata et al., 2007). Therefore, during low energetic support conditions as in epileptic attacks, hypoxia or ischemia, the production of adenosine is much increased (Latini and Pedata, 2001). In the second case, the biosynthetic pathway takes place exclusively at the intracellular level and involves the hydrolysis of S-adenosyl-homocysteine (SAH) by the SAH hydrolase enzyme.

Under physiological conditions, extracellular adenosine concentration is 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).

Cellular carriers mediate adenosine transport across the cytoplasmic membrane, as its hydrophilic structure does not allow its direct crossing. Nucleoside transporters are classified according to their functional characteristics and their molecular structure into: equilibrative transporters and concentrative transporters (Cass et al., 1998).

Equilibrative nucleoside transporters (ENTs) operate through the mechanism of passive transport and facilitate the diffusion of adenosine according to its concentration gradient between the intra- and extracellular compartment. Currently, four types of equilibrative transporters have been identified: ENT1, ENT2, ENT3 and ENT4 (Podgorska et al., 2005). Both ENT1 and ENT2 are widely expressed in the brain and are found in neurons and astrocytes.

Concentrative nucleoside transporters (CNTs) promote the flow in the cells of nucleosides against their concentration gradient through Na^+-dependent active transport mechanisms. They are divided into three subtypes: CNT1, CNT2, and CNT3, based on their selectivity for different substrates. In particular, CNT2 and CNT3 are involved in adenosine transport (Gray et al., 2004). CNT1 transporters selectively transport pyrimidines, but have also the ability to link purines as well.

After exerting its effects as a neuromodulator or signalling molecule, adenosine can become a substrate of two different enzymes: either cytosolic, after its reuptake, or extracellularly located, for example at the synaptic terminal. The two enzymes are adenosine kinase (AK) which determines its phosphorylation to AMP, and adenosine deaminase (ADA) which determines its deamination to inosine (Latini and Pedata, 2001). The processing of adenosine by one or the other enzyme essentially depends on its concentration. The AK enzyme possesses a low affinity constant (Km) for its substrate, so it is active even at low concentrations of adenosine; On the other hand, ADA, having a high Km and therefore a lower affinity for the substrate, is activated when adenosine levels increase (Meghji et al., 1988). In physiological conditions, adenosine inactivation pathway through phosphorylation prevails, while deamination becomes important in conditions of high adenosine concentration, such as during an ischemic event.

1.2 Purinergic receptors

The existence of adenosine receptors was unequivocally proved when first cloning was achieved in 1990 (Maenhaut et al., 1990). Based on the responses of various tissues to purines, Burnstock proposed the existence of distinct receptor subtypes able to bind adenosine or ATP, named P1 and P2 receptors, respectively (Burnstock et al., 1978). Originally, the “P” in P1 and P2 was meant to designate “purine”. However, it was discovered that some of the P2 receptors bind pyrimidines, UTP or UDP, preferentially to the purine ATP. Hence, the “P” in P2 is now used to designate “purine or pyrimidine”. Various synthetic adenosine analogues activate P1 receptors, but not P2, and synthetic ATP or UTP analogues activate P2, but not adenosine receptors. P1 receptors were initially distinguished in two classes (A_1 and A_2 receptors, ARs) based on their excitatory or inhibitory actions on adenylyl cyclase

(Van Calker and Hamprecht, 1979). Later work defined four different subtypes of P1 receptors: A_1, A_{2A}, A_{2B} and A_3 adenosine receptor (A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR) (Fredholm et al., 2001). On the other hand, P2 receptors appeared to be more heterogeneous and in 1994, Abbracchio and Burnstock have classified these receptors in two major families: (i) P2X ligand-gated ion channel receptors and (ii) P2Y G-protein coupled receptors (Abbracchio and Burnstock, 1994). Receptors for ATP and adenosine are widely distributed in the nervous system as well as in other tissues and physiological effects of purines on almost all tissues have been described. The development of synthetic compounds that activate P1 or P2 receptors is important for elucidating how these receptors act. Indeed, some of these newly synthetized compounds are more potent and selective than the parent purines, moreover, they are more stable than the short-lived endogenous compounds adenosine and ATP.

1.2.1 P1 purinergic receptors

Adenosine receptors are widely distributed throughout the body, including the CNS, cardiovascular system, respiratory system, gastrointestinal tissue, and immune system, indicating that adenosine has a significant impact on physiological functions. (Peleli et al., 2017; IJzerman et al., 2022).

As mentioned above, in the early 1990s, adenosine receptors have been cloned and pharmacologically characterized. The four adenosine receptors, A_1, A_{2A}, A_{2B} and A_3, have all been cloned already, at the very least from rats, mice, and humans. Adenosine receptors of the same subtype are reported to be highly comparable across mammalian species, with the exception of A_3ARs. This subtype is the latest cloned and pharmacologically characterized and presents a considerable structural variability among different species. For instance, almost 30% of difference in the amino acid sequence is found between humans and rat (Linden et al., 1993). All ARs are metabotropic GCPR, with the typical structure represented by a *core* of seven plasma membrane-crossing domains, with an extracellular N-terminus, an intracellular C-terminus, plus three intracellular and three extracellular loops of different lengths and functions among the different ARs subtypes (Fredholm et al.,

2000). These domains provide specific crucial features for the interaction between receptor and ligand.

All ARs are coupled to G proteins and they can stimulate different transduction pathways, depending on cell type (Fredholm et al., 2001). Particularly important in conferring specificity of binding of the four receptors with the different G proteins is the structure of the third cytoplasmic loop and of the carboxy-terminal end. Several phosphorylation and palmitoylation sites, located in the carboxy-terminal end, play a role in the phenomena of receptor decoupling from the G protein, that are at the basis of desensitization and internalization mechanisms (Borea et al., 2018). In particular, A_1AR and A_3AR subtypes are associated with Gi activation, adenylyl cyclase (AC) inhibition and decrease of intracellular cAMP levels, while A_{2A}ARs and A_{2B}ARs are linked to Gs proteins that activate the same enzyme increasing cAMP concentration in the cytosol. However, ARs have also been reported to couple to other G-proteins than, modulating different second messenger systems. For instance, in addition to their effects on adenylate cyclase (and contrary to adenosine A_{2A} receptors) A_1ARs, A_{2B}ARs and A_3ARs are also characterized by their stimulatory effect on phospholipase C (PLC) (Abbracchio et al., 1995; Feoktistov and Biaggioni, 1997). Furthermore, A_1ARs and A_3ARs can also activate phospholipase D (PLD) (Fredholm et al., 2001). Several types of Ca^{2+} and K^+ channels are also activated (either by a direct G protein-channel interaction or by second messenger systems) after adenosine receptor stimulation, such as the inward rectifier GIRK channel Kir3.0, that is positively modulated by adenosine A_1AR activation (Takigawa and Alzheimer, 2002) (Figure 1).

Moreover, it is important to underline the different affinity of the AR subtypes for the endogenous ligand. As stated above, under physiological conditions, extracellular adenosine concentrations are estimated to be between 20 and 300 nM (Latini and Pedata, 2001). These levels are sufficient to activate the high affinity A_1ARs, A_{2A}ARs and A_3ARs subtypes. While, the A_{2B}AR requires higher concentrations (micromolar range) of adenosine to be activated (Frenguelli et al., 2007; Latini and Pedata, 2001). Therefore, high adenosine concentrations are only reached under pathological conditions, such as during hypoxia or ischemia *in vivo* (Pedata et al., 2001) and *ex-vivo* (Latini et al., 1999b). Hence, adenosine receptors can be considered important targets for drug development in numerous pathologies such as cerebral ischemia,

pain, cardiovascular and neurogenerative diseases, so a big interest has developed in the study and synthesis of novel selective and potent molecules targeting these receptors. Indeed, some adenosine receptor binding drugs are already successfully administered today, such as *Adenocard™* that is used to treat patients suffering from paroxysmal tachycardia. Moreover, Istradefylline, a selective A_{2A}AR antagonist, used as adjunctive therapy in Parkinson's disease, was marketed as *Nouriast™* in Japan in 2013 and as *Nourianz™* in the United States in 2019.

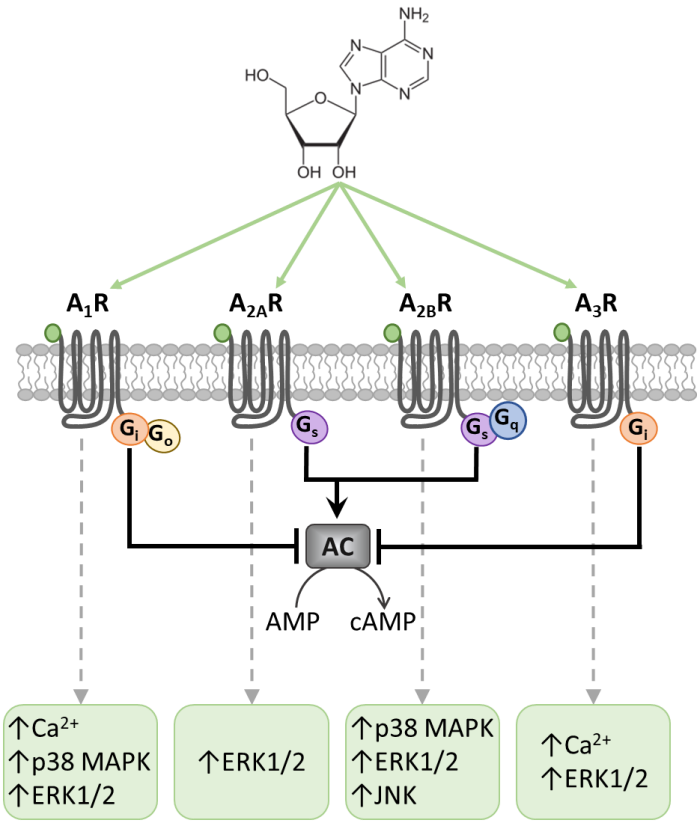


Figure 1. ***Adenosine P1 receptors***. Subtypes A_1 and A_3 inhibit adenylate cyclase and are associated with the stimulation of phospholipase C, resulting in an increase in the production of inositol 3-phosphate (IP3) and mobilization of Ca^{2+} from intracellular deposits. In addition, through coupling to an unidentified G protein, the A_1 subtype is also able to promote the escape of K^+ ions from the cytoplasm, with consequent hyperpolarization of the cell membrane. The A_{2A} and A_{2B} subtypes are generally coupled to Gs proteins that activate adenylate cyclase, resulting in an increase in cAMP levels. (Modified from: Coppi et al., 2021b).

1.2.1.1 A_1 adenosine receptor (A_1AR)

A_1 adenosine receptors are extremely conserved proteins with a sequence homology of around 87% - 92% between the different species, despite slight differences in coupling to G proteins and a species-specific tissue distribution were highlighted. The Gi-coupled A_1ARs decrease cAMP level by inhibiting AC activity. Moreover, they are Gq-coupled and promote PLC-β stimulation, thus rising diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). In addition, A_1ARs recruit pertussis-toxin (TTX)-sensitive potassium (K) and KATP channels, expressed in neurons and myocardium, while reduces Ca^{2+} channels of Q, P, and N type (Mei et al., 2018). Furthermore, it was demonstrated in transfected CHO cells that the A_1ARs are able to activate ERK1/2 at physiologically relevant concentrations of the endogenous agonist (Schulte and Fredholm, 2000).

In the brain A_1ARs are highly distributed in different regions, including the cortex, hippocampus, cerebellum and spinal cord, autonomic nerve terminals, and glial cells (Chen et al., 2013; Ballesteros-Yáñez et al., 2018), suggesting a particularly important role of adenosine in brain functions. In neurons, A_1ARs are localized both pre- and postsynaptically (Deckert and Jorgensen, 1988); in particular, in the hippocampus subcellular analysis of nerve terminals revealed that A_1ARs immunoreactivity is strategically located in the active zone of presynaptic terminals, as expected on the basis of the ability of A_1ARs agonists to decrease neurotransmitter release.

1.2.1.2 A_{2A} adenosine receptor (A_{2A}AR)

A_{2A} adenosine receptors are highly conserved with 90% of sequence homology between different species. They are Gs-coupled receptors; thus, they stimulate AC activity, increasing cAMP levels which acts as a second messenger by activating protein kinase A (PKA) (Antonioli et al., 2019). Interestingly, it was demonstrated that in the brain, A_{2A}ARs regulate a specific neuron type of Gs protein named Golf, which is also related to AC (Kull et al., 2000).

Moreover, A_{2A}ARs play a role in the regulation of MAPK, affecting the transduction pathway of several cells from different organs and tissues (Baraldi et al., 2008; Chen et al., 2013). In particular, differently from the A_1ARs, that promote the activation of

ERK1, ERK2, p38 MAPK and JNK, the A_{2A}Rs are involved only in the stimulation of ERK1 and ERK2 signalling (Coppi et al., 2021a).

These receptors are highly expressed in the brain and in peripheral immune cells. In particular, they are widespread in the caudate and putamen, in the nucleus accumbens, as well as in the olfactory tubercle with particularly high levels found in the striatum/caudate-putamen nuclei (Peterfreund et al., 1996). Concerning the periphery, they are widely expressed on blood vessels and inflammatory/immune cells (Yu L. Q. et al., 2004).

In the SNC, differently from A_1ARs, A_{2A}ARs have an excitatory role. Specifically, they enhance glutamate release and promote cell excitability (Goncalves and Ribeiro, 1996; Lopes et al., 2002). Accordingly, during an *ex-vivo* ischemic-like insult generated by oxygen and glucose deprivation (OGD), A_{2A}ARs activation contributes to excitotoxic damage caused by extracellular glutamate overflow (Colotta et al., 2012; Maraula et al., 2013; Maraula et al., 2014). Regarding its peripheral functions, adenosine is one of the most potent endogenous anti-inflammatory substances because of its effects on this receptor subtype (Antonioli et al., 2019). Indeed, A_{2A}ARs are highly expressed in lymphocytes, granulocytes, and monocytes/macrophages, where their activation inhibits the release of pro-inflammatory cytokines and increases the release of anti-inflammatory mediators like interleukin-10 (IL-10) (Varani et al., 2011; Bortoluzzi et al., 2016).

1.2.1.3 A_{2B} adenosine receptor (A_{2B}AR)

The A_{2B}AR subtype is considered the most enigmatic receptor due to the scarcity of selective ligands and to the low affinity for adenosine (EC50 = 5-20 μM; Beukers et al., 2000; Fredholm et al., 2001; Sachdeva and Gupta, 2013). Hence, in physiological conditions they are not activated due to the low affinity for the endogenous ligand, while they are recruited during pathological conditions and tissue damage, when adenosine reaches micromolar concentrations. For these reasons, the importance to study this receptor subtype and its involvement in several pathologies has recently gained attention.

A_{2B}ARs, as the A_{2A}ARs, are Gs-coupled and activate AC, promoting phosphorylation of PKA and the recruitment of various effectors directly stimulated by cAMP. In

addition, they can be coupled to Gq protein, and promote PLC stimulation (Gao et al., 1999; Linden et al., 1999; Panjehpour et al., 2005), to the MAPK and arachidonic acid pathway and to regulate the membrane channels, probably by the βγ subunit of G protein (Schulte and Fredholm, 2003).

As mentioned above for the cognate A_{2A}ARs subtype, A_{2B}ARs activation within the CNS is reported to increase glutamate release (Goncalves et al., 2015; Fusco et al., 2019), but the mechanism is at variance from the former.

In the periphery, A_{2B}ARs are present on hematic cells, such as lymphocytes and neutrophils, with the highest expression levels found on macrophages (Gessi et al., 2005; Yang et al., 2006). Here, A_{2B}ARs in most cases are co-expressed with A_{2A}ARs and their activation exerts anti-inflammatory effects by inhibiting migration and vascular adhesion (Yang et al., 2006) of inflammatory cells (Wakai et al., 2001; Eckle et al., 2008).

1.2.1.4 A_3 adenosine receptor (A_3AR)

The A_3 adenosine receptor subtype shows significant differences among the various species concerning its sequence, pharmacological profile, distribution and function. In particular, they have only 74% of sequence homology between rat and human (Koscso et al., 2011).

The Gi-coupled A_3AR inhibits AC, thus reducing cAMP accumulation, while through Gq-coupling, stimulates PLC and induces the intracellular Ca^{2+} mobilization.

A_3ARs are present in a variety of cells and organs; their expression differs among tissues, either centrally or peripherally. They are sparsely present in the thalamus, hypothalamus, and hippocampus. Motor nerve terminals, microglia, astrocytes, cortex, and retinal ganglion cells express A_3ARs while in the pial and intracerebral arteries they are present in the vasculature (Janes et al., 2014; Borea et al., 2016). Their activation can lead to both protective and harmful effects (Cheong et al., 2013); in non-neuronal cells, it was observed that a non-excessive activation prevents apoptosis mechanisms, while a persistent and intense activation induces toxic effects (Yao et al., 1997).

1.2.1.5 Adenosine receptor heteromers

Homomer, oligomer, and heteromer formation has been recently recognized as a common phenomenon affecting numerous GPCRs, including adenosine receptors. The interaction between different receptors generates a structure that has distinct properties in respect to those of the single receptor (Franco et al., 2021).

The first identified interaction between adenosine receptors is that between A_1AR - A_{2A}AR. This heteromer changes its signalling pathway based on adenosine levels, activating the Gi- coupled or Gs-coupled signalling. In particular, in the presence of high concentrations of adenosine, A_{2A}AR is activated and the C-terminal domain of the receptor is positioned to prevent Gi activation. On the contrary, in the presence of low level of adenosine, Gi signalling prevails (Navarro et al., 2018; Franco et al., 2021).

There are only few papers (to current knowledge two: Hinz et al., 2018; Gnad et al., 2020) describing the interaction between A_{2A}AR - A_{2B}AR. Interestingly, Hinz et al. (2018) observed an extremely different pharmacology of A_{2A}ARs when co-expressed with the A_{2B}ARs in CHO cells. In particular, with several *ex-vivo* techniques, they show that, in the presence of high levels of A_{2B}ARs expression, the A_{2A}AR-mediated signalling results decreased and they lost their high-affinity for selective ligands.

The existence of the A_{2A}AR - A_{2B}AR heteromer is interesting and must be further investigated as promising agonists, displaying high *ex-vivo* affinity for the A_{2A}ARs, may become inefficacious in presence of the heteromer (Hinz et al., 2018; Franco et al., 2021).

Finally, concerning A_{2A}AR-A_3AR heteromer, actually there are only few papers that describes its presence in primary cortical neurons (Lillo et al, 2020; Lillo et al., 2022). In presence of this heteromer there is a strong reduction of Gi signalling in normal condition, while the presence of an A_{2A}AR antagonist releases the A_3AR from the blockade (Lillo et al., 2020; Franco et al., 2021) (Figure 2).

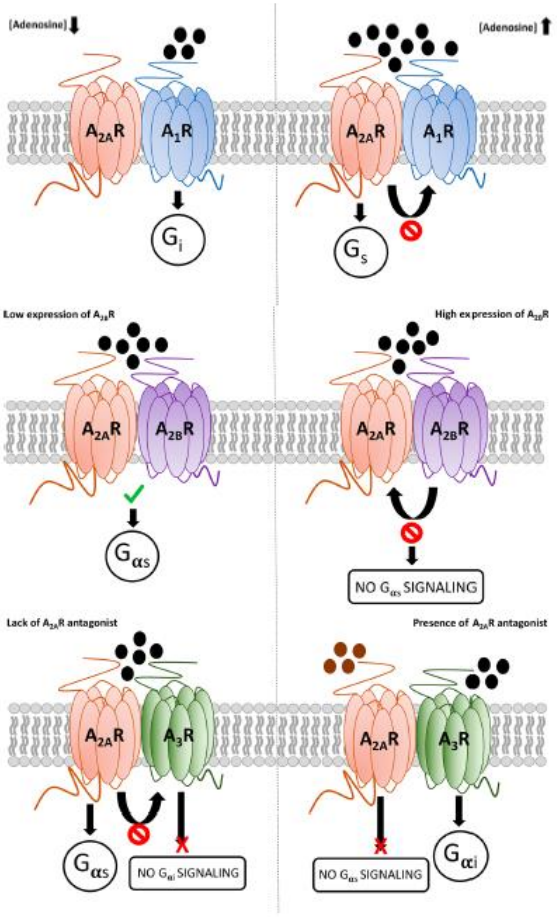


Figure 2. ***Adenosine receptors heteromers***. Schematic representation of A_1– A_{2A}, A_{2A}– A_{2B}, or A_{2A}– A_3 adenosine receptor heteromers. Receptors are depicted as monomers for simplicity. (Taken from: Franco et al., 2021).

1.2.1.5.1 Multi-target drugs

For a long time, pharmacological studies focused on the research for substances whose mechanism of action involved a univocal correspondence between drug and target molecule according to the "one drug for one target" principle. Although the use of most drugs is consistent with the above principle (Wermuth, 2004), in recent years, the possibility of identifying molecules with a multi-target pharmacological profile, has aroused lot of interest. Multi-target drugs, first proposed by Morphy et al. and Roth et al. in 2004, refer to drugs that simultaneously modulate the activity of multiple targets at clinically relevant concentrations, and that in the last few years have gained the interest of scientific community. This shifting from single- to multi-target approach is exemplified by the fact that multi-target drugs represent 21% of the new molecular entities approved by FDA from 2015 to 2017 (Ramsay et al., 2018).

The application of multi-target drugs is represented by the administration of a cocktail of drugs aimed to recognize the main molecules involved in the etiopathogenesis of a disease. Combined drug therapy, already in vogue for several years, has shown considerable clinical results in the treatment of various pathological states such as depression, schizophrenia, Alzheimer disease, Parkinson disease, cancer and infectious diseases (Goedert and Spillantini, 2006; Silver, 2007; Millan, 2009). Despite the effectiveness of the poly-pharmacological treatment, the use of a single drug capable of multiple physiological manipulations would have numerous advantages: for example, the resolution of problems related to the differences in bioavailability, pharmacokinetics and metabolism between the different substances used, ensuring a simultaneous action on all targets of interest. Furthermore, it would eliminate the risk of possible drug-drug interactions and reduce the possibility of incurring side effects. This would allow easily determining the drug dosage and consequently enhancing compliance and therapy efficacious (Bottegoni et al., 2012). Despite the presence of several drugs that are multifunctional ligands, few of these were intentionally designed to act as such, and generally, the mechanisms of action were studied retrospectively. At the same time, there are non-selective ligands whose unintentional cross-reactivity is often responsible for the side effects associated with some drugs (Morphy and Rankovic, 2005).

The use of multi-target ligands could be relevant for the treatment of comorbidity pathological conditions, such as neurodegenerative diseases, whose onset is the result of promiscuous and not always well-defined and identified alterations between genetic, epigenetic and physiological factors. Depression, schizophrenia and bipolar disorders present polygenic origin, making the development of molecules with a broad spectrum of action advantageous, replacing traditional single-target molecular therapies, which have often proved unsuccessful. The main reason why effective therapies are still under development to counteract, if not prevent, the onset of neurodegenerative diseases is due the fact that often the alterations in the phenotype do not correspond to a single, if any, mutation in the genotype. Indeed, the etiopathogenesis is often to be identified among the mechanisms of epigenetic regulation, such as DNA methylation, post-translational modifications of histone proteins, non-coding RNAs and in particular miRNA (Uher, 2011; Millan, 2013).

To date, there are several approaches (Figure 3) used for the synthesis of multi-target molecules, one of which involves the use of "medicinal chemistry hybridization" (MCH), where two or more highly selective ligands are chemically covalently linked. The linker is designed to be independently metabolized and may release the two separate ligands able to interact independently on each target (Bansal and Silakari, 2014; Morphy and Rankovic, 2005). This technique is also called "designing in" and the drugs obtained are called hybrids. Another approach is that of "balancing", which involves the fusion of two selective ligands in order to improve their activity at "sub-threshold" doses for receptor activation. The obtained molecules are named chimeric drugs. Finally, the "designing out" was proposed in which we proceed by removing the non-specific activity of a non-selective ligand while leaving the specific activity for the target of interest unchanged (Morphy and Rankovic, 2005).

Although the theory of multi-target drugs is captivating and revolutionary from a conceptual point of view, it is nevertheless not excluded that the realization of these molecules may prove to be chemically not always feasible. To date, encouraging data have been obtained in preclinical experimentation models (Millan, 2014), unfortunately not always supported by clinical studies.

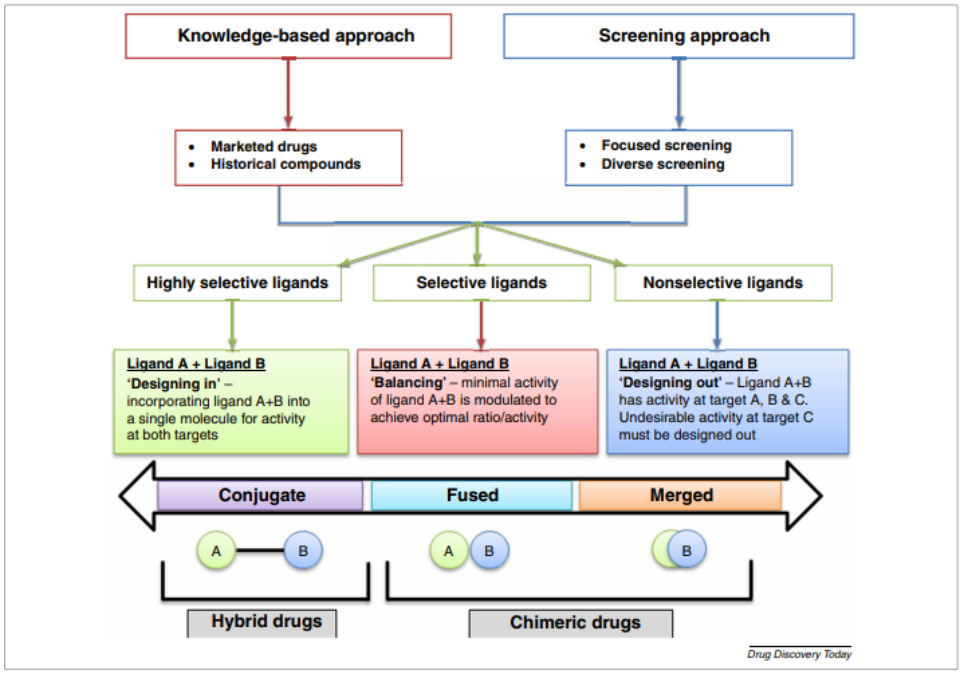


Figure 3. ***Schematic representation of the main experimental approaches to synthesize multi-target drugs.*** (Taken from: Bawa et al., 2016).

In the first part of this Thesis, we evaluated the effects of new multi-target compounds synthetized by the group of Professor Vittoria Colotta at the University of Florence. The new derivatives were obtained by a molecular hybridization approach that was mentioned above as "designing in", allowing to obtain new chemical entities by combining two or more pharmacophoric units from different bioactive compounds into a single molecule (Ivasiv et al., 2019). NPD compounds were used: NPD151 is a selective A_{2A}AR antagonist, while NPD168, NPD149 and NPD133 are mixed multi-target compounds formed by an A_{2A}AR antagonistic component (triazolopyridine) (Falsini et al., 2019), conjugated to an antioxidant molecule, either edaravon (EDA), 2-oxothiazolidin-4-carboxylic acid (OTC) or α-Lipoic acid (ALA), respectively. Moreover, in this Thesis was tested the new mixed A_{2A}-A_{2B}AR antagonist (P626), designed by molecular hybridization. It was synthesized by the research group of Pharmaceutical Chemists of the Department of Neurofarba, at the University of Florence in collaboration with other universities. In the study, conducted by Professor Varano et al. (2020), the core of thiazole [5,4-d] pyrimidine with antagonist/inverse agonist activity for the A_{2A}AR was combined in the same molecule with the benzene-sulfonamide group, characteristic of CD73 inhibitors (an ectonucleotidase involved in the conversion of ATP into adenosine following abnormal accumulation of ATP). The SO2NH2 fraction appears to interact with a Zn2+ ion present in the catalytic site of the enzyme, thus strengthening the inhibitory activity of the compounds. Starting from this principle, different molecules were synthesized with different length and flexibility of the linker and to amplify the structure/activity relationship, molecules bearing a saccarinyl substituent in position 5 were synthesized, others containing a group of ethyl benzoate. The newly synthesized thiazolopyrimidines were evaluated both for their affinity for the A_{2A}AR expressed in CHO cells and for their inhibitory activity on human CD73 recombinants. Among the molecules obtained with this hybridization technique, the molecule named P626 (compound 2 in Varano et al., 2020) not only showed an interesting dual profile with A_{2A}-A_{2B}AR antagonist/reverse agonist activity, but also was also highly selective for the adenosine receptor subtypes A_1 and A_3. On the contrary, this molecule does not seem to exert a high inhibitory action for CD73.

Recently, a potent multi-target A_{2A}-A_{2B}ARs agonist (C2-substituted compound, MRS3997) has been developed by Professor Kennet Jacobson's group, from the Institute of National Health (NIH) of Washington. MRS3997 has a high potency for both A_{2A}AR and A_{2B}AR, with an EC50 of 39.7 (A_{2A}) and 109 (A_{2B}) nM for the stimulation of adenyl cyclase in CHO cells transfected with human receptors (Gao et al., 2014). It has the advantageous to act as a full agonist on A_{2B}AR, differently from BAY60-6583 that is a partial A_{2B}AR agonist (Hinz et al., 2014), thus offering the possibility of a correct interpretation of results.

2 Neurotransmission and synaptic plasticity

The essential mechanism that facilitates communication between neurons and their targets is called neurotransmission. It controls the CNS excitatory and inhibitory activities, supports sensory processing and controls autonomic and motor processes in a variety of animals. The synapse, a specially designed area between neurons and their targets, is where neurotransmission takes place. It is an extremely specialized contact between a presynaptic and a postsynaptic cell, which is implicated in encoding the information. At nerve terminals, synaptic transmission consists in repeated cycles of synaptic vesicle (SV) release by exocytosis followed by neurotransmitter (NT) reuptake by SVs endocytosis or selective transport. At these synapses, voltage-gated Ca^{2+} channels are opened by an action potential (AP) that is generated close to the cell body and propagates down the axon. In particular, SVs that are part of the reserve pool are bound to the actin filaments of the cytoskeleton by synapsin, while those that can be readily released are anchored directly to the presynaptic membrane by synaptotagmin. The entry of Ca^{2+} into the synaptic terminal determines the activation of synaptotagmin with consequent fusion of the vesicles to the presynaptic membrane and exocytosis of the vesicular contents. Furthermore, Ca^{2+} activates the Ca^{2+} -calmodulin-dependent protein kinase that phosphorylates the synapsin causing the release of the vesicles from the filaments of the cytoskeleton with consequent anchoring to the presynaptic membrane. Each vesicle contains a "quantal" neurotransmitter amount; so, the postsynaptic response produced by the release of a single vesicle, or quantal response, is relatively uniform (Fatt and Katz, 1952) and quantifiable for each NT.

Once the neurotransmitter has been released, it is finally recognized by receptors on the postsynaptic cell. As mentioned above, almost all types of synapses are modulated by a number of transient or permanent processes, some of which reduce synaptic strength and others that increase it.

It has been known for sixty years that synapse strength, or their efficacy in carrying the information, is not constant but may change during different activities. The ability to be modified indicates that the brain is plastic, i.e. it is programmable. The phenomenon by which the nervous system adapts to external stimuli is called synaptic plasticity and it is mediated through changes in the number and/or strength of synapses. It is fundamental in a vast range of events such as the development of the nervous system, the processes of memory and learning, and the ability to modify behavior in relation to environmental stimuli. Moreover, altered synaptic plasticity is involved in pathologies affecting the nervous system, such as epilepsy, and its recovery is associated with remission of neurological symptoms.

2.1 Facilitation and depression of synaptic transmission

There are numerous mechanisms responsible for an increase or depression of neurotransmission. Focusing on the main ones, synaptic facilitation has to be surely mentioned. It is a process of short-term synaptic enhancement, which can last from a few milliseconds (msec) to several minutes (min). Synaptic facilitation was observed in various organisms, from the simplest invertebrates to mammals. Indeed, it appears that facilitation is a short-term adaptation to sensory inputs and transient changes in behavioral states. This phenomenon occurs when, by providing two stimuli after a certain time interval Δt, the second excitatory post-synaptic potential (EPSP) recorded has an intensity up to five times greater than the first (Figure 4).

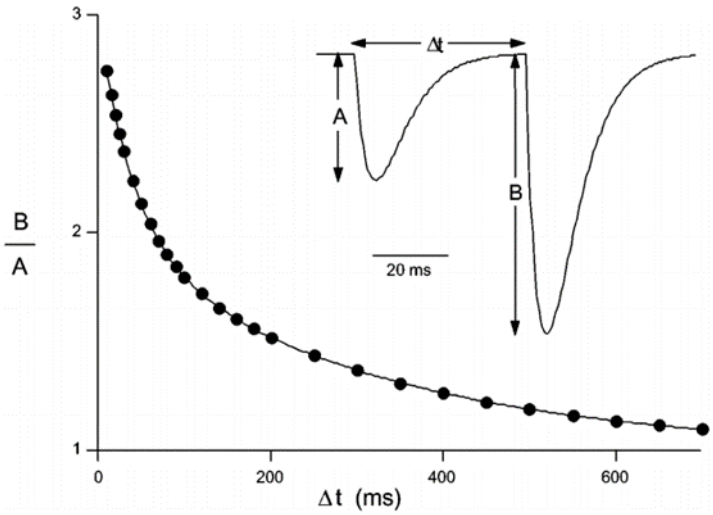


Figure 4. ***Schematic protocol of short-them synaptic plasticity enhancement.*** As shown in the inset, activation with pairs of stimuli separated by time Δt evokes synaptic currents with the second response (B) larger than the first (A). As shown in the plot of B/A versus Δt, the magnitude of facilitation decreases as the interpulse interval is increased. (Taken from: Zucker and Regehr, 2002).

Another process that intensifies synaptic transmission is the Post-Tetanic Potentiation (PTP) that gradually increased in relation to the number of stimuli in a train. Since this process takes place over a period ranging from five seconds to several min, each AP increases synaptic strength, on average, by 1–15% but the cumulative effect of a train of hundreds of pulses can result in a multifold improvement. On the basis of its duration, which lasts for 30 seconds to several minutes as mentioned above, PTP can be separated from processes like augmentation, which grows and decays within a time span of 5–10 seconds.

The molecular mechanism responsible for synaptic facilitation is based on the "residual Ca^{2+}" hypothesis of Katz and Miledi (1968), in which a transient accumulation of Ca^{2+} occurs in the presynaptic nerve terminal between the two stimuli. A small fraction of these ions, which enter the synaptic terminal as a consequence of the first AP, accumulates for several hundred msec before they can be "cleaned up" by the various Ca^{2+}-reuptake mechanisms inside the cell. Although this residual number of Ca^{2+} ions is not sufficient to trigger the release of the neurotransmitter, it nevertheless significantly adds to the Ca^{2+} that enters the terminal during the second AP. This event causes a significant increase in the probability of SV fusion to the plasma membrane compared to that observed during the first AP.

Beyond facilitation, the accumulation of Ca^{2+} at presynaptic level is indeed involved in various forms of short-term plasticity (Katz and Miledi, 1968; Zucker and Stockbridge, 1983), such as augmentation (Zengel et al., 1980), PTP (Delaney et al., 1989), and recovery from presynaptic depression (Dittman and Regehr, 1998).

Another evidence of Ca^{2+} involvement in synaptic enhancement is the fact that the selective block of Ca^{2+} channels by toxins or divalent ions alters the presynaptic response. In studies on synaptic plasticity, it has been demonstrated that a reduced Ca^{2+} influx decreases synaptic facilitation and/or augmentation (Wang and Quastel, 1991; Doussau et al., 1998).

The role of retrograde messengers by which the postsynaptic cell influences the release of vesicles by the presynaptic terminal is increasingly emerging in the mechanisms of synaptic plasticity. Indeed, different types of dendrites can release a variety of messengers that act by G protein-coupled receptors located on presynaptic terminal. Some retrograde messengers are released also by non-vesicular mechanisms: endogenous cannabinoids, such as anandamide and arachidonylglycerol (2-AG), are produced by the cleavage of phospholipids and are detected by CB1 receptors on the presynaptic terminal. Retrograde signalling of non-vesicular endogenous cannabinoid release has been shown to silence inhibitory synapses in the hippocampus and both excitatory and inhibitory synapses in the cerebellum (Wilson and Nicoll, 2001; Wang and Zucker, 2001). The vesicular and non-

vesicular release of retrograde messengers is however influenced by the increase in intracellular Ca^{2+} levels.

Many presynaptic terminals express ionotropic receptors, which also may contribute to short-term synaptic plasticity (MacDermott et al., 1999). These receptors include Ca^{2+} permeable receptors, such as NMDA and α7-nicotinic receptors, as well as Ca^{2+} impermeable receptors, such as GABAA receptors and the glycine receptor, coupled to Cl^- permeable channels. The activation of presynaptic ionotropic receptors can increase or decrease the release of neurotransmitters by various mechanisms.

At the level of CA3 pyramidal muscoid fibers of the hippocampus, glutamate release can contribute to the opening of kainate autoreceptors located on the presynaptic terminal, leading to synaptic enhancement (Schmitz et al., 2001). However, these results are difficult to interpret since excessive levels of kainate may instead contribute to synaptic depression due to excitotoxicity (Schmitz et al., 2000). The block of these autoreceptors, as well as their genetic deletion, reduces the extent of synaptic enhancement during a train of stimuli.

The synaptic depression is commonly observed in synapses receiving low intensity stimuli at short intervals (Δt = 40 ms). This phenomenon probably depends on the inactivation of voltage-dependent Na^+ channels and some subtypes of Ca^{2+} channels, or on a transient depletion of the vesicle pool. Specifically, periods of increased activity frequently result in a loss of synaptic strength and, as mentioned, this can be attributed to several causes. The most prevalent mechanism seems to be a presynaptic reduction in neurotransmitter release, which is probably due to the depletion of a pool of vesicles that are ready for release. Additionally, the activation of presynaptic terminals, postsynaptic cells, or nearby cells may release modulatory substances, resulting in a reduction of synaptic strength. Finally, the target neuron may become less sensitive to neurotransmitter due to postsynaptic characteristics, such as desensitization of ligand-gated ion channels (Zucker and Regehr, 2002).

2.1.1 Paired-pulse facilitation

Paired-pulse facilitation (PPF), and its counterpart Paired-Pulse Depression (PPD), are considered the simplest forms of synaptic plasticity. These types of short-term plasticity are defined when two stimuli are delivered within a short interval and the response to the second stimulus can be either enhanced (PPF) or depressed (PPD), relative to the time interval from the first stimulus (Katz and Miledi, 1968; Zucker and Regehr, 2002). Many synapses frequently exhibit PPD at short (less than 20 ms) inter-stimulus intervals, which most likely results from the inactivation of voltage-dependent calcium channels or from a temporary depletion of the pool of vesicles docked at the presynaptic terminal that are ready for release. At variance, longer inter-stimulus durations (20-500 msec) cause PPF at many synapses, for example in the hippocampus; inter-stimulus intervals of about 40-50 msec are known to maximize PPF, while facilitation declines over a period of 500 msec. It is possible that other mechanisms concur to the "residual Ca^{2+} "hypothesis in order to explain PPF: the calcium left over from the first action potential leads to increased SV release during the second stimulation and probably protein kinases that control the activity of presynaptic phosphoproteins may be activated in these processes.

It was established that synapses with a very high "basal" NT release have a tendency to suppress their response to the second pulse because of saturation of the response (Dobrunz and Stevens, 1997). Instead, synapses with a low basal NT release, when exposed to the second stimulus, typically show an increased release. In line with this theory, when the activation of presynaptic inhibitory (i.e. Gi coupled) autoreceptors and other events that reduce NT release, are "basically" involved we usually observe a remarkable PPF or even a change from PPD to facilitation.

As mentioned above, PPF is a phenomenon closely dependent on the presynaptic modulation of NT release (Regehr, 2012). Indeed, the assumption of PPF is that neuromodulators, or drugs that inhibit the release of excitatory neurotransmitters, cause an increase in PPF. Consequently, substances that stimulate presynaptic release cause a reduction of this phenomenon (Regehr, 2012).

Several studies on short-term plasticity have been conducted in the hippocampus, where the extracellular recording technique is used to measure the synaptic activity

in neuronal population after stimulation of a large number of axons. The limit of this technique is that the variation in the amplitude of the single post-synaptic currents (EPSC) in response to changes in quantal contents are difficult to record as they are dependentent on the NT release from many pre-synaptic fibers. Techniques of "minimal extracellular stimulation" have been developed to activate the release from a single axon for "in depth" studies on the cellular basis of PPF or PPD (Hess et al., 1987). However, this technique does not allow appreciating whether the lack of presynaptic NT release is due to phenomena of plasticity or to the inability of the stimulus to trigger an AP in the axon. Finally, another common technique is the recording of currents from pairs of monosynaptically connected neurons, which allowed to discriminate that, in monosynaptically connected hippocampal CA1-CA3 pyramidal cells, the amplitude of the second EPSC recorded depends on the amplitude of the first one (Debanne et al., 1996).

2.2 Role of adenosine receptors in neurotransmission

The role of adenosine as a neuromodulator has been postulated from '70. Indeed, many P1-mediated modulatory actions on presynaptic neurotransmitter release or postsynaptic neuronal excitability have been described. Under physiological conditions, adenosine exerts a tonic inhibition of synaptic transmission both *ex-vivo* and *in vivo* by stimulating A_1AR, as demonstrated in several brain regions such as the hippocampus, striatum and olfactory cortex (Latini and Pedata, 2001; von Lubitz, 1999). In particular, these mechanisms have been studied in brain regions with a high A_1AR expression, such as the hippocampus. The inhibitory effect of A_1AR stimulation has a pre- and postsynaptic component. The activation of presynaptic A_1ARs reduces Ca^{2+} influx through the preferential inhibition of N-type and, probably, Q-type channels (Wu and Saggau, 1994; Yawo and Chuhma, 1993). The inhibition of presynaptic calcium currents decreases transmitter release (Prince and Stevens, 1992) and adenosine, by stimulation of A_1ARs, has been found to inhibit the release of many classical neurotransmitters: glutamate, acetylcholine, dopamine, noradrenaline and serotonin (Fredholm and Dunwiddie, 1988). In particular, a powerful suppression of glutamate release from presynaptic terminals has been described in the hippocampus (Burke and Nadler, 1988; Corradetti et al., 1984),

where adenosine A_1AR activation reduces the number of quanta released in the Schaffer collateral-commissural pathway (Lupica et al., 1992). The postsynaptic effect of A_1AR activation consists in a direct hyperpolarization of neurons via activation of GIRK channels (G protein-coupled inwardly-rectifying potassium channels) at the postsynaptic site (Takigawa and Alzheimer, 2002, 1999). It is demonstrated that the exogenous application of the selective A_1ARs antagonist DPCPX causes a 15% increase of synaptic potential amplitude in *ex-vivo* brain slices (Latini et al., 1999b). This is an expected result in a brain region where extracellular adenosine concentration is found to be around 10-50 nM (Latini et al., 1999b; Latini et al., 1998) and A_1ARs, whose affinity for adenosine is in the low nanomolar range, are highly expressed. These data are confirmed by the fact that, in slices taken from homozygous A_1ARs knockout mice, no evidence is found for an endogenous inhibitory action of adenosine in the Schaffer collateral pathway in the CA1 region of the hippocampus or at the mossy fibre synapses in the CA3 region (Moore et al., 2003). In addition, no inhibition of synaptic transmission is elicited by the application of exogenous adenosine (Johansson et al., 2001).

At variance from A_1AR -mediated synaptic inhibition are the effects elicited by A_{2A}ARs activation that has been shown to mediate excitatory actions in the nervous system (Latini et al., 1996; Pedata et al., 1984; Sebastião and Ribeiro, 1996; Spignoli et al., 1984). Electrophysiological investigations of the role of A_{2A}ARs in synaptic function under physiological conditions have shown that they increase synaptic neurotransmission. Indeed, experimental evidence demonstrated that A_{2A}AR stimulation leads to an increase in long-term potentiation (LTP), a phenomenon associated to learning and memory; on the contrary, a reduction of LTP was observed in A_{2A}ARs knock-out mice in the nucleus accumbens (D'Alcantara et al., 2001). Indeed, the A_{2A}ARs stimulation in the hippocampus results in a Ca^{2+}-dependent release of acetylcholine (Cunha et al., 1995; Pedata et al., 1984). Furthermore, the application of the selective A_{2A}AR agonist CGS21680 decreases the ability of A_1AR agonists to inhibit excitatory neurotransmission (Cunha et al., 1994; O'Kane and Stone, 1998). This effect supports the hypothesis that A_{2A}AR stimulation increases synaptic transmission through A_1AR desensitization (Dixon et al., 1997). Alternatively, another current theory is that A_{2A}ARs increase excitatory amino acid release. Indeed, the

selective stimulation of adenosine A_{2A}AR enhances the amount of glutamate released in hippocampus and striatum of young rats (Corsi et al., 1999; Popoli et al., 1995). In spite of the excitatory A_{2A}ARs role in neurotransmission, the net effect of adenosine is an inhibitory tonus on neurotransmission, in accordance with observations suggesting that the activation of A_{2A}ARs requires a protracted stimulation to induce evident effects on synaptic transmission (Latini et al., 1999a). It is interesting to note that the role of A_{2A}ARs in the striatum has gained attention in the field of Parkinson's disease (PD) because their heterodimerization with D2 dopamine receptors may modulate the activity of the latter. Indeed, the interaction between A_{2A}ARs and D2 receptors results in an antagonistic action of one receptor subtype on the other, an effect which provided a rationale for evaluating A_{2A}AR-selective antagonists to counteract PD's symptoms. This hypothesis was further supported by epidemiological evidences indicating an inverse relationship between caffeine consumption and the risk of developing this pathology (Ascherio et al., 2001; Ross et al., 2000; Palacios et al., 2012; Shao et al., 2018).

Little is known about A_{2B}AR effects in the CNS, where they are uniformly, although scarcely, expressed. However, as they are coupled to Gs proteins, their actions are often similar to those elicited by the A_{2A}AR subtype. Indeed, Cunha and co-workers demonstrated that the A_{2B}AR selective agonist BAY60-6583 attenuates the predominant A_1AR-mediated inhibitory control of synaptic transmission in the CA1 hippocampus (Goncalves et al., 2015). These data are consistent with the relatively abundant expression of A_{2B}ARs in hippocampal presynaptic sites, demonstrated by means of synaptosome preparation and reported by the same authors (Goncalves et al., 2015). The facilitatory effect of A_{2B}ARs on glutamatergic neurotransmission was confirmed by our group in acute hippocampal slices by using the electrophysiological protocol of paired pulse facilitation (PPF) (Fusco et al., 2019).

In literature, discrepancies about the role of A_3ARs in the CNS are present. An excitatory role of this receptor subtype has been supported by evidences 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 A_3ARs are provided by studies carried out by Pugliese et al. (2007). In the same brain area, A_3AR activation through

a selective agonist has been shown to antagonize the A_1ARs -mediated inhibition of excitatory neurotransmission (Dunwiddie et al., 1997). However, further electrophysiological studies refused this hypothesis, since several authors demonstrated that no significant interaction between A_1ARs and A_3ARs occurs in the rat cortex and hippocampus (Brand et al., 2001; Lopes et al., 2003). Brand et al. (2001), demonstrated that, in rat cortical neurons, the selective activation of this subtype is involved in the inhibition of excitatory neurotransmission, suggesting a synergic action with the inhibitory effect mediated by A_1ARs activation. Despite results obtained by A_3ARs stimulation, there are evidences that the selective block of these receptors does not affect neurotransmission in the CA1 region of the hippocampus under normoxic conditions, indicating that endogenous adenosine at physiological concentration does not exert tonic activation of A_3ARs (Dunwiddie et al., 1997; Pugliese et al., 2003).

We characterized the role of A_{2A}ARs and A_{2B}ARs in the CA1 region of rat hippocampus using for the first time multi-target ligands able to simultaneously activate or antagonize these receptors.

2.3 Hippocampus

The hippocampus is one of the most studied and characterized brain structures since 1957, the year of the H.M case study, who underwent the surgical removal of the hippocampus and surrounding brain structures to treat a severe form of epilepsy. Following this operation, the patient lost his ability to process new memories, irreparably compromising his memory storage (Knierim, 2015).

2.3.1 Anatomy and synaptic circuits

The hippocampus is an elongated anatomical structure situated in the medial temporal lobe; it is part of the Limbic System, which is mainly involved in processing emotions, learning and memory. The name Hippocampus derived from its shape close to that of a seahorse, organism belonging to the genus Hippocampus. In rodents, this structure is quite large, with a banana-shape structure, situated just beneath the neocortex, while in humans and other primates the hippocampus is positioned along the anterior-posterior plane of the brain (Figure 5).

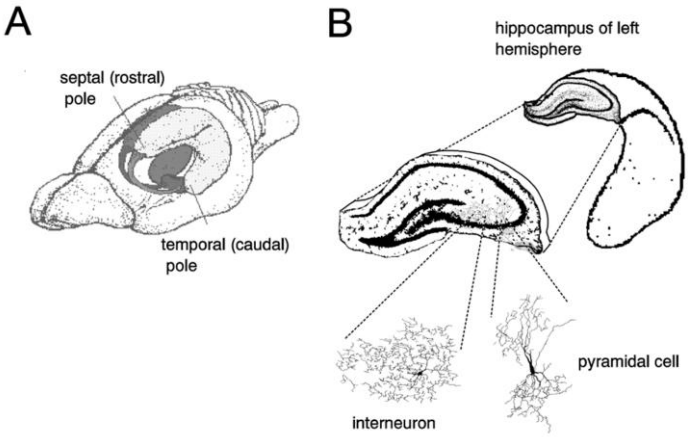


Figure 5. ***Location of hippocampus in the rat brain***. A) Schematic representation of hippocampus in the whole rat brain, excluding the thalamus and overlying neocortex. B) Isolated hippocampus and transverse sectioning of slice preparations. (Modified from: Lämsä, 2010).

The hippocampus is characterized by an easy identifiable trisynaptic circuits and its principal region, called *cornus ammonis*, (CA) is divided into CA1, CA2 and CA3. The other regions of the hippocampus are the dentate gyrus (DG), the subiculum and the presubiculum. The main afference to the hippocampus is the entorhinal cortex, which in rodents is divided into medial and lateral (Figure 6). Cells from the upper layers of the entorhinal cortex provide the greatest cortical input to the hippocampus via the perforant pathway that projects to the DG. Neurons in the DG, called granular cells, project to pyramidal cells in the CA3 region by the muscoid fibers; axons from the CA3 pyramidal neurons project to the CA1 region via the collateral-commissural Schaffer pathway. These CA1 pyramidal neurons constitute the main source of excitation of the subiculum. From this point, the organization of intrinsic connections becomes much more elaborate: CA1 projects not only to the subiculum, but also to the entorhinal cortex. In addition, although the subiculum projects to the presubiculum and the parasubiculum, its major cortical projection is directed to the entorhinal cortex completing the cycle. An important aspect, in addition to the classic trisynaptic circuit, is that CA3 axons, beyond their projections to CA1, project collateral fibers that create synapses on other CA3 neurons. The entorhinal cortex not only projects to the DG, but also to the CA3 and CA1 regions. CA3 provides a feedback projection to the DG, through the excitatory muscoid cells of the DG, thus overturning the previous notion that the processing of the hippocampus is exclusively unidirectional (Knierim, 2015).

In the CA1 and CA3 regions, bodies of pyramidal neurons are situated in a simple structure of continuous layers called the pyramidal cell layer and they use L-glutamate as their principal neurotransmitter. Pyramidal cells have two dendritic ramifications that emerge from opposite sides of the cell body: the basal dendrites originate from the side that gives rise to the axon, while the apical dendrites derive from the opposite side of the cell body. The excitatory input that reaches CA1 pyramidal neurons originates from the CA3 region. About 5000 axons from CA3 pyramidal cells converge on a single CA1 cell. The connections formed by the Schaffer collateral fibers are called *en passant* synapses because the CA3 axons continue along the *stratum radiatum*, making contact with the dendrites of many other CA1 pyramidal cells.

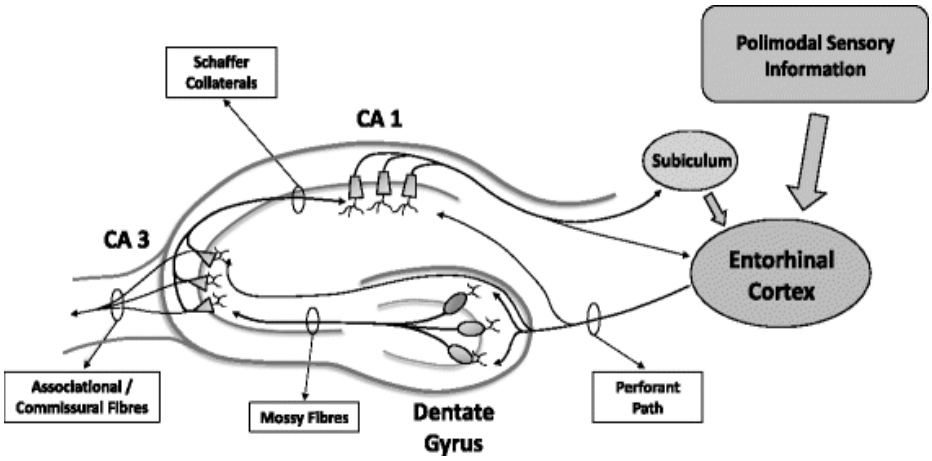


Figure 6. ***Schematic synaptic circuit of hippocampal slice.*** The black lines represent the classic continuous "trisynaptic" circuit. (Modified from: Lämsä, 2010).

The hippocampus is a brain area particularly susceptible to several insults such as epilepsy, hypoxia, ischemia, or encephalitis. Moreover, entorhinal cortex is usually the first area of the brain showing the typical depositions of the Alzheimer's disease. Furthermore, hippocampal damages are responsible of deficits in memory and learning processes. Indeed, one of the main functions of the hippocampus and the adjacent areas of the brain is to support the creation of new declarative memories, divided in episodic and semantic memory. Episodic memory refers to the memory of a specific event from the past, linked to a specific time and place; while semantic memory refers to own general knowledge of the world. Several studies have shown that the hippocampus is crucial for generating new episodic memories; while the

other regions of the medial temporal lobe are involved in the formulation of semantic memory. According to what mentioned above, some patients with hippocampal damage presented deficits in processing memories related to events in their daily life, but they seemed to have an intact semantic memory.

2.3.2 Hippocampal short-term plasticity

At least two types of short-term facilitation have been described in mammals at the hippocampal level: EPSP facilitation known as PPF (Fujita and Sakata, 1962; Bliss and Gardner-Medwin, 1973) and frequency-facilitation (Andersen and Lomo, 1967). The latter expects that, during the last repetitive response within a wide range of frequencies (approx. 1-30/s), the amplitudes of responses continue to increase during the initial part of the train and then remain facilitated for a certain period.

In the hippocampus, PPF has been studied mainly in the CA1 and CA3 areas (Creager et al., 1980; Manabe et al., 1993). At the level of the synapses between Schaffer's collateral fibers and the CA1 region, the reduction of PPF reflects an increase in glutamate release (Regher, 2012). Since, as mentioned above, the A_1ARs are widely expressed in the presynaptic terminals of the hippocampus, where they inhibit glutamate release, adenosine potently increases the PPF phenomenon through the activation of this receptor subtype (Fernandez-Fernandez et al., 2015).

On the contrary, it is known that the activation of A_{2A}ARs at the hippocampal level is associated to an increase in presynaptic glutamate release. Indeed, Lopes et al. (2002) studied the effects of the selective A_{2A}AR agonist, CGS21680, on synaptic transmission and PPF in rat hippocampal slices. Their results demonstrated that CGS21680 facilitates hippocampal synaptic transmission and reduces PPF ratio indicating an increase in glutamate release (Lopes et al., 2002) (Figure 7).

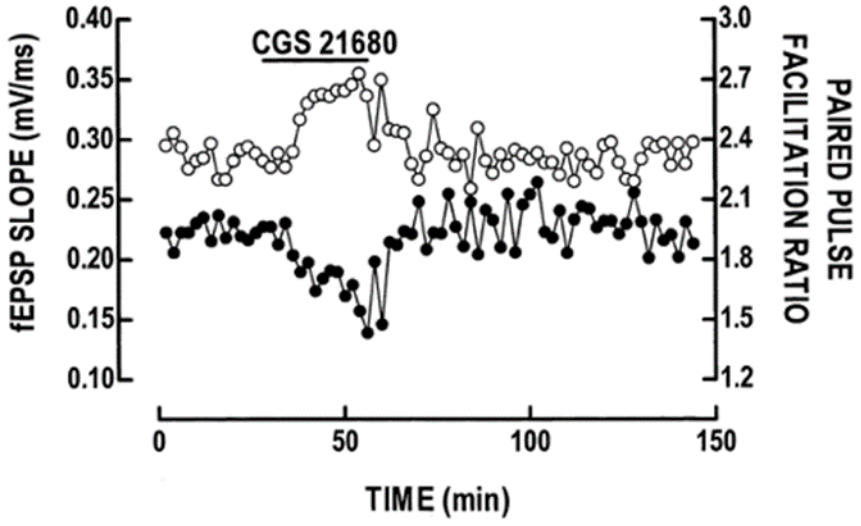


Figure 7. ***Effects of CGS21680 on basal synaptic transmission and paired pulse facilitation recorded in rat hippocampal slices.*** The empty circles are the averages of the slope of eight consecutive field Excitatory Postsynaptic Potentials (fEPSPs) evoked by basal Shaffer collateral-commissural stimulation and recorded in the CA1 area of a hippocampal slice that was perfused with CGS21680, as indicated in the upper bar. The filled circles represent the average of eight PPF traces (50 ms interval), quantified as the P2 / P1 ratio. (Taken from: Lopes et al., 2002).

Similarly, the activation of hippocampal A_{2B}ARs also induces an increase in the glutamate release, as confirmed by the fact that the selective agonist of these receptors, BAY60-6583 (200 nM), inhibits PPF in the CA1 region of mouse hippocampus (Gonçalves et al., 2015). Our research group confirmed these results in the rat hippocampus (Figure 8) (Fusco et al., 2018). Of note, the effect of A_{2B}AR on PPF is sensitive not only to selective antagonists PSB603 or MRS1754 (figure 8B, the two central panels), but also to the selective A_1AR blocker, DPCPX (Gonçalves et al., 2015; Fusco et al., 2018), indicating that A_{2B}ARs inhibit PPF *via* the inhibition of the A_1AR subtype. In other words, the A_{2B}ARs selective activation reduces the predominant A_1AR-mediated inhibitory control of synaptic transmission in the CA1 hippocampus due to endogenous adenosine acting on this latter receptor subtype at basal conditions (Goncalves et al., 2015). These data are consistent with the expression of A_{2B}ARs in hippocampal presynaptic sites, demonstrated by means of synaptosome preparation, reported by the same authors (Goncalves et al., 2015)

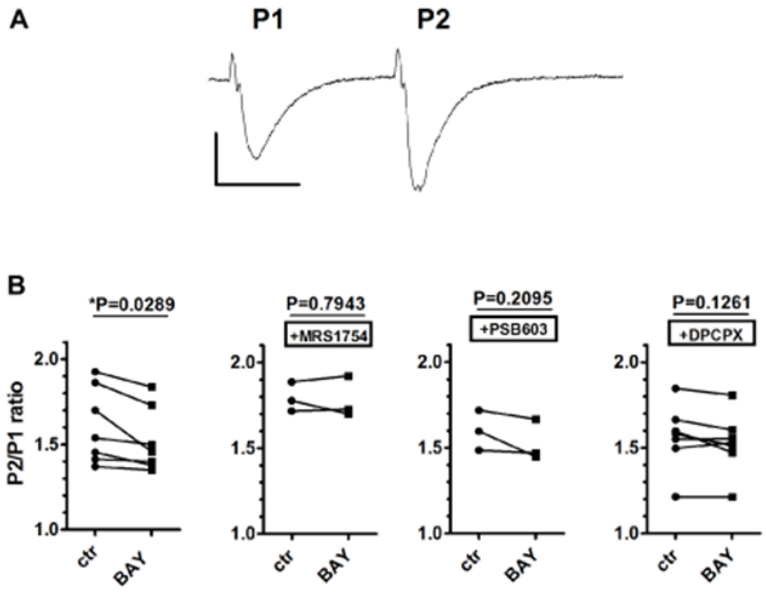


Figure 8. ***The selective stimulation of A_{2B}ARs reduced paired-pulse facilitation (PPF) in rat hippocampal slices.*** (A) Trace of fEPSP responses to PPF protocol (40-ms interval), recorded during a typical experiment. Calibration: 0.5 mV, 20 ms. (B) Each graph shows PPF quantified as the ratio (P2/P1) between the slope of the second fEPSP (P2) and the slope first fEPSP (P1). The effect of BAY60-6583 (BAY, 200 nM) on PPF was investigated in the absence (n = 7) or in the presence of MRS1754 (500 nM, n = 3) or PSB603 (50 nM, n = 3), selective A_{2B}AR antagonists, or in the presence of DPCPX (100 nM, n = 7), a selective A_1AR antagonists. (Taken from: Fusco et al., 2018).

Moreover, the effects of two newly synthesized A_{2B}AR agonists, P453 (50 nM) and P517 (500 nM) (Betti et al., 2018), were studied on PPF in the CA1 area of the rat hippocampus. Results obtained with these two newly synthetized compounds were superimposable to those observed in the presence of the prototypical A_{2B}AR agonist BAY60-6583 and confirmed that the activation of A_{2B}AR receptors reduces PPF, thus corroborating the facilitating role of A_{2B}AR stimulation in glutamate release at presynaptic level in this brain area (Fusco et al., 2019).

3 Cerebral ischemia

Stroke is the most common cerebral vascular disease and a leading cause of permanent disability and death worldwide today. The word “ischemia” derives from Greek “ισχαιμία” (“Reduction of blood”), meaning the total lack of blood flow in an organ. The traditional definition of stroke is based on the sudden onset of the loss of focal neurological function due to infarction or hemorrhage in the relevant portion of the brain. Ischemia is not a disease ascribable to a unique cause. There are many

factors that cause reduction or total arrest of blood flow: tachycardia, hypotension, extravascular compression, atherosclerosis, thromboembolism, gas embolism, vasoconstriction and adherent bridles, following surgery or chronic inflammation. Rarely, an infection can lead to strokes. Notably, an increased incidence of stroke has occurred with COVID-19 (Zhou et al., 2020a, 2020b). In addition, there are ischemic events defined cryptogenic, that seem to be without apparent cause, but normally at the bases of this condition, there are many others diseases, such as obesity, solid and blood tumors, myocardial infarction, anemia and granuloma. An unhealthy life style, such as smoking or consuming alcohol, considerably alter the predisposition to this pathological condition. Cerebral ischemia could be derived from a hemorrhagic or occlusive event, depending on the break of vessel and blood leak otherwise vessel occlusion due to presence of cellular material.

In 1970, cerebral ischemia has been defined by World Health Organization (WHO) as "Neurological focal or global cerebrovascular injury syndrome persisting beyond 24 h or leading to death within 24 h" (24 h were chosen arbitrarily for distinguish it from transient ischemic attack) (Figure 9). Thanks to this definition, it could be presumed that the nervous tissue subjected to ischemic damage, if reached in appropriate time, can recover their neuronal activity. For this reason, rapidity of intervention is indispensable and, given the high risk of death and disability which ischemia implicates, this disease is considered a medical emergency for which an immediate diagnosis is essential to be able to formulate an appropriate therapeutic intervention. Ischemia could involve both large and small vessels but the symptomatology is usually the same and consists of feeling oppression, asphyxiation, spasm, formication (paraesthesia), aphasia, dysarthria, hemiparesis, hemianopia and migraine. Medical treatments known for cerebral ischemia are unfortunately too few and many drugs that have good results in the preclinical studies, are ineffective in clinical trials (De Keyser et al., 1999). Now, the only drug approved to treat ischemia is tissue plasminogen activator (tPA), a specific enzyme that cleaves proteins which are present in the blood, such as fibrin, an essential protein in blood clotting, and thrombi. The main issue is that in order to perform such a vital function tPA must be necessarily administered in the first 3 h after the manifestation of the symptomatology.

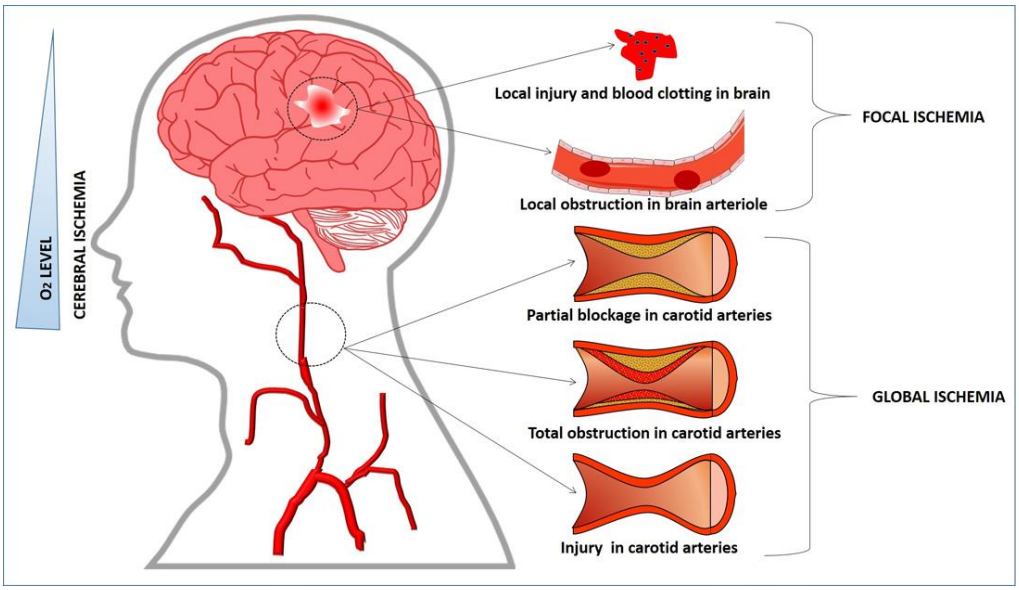


Figure 9. ***Pictorial representation of the types of cerebral ischemia.*** (Taken from: Sarkar et al., 2019).

3.1 Epidemiology

According to data of the Italian Ministry of Health, (www.ministerodellasalute.it) worldwide cerebral ischemia is at the second place among lethal diseases in the industrialized countries and it is the third cause of disability among the elderly. In Italy, cerebral ischemia is the third cause of death, after cardiac ischemia and neoplastic diseases: it causes 10-12% of annual deaths and it is the first ranked among invalidating diseases. Every year, 196.000 cases of stroke occur in Italy, 20% of which are recurrent. The remaining 80% are all new recordable cases. Between the 10 and 20% of people affect by cerebral ischemia die within a month and a 10% die within the first year. Hemorrhagic stroke mortality within the first month equals to 50%. Only 25% of patients surviving a stroke event recover completely, 75% survive with a certain form of disabilities, half of whom with a deficit as serious as they lost their selfsufficiency. The age in which cerebral stroke appears more frequently is around 55-60 years and its incidence increases with aging: young cases of cerebral stroke are much less frequent, about 7 cases/100.000/for year. The incidence rate among the elderly is around 6.5% and it mainly occurs in men than in women, 7.4% vs 5.9 respectively. It was evaluated that demographic evolution, characterized by sensible

aging of the population will lead to a significant increase in stroke cases in the next future, if the incidence should remain constant.

3.2 Neuropathology of cerebral ischemia

The ischemic process results from a sequence of physiopathologic effects (Figure 10) which progress drastically through time and space, leading to cells death and consequently to the subsequent decline of brain damage. The mechanisms that occur during cerebral ischemia, in addition to the reduction of cerebral blood flow, are hippocampal damage, white matter lesions, oxidative stress, excitotoxicity, inflammation, Ca^{2+}overload, cytotoxic oedema, a reduction of ATP, malfunctioning of Na^{+}/K^{+}-ATPase, neuronal cell death due to necrosis and apoptosis and the blood-brain barrier (BBB) disruption (Dirnagl et al., 1999).

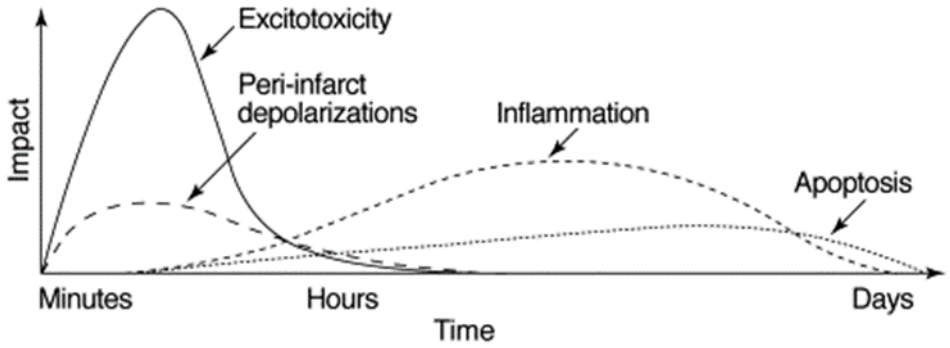


Figure 10. ***Representation of the principal phases of an ischemic insult***. Reported according to the time and intensity of the event (Taken from: Dirnagl et al., 1999).

3.2.1 Alterations of cellular homeostasis

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

Because of the reduction of cerebral blood flow and subsequent oxygen depletion during ischemia, mitochondrial oxidative phosphorylation is interrupted, thus resulting in a rapid decline in cellular level of ATP (Katsura et al., 1994; Martin et al., 1994; Rama & Garcia Rodriguez, 2012). The reduction of blood flow values caused by ischemia induce energy failure, thus an increase of extracellular potassium ions and increase of sodium ions ensue into cells with subsequent plasma membrane depolarization (Rama and Garcia Rodriguez, 2012). Furthermore, reduction in ATP level stimulates the glycolytic metabolism of residual glucose, which leads to protons and lactate accumulation and thus to rapid intracellular acidification and further reduction in cellular ATP production (Martin et al., 1994). Indeed, it has been observed that the lactate content during ischemia rises to ~20 mM (normal conditions ~15-20 mM), leading to a pronounced acidosis (Katsura et al., 1999). The accumulation of lactic acid in ischemic brain greatly disturbs the normal H^+ homeostasis, and it was suggested that excessive lactic acidosis facilitates ischemic mechanisms leading to cerebral infarction (Plum, 1983).

Several studies have underlined the importance of acidosis to the evolution of ischemic brain injury, since pH regulates several cellular processes (Busa and Nuccitelli, 1984) and modulates the activity of many enzymes and ion channels (Heales, 1999).

In the brain, pH is strictly regulated by active (ion pump transport) and passive (ion channel transport, intracellular buffer solution) mechanisms, which maintain brain pH at approximately 7.2 (Casey et al., 2010). The main mechanisms underlining pH regulation are summarized in Figure 11.

The ischemic insult causes phosphocreatine stores depletion, intracellular CO_2 accumulation and ATP and glucose reduction, leading to excessive production of H^+, and thus pH may fall to 6.5. Brain pH alteration after ischemic damage has been well documented by Back and co-workers in a model of permanent middle cerebral artery occlusion (MCAo), in which areas of acidification and alkalization can be detected in ischemic penumbra surrounding the markedly acid core (Back et al., 2000).

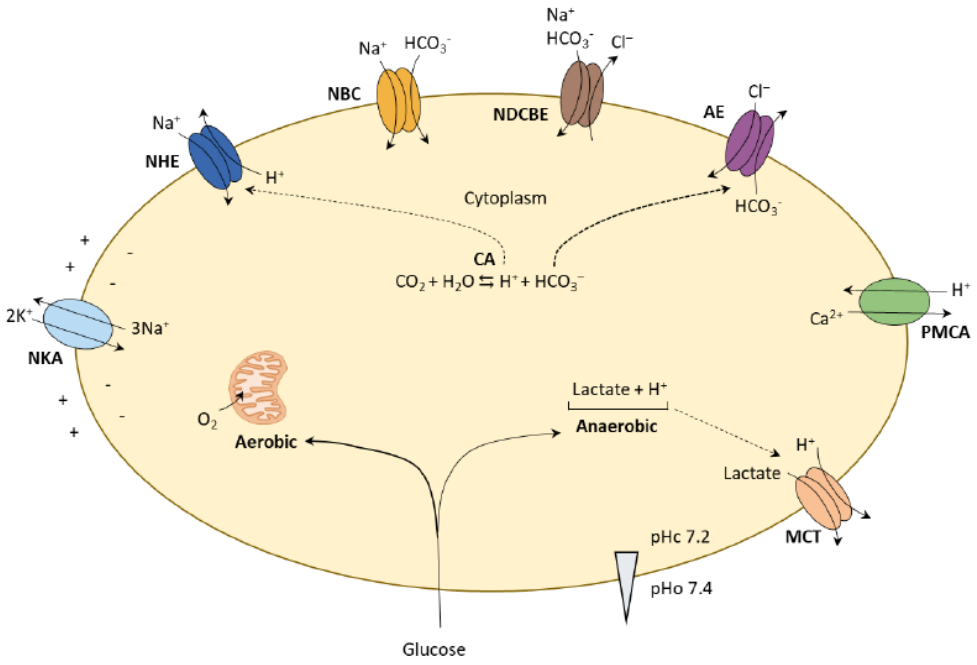


Figure 11. Cytoplasmatic pH regulation. The cytoplasm tends to acidify owing to the activities of various metabolic pathways, such as the ATP production in the cytoplasm by glycolysis that generates lactate (the anaerobic component in the figure) and the oxidative phosphorylation in mitochondria that produces CO_2 (the aerobic component shown in the figure). The predominant pH-regulatory transporters that are responsible for alkalinization are the plasma membrane Na^+ –H^+ exchangers (NHEs) and Na^+–HCO^{3-}– co-transporters (NBCs). A limited number of cell types also alkalinize their cytosol through the actions of Na^+-dependent Cl^-/HCO^{3-} – exchangers (NDCBEs). These transporters use the energy stored in the inwardly directed electrochemical Na+ gradient that is established by Na+–K+-ATPase pumps (NKAs) to drive solute transport. The actions of plasma membrane Cl^-/HCO^3– or anion exchangers (AEs), which acidify the cell, counterbalance these alkalinizing mechanisms. Plasma membrane Ca^{2+}-ATPases (PMCAs), which exchange cytosolic Ca^{2+} for extracellular H^+, also acidify the cytosol in response to stimuli that elevate intracellular Ca^{2+}. In tissues undergoing anaerobic metabolism, monocarboxylate–H^+ co-transporters (MCTs) favor alkalization. CA, carbonic anhydrase; pHc, cytosolic pH; pHo, extracellular pH. Modified from (Casey et al., 2010).

3.2.1.1 Role of carbonic anhydrase inhibitors in cerebral ischemia

The carbonic anhydrases (CAs) are a family of ubiquitous metalloenzymes present in most organisms all over the phylogenetic tree. To date, eight CA classes are known: α-, β-, γ-, δ-, ζ-, η-, θ-, and ι-CAs (Mishra et al., 2020), the last three recently discovered (Del Prete et al., 2014; Jensen et al., 2019; Kikutani et al., 2016). CAs present in animals belong to α-class (Aspatwar et al., 2014; Supuran, 2007), plants and algae have α-, β-, γ-, δ- θ- and ι-classes; fungi encode for α-and β-CAs; protozoa for α-, β- and/or η-CAs; bacteria for α-, β-, γ- and ι-CA classes (Capasso and Supuran,

2015). Vertebrates only encode α-CAs, and a large number of α-CA isoforms have been described: 15 in humans and other primates, and 16 in other mammals, with different catalytic activity, subcellular localization, tissue distribution and expression levels. α-CAs can be classified into four groups based on the subcellular localization: there are five cytosolic forms (CA I, CA II, CA III, CA VII and CA XIII), five membrane-bound enzymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial forms CA (Va and CA Vb), and a secreted CA isozymes (CA VI). Three cytosolic a-catalytic forms are also known, which are denominated CA related proteins (CARP): CARP VIII, CARP X, and CARP XI. The role of CAs is to catalyze the reversible hydratation of carbon dioxide into a bicarbonate ion and a proton ($CO_2+H_2O \leftrightarrows HCO_3^- + H^+$) by ensuing a two-step reaction process (except the three acatalytic isoforms). Hence, they play a pivotal role in pH regulation and several metabolic pathways such as lipogenesis, gluconeogenesis and urogenesis (Hewett-Emmett, 2000; Supuran and Scozzafava, 2002).

As stated above, the oxidative phosphorylation of glucose is impeded during hypoxic/ischemic situation, thus the anaerobic glycolytic route provides the majority of energy (Siesjö, 1981; Katsura et al., 1991). This pathway results in the accumulation of protons and lactate as well as the subsequent acidification of the surrounding environment. Indeed, min after a stroke begins, brain pH during cerebral ischemia drops from 7.2 to below 6.5 (Dirnagl et al., 1999; Melani et al., 2014). Studies conducted *ex-vivo* have revealed that neurons and glial cells exhibit a drop in pH in hypoxic/anoxic circumstances (Adams et al., 2007). By producing free radicals that affect glutamate reuptake, glial cell activation, and neuronal apoptosis (Chen et al., 2014; supuran et al., 2008), brain acidosis itself damages neurons and exacerbates ischemic brain injury (Neri et al., 2011; Mishra et al., 2020), which results in cerebral infarction symptoms like edema and blood-brain barrier (BBB) dysfunction (Obara et al., 2008; Wykoff et al., 2000).

Recently, Di Cesare Mannelli et al. (2016) evaluated the effect of several newly synthetized sulphonamide and coumarin CAIs in the permanent MCAo (pMCAo) model of cerebral ischemia in the rat. They found that repeated subcutaneous injections (5 and 20 min after surgery) of CAIs at the dose of 1 mg/kg were able to significantly reduce the neurological deficit 24 h after pMCAo, whereas the

prototypical CAI, ACTZ, 30 mg/kg subcutaneously was ineffective in reducing the neurological deficit. In addition, it has been reported that ACTZ at the dose of 100 mg/kg injected into the femoral vein 30 min after transient (1.5 h) MCAo, 22 h thereafter, reduced the infarct volume in male Wistar rats (Han et al., 2020).

Moreover, hypothesizing a potential CA significance in ischemia is made conceivable by the data that two CA isoforms, IX and XII, increase under hypoxic circumstances via the hypoxia inducible factor (Wykoff et al., 2000; Stiehl et al., 2006; Supuran, 2017; Ebbesen et al., 2009). Therefore, CA inhibitors (CAIs) may support pH homeostasis during brain ischemia by lowering hydrogen ion concentrations (Supuran et al., 2008; Supuran et al., 2018). However, CA inhibition has a variety of benefits that are not just connected to the diuretic action and may be helpful in brain pathologies.

3.2.2 Hippocampal damage

The hippocampus is one of the most vulnerable brain areas to an ipoxic-ischemic insult. During cerebral ischemia, the reduction of blood flow leads to neuronal damage, which starts with the CA1 pyramidal neurons of the hippocampus and is followed by dorsoventral striatal neurons and pyramidal neurons in the neocortex (Pappas et al., 1996; Farkas et al., 2007; Damodaran et al., 2014). Another event that occurs in the CA1 hippocampal region is the activation of microglia and astrocytes (Pappas et al., 1996; Farkas et al., 2004). Butler et al. (2002) used modified fluro-jade staining to study neurodegeneration in the hippocampus, following MCAo, after 6 and 12 h and 1, 4, 7, 14, and 21 days. Degeneration started 6 h after damage in the striatum, while, the hippocampus suffered neuronal degeneration between 12 h and 7 days after injury, with a peak in neuronal death at 4 days (Butler et al., 2002).

In 2008, Raval and his group studied the impact of cerebral ischemia in the striatum, the neocortex, and the hippocampal CA1 region, that are three cerebral areas particularly susceptible to an ischemic insult. They discovered that 4 min after cerebral ischemia was induced, neuronal death started. Ten min after the insult, over 80% of CA1 pyramidal neurons and neocortical neurons were vulnerable to damage. Moreover, more than 90% of CA1 neurons and 5–10% of neocortical neurons died after 15 min - ischemia, whereas after 20 min of an ischemic insult the 30% of

neocortical neurons and CA3 region neurons died (Raval et al., 2008). Moreover, in 2018, our research group studied the effect of an *ex-vivo* ischemic- insult on the CA1 rat hippocampal region. It was observed, by immunohistochemical analysis, that a severe oxygen and glucose deprivation significantly decreased the number of the CA1 neurons both 1 h and 3 h after the end of the insult. In particular, at the same time point, was observed that these hippocampal neurons expressed cytochrome C, an apoptotic marker (Fusco et al., 2018). Concerning the astrocytes, after 3 h of the end of the ischemic-like insult, was observed a strong astrogliosis in the CA1 hippocampal region, probably due to an increase in glutamate release (Fusco et al., 2018).

3.2.3 Oxidative stress

The oxidative stress is one of the main causes of neuronal damage after brain ischemia. Superoxide anions (O_2-), hydroxyl O radicals (-OH), and hydrogen peroxide (H_2O_2) are examples of free radicals that are produced in excess during an ischemic insult. Their high production causes lipid, protein and DNA dysfunctions that resulted in neuronal damage and death (Kim et al., 2015). They are not directly involved in cell damage, but these substances trigger other pathways involved in the development of brain oedema. One of the main causes of free-radical caused cell damage is lipidperoxidation that affects the lipid components of neuronal membranes. Lipoperoxidation induced an increase in inflammatory cells, inflammatory mediators, and matrix metalloproteinases (MMPs), that are involved in the pathogenesis of vasogenic brain oedema through increased vascular endothelial permeability and disruption of BBB, caused by degradation of the extracellular matrix in blood vessels (Sitrin et al., 2000; Marikky et al., 2003). It was also known that the early stages of chronic hypoperfusion also involve modifications in the antioxidant system. Therefore, an increase in ROS production contributes to a decrease in antioxidant enzymes in the brain.

3.2.3.1 Antioxidant and oxidative stress

As mentioned above, free radicals are important mediators of tissue injury in cerebral ischemia (Chan et al., 2001). Therefore, it is important to counteract this harmful effect by re-establishing the antioxidant enzyme system.

The free radical scavenger edaravon eliminates free radicals produced during ischemic reperfusion in various experimental models and has inhibitory effects on MMP-9 expression in the ischemic brain (Watanabe et al., 1988; Yamamoto et al., 1997; Abe et al., 1988; Nishi et al., 1989; Yagi et al., 2009). Edaravon is expected to alleviate neurotoxicity (Kelly et al., 2008; Montaner et al., 2001; Castellanos et al., 2003; Kim et al., 2006) and for this reason, it is a drug used in brain ischemia therapy. In a clinical trial performed by Isahaya et al. (2012) it was demonstrated that edaravon suppress circulating MMP-9 levels in patients with acute ischemic stroke (Isahaya et al., 2012). MMP-9 expression has been correlated with increased oxidative stress in the clinical study, and has been related to the size and severity of acute cerebral infarction, development of concomitant haemorrhagic infarction, and aggravation of symptoms (Isahaya et al., 2012). Moreover, Zhang et al., (2017) demonstrated that the activation of the ERK/Nrf2 signalling pathway was related to oxidative stress induced neuronal injury and apoptosis in the rat hippocampus of 2VO-induced cerebral hypoperfusion model (CCH). Treatment with edaravon activates the ERK signalling pathway and up regulates the Nrf2/HO-1 gene expression, thus attenuating the neuronal injury during CCH (Zhang et al., 2017).

Glutathione (GSH), also known as the thiol tripeptide γ-L-glutamyl-L-cysteinyl-glycine, is the most important antioxidant synthesized in cells. It acts as a major mechanism for intracellular antioxidant defence against oxidative damage and maintaining redox homeostasis (Forman et al., 2009). When GSH detoxifies free reactive oxygen species (ROS), GSH itself is converted to glutathione disulphide (GSSG) (oxidized GSH) (Mari et al., 2009). The depletion of GSH impairs mitochondrial ATP production and induces cell death signalling pathways (Redza-Dutordoir and Averill-Bates, 2016). Many studies have shown that glutathione antioxidant system plays an important role in cerebral ischemia (Schulz et al., 2000). GSH levels are decreased in diseases with oxidative stress, including stroke (Choi et al., 2000), and a low level of GSH in the brain

can increase the risk for stroke (Namba et al., 2001; Park et al., 2000). Ubiquilin-1 (Ubqln1), an ubiquitin-like protein, functions as an ubiquitin receptor that interacts with polyubiquitinated (polyUb) proteins and delivers them to the proteasome for degradation (Ko et al., 2004). It was observed that L-2-oxothiazolidine-4-carboxylic acid (OTC), a synthetic cysteine precursor, is a GSH booster and Ubqln1 inducer. It is also demonstrated that OTC has a neuroprotective role in both *ex-vivo* and *in vivo* models of ischemic stroke (Liu et al., 2020). OTC is a cyclic form of L-cystein that is a prodrug. OTC is transformed in cysteine by the intracellular ubiquitous enzyme 5-oxoprolinase (Kim et al., 2012). Cysteine is an antioxidant that inactivate hydroxyl radicals or maintain sulfidic groups free. Cysteine generates glutathione, so OTC supplies the cells of this important antioxidant. Some studies have demonstrated that OTC improve the capacity of dopaminergic agonists, such as pramipexol, to pass the BBB. In particular, given the easy oxidability of cysteine, these compounds have been derivatized with OTC and, thanks to the presence of this cysteine portion, the molecules can be recognized from transporters, like LAT1 and EAAT, reaching a better central bioavailability (Dholkawala et al., 2016).

3.2.4 Excitotoxicity

Cells consume a substantial amount of oxygen and glucose, through catabolic processes to obtain the energy necessary for survival in the form of ATP. At the moment of the reduction of physiological concentration of oxygen and glucose, that occurs during an ischemic insult, the cell necessarily looks for stock of energy due to the fact that it cannot produce energy by itself, and finds it in ATP. Given the disequilibrium between energy consumption and production, the cell is forced to initiate the anaerobic metabolism that causes ATP depletion, transformation to AMP and the consecutive extracellular accumulation of adenosine. ATP has essential functions at intracellular level, of which is fundamental the operation of ATP-dependent pumps, such as the Na^+/K^+ which allows the active transport of Na_+ and K^+ through the plasma membrane. The decrease in ATP causes a block of the pump and consecutively a block of ions transport: thus, Na^+ accumulates in the intracellular side. This ionic distribution, strongly unbalanced, provokes a vast depolarization at cellular level, making the membrane potential more positive and causing the opening

of other ion channels, such as the Ca^{2+}channel. Ione calcium, once inside the cell, contributes to neurotransmitter release through the process of vesicle fusion, mediated by the proteins of SNARE complex. The most extensively released neurotransmitter in the ischemic process is glutamate, an amino acid and excitatory neurotransmitter that, if released in high concentration, leads to cellular toxicity. Glutamate, at postsynaptic level, activates AMPA, NMDA and Kainate receptors that increase the intracellular concentrations of Na^+, K^+ and $Ca2^+$, which further depolarize the cell membrane. The resulting cerebral oedema is caused by the increase of intracellular ionic concentration, which hence recalls water from outside. Moreover, the energy-dependent glutamate transporters are blocked and this contributes to the permanence of glutamate within inter-synaptic fissure and results in continuous receptors stimulation.

In addition, the intracellular messenger Ca^{2+} activates a cascade of events (such as the synthesis of nitric oxide, characteristic of inflammation) which leads to the onset of tissue damage (Beckman and Koppenol, 1996; Iadecola, 1997) and to the subsequent necrosis of the tissue (Dirnagl et al., 1999). Among the most remarkable events of ischemia, there are the activation of enzymes that break down the cytoskeleton protein (Furukawa et al., 1997), lipases activation, such as the phospholipase A2 (PLA2) and consequently activation of cyclooxygenase (COX2, involved in inflammation). It is possible to identify two main ischemic areas (Figure 12): the focal area which undergoes a severe reduction of cerebral blood flow (CBF) and where neurons are continuously exposed to depolarization is defined the "ischemic *core*" (Hossmann, 1994). This causes rapid necrosis due to cytoskeleton destruction or to proteolysis. The perifocal area, named ischemic penumbra or "penumbra", can maintain, in the first periods after the ischemic attack, the physiologic metabolism of the tissue thanks to the perfusion of collateral anastomotic vessels (Hossmann, 1994; Obrenovitch, 1995). Lacking a properly pharmacologic treatment or reperfusion, the ischemic penumbra may advance toward an infarcted condition caused by ischemia and apoptosis.

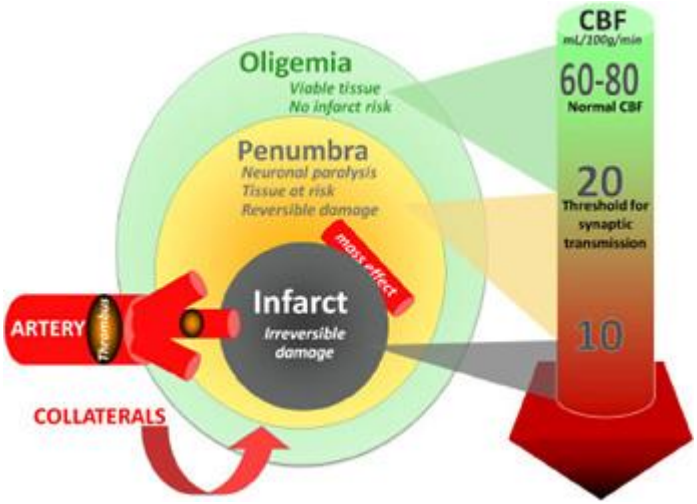


Figure 12. ***Pathophysiology of stroke***. The occlusion of the artery leads to hypoperfusion of the tissue segment supplied by that vessel. The affected parenchyma usually consists of a severely hypoperfused (CBF ≤ 10 ml/100 g/min) central infarct core where the damage is irreversible. It is bordered by the critically hypoperfused (CBF 10–20 ml/100 g/ min) ischemic penumbra (tissue-at-risk), where the injury may be reversed if timely reperfusion occurs. Collaterals aim at preserving as much penumbral tissue as possible. With time (min to h) the infarct core expands at the expense of the penumbra. This is also helped by its mass effect on the neighbouring arteries. The penumbra is surrounded by tissue, which is not at risk of infarction, the so-called 'benign oligemia' (CBF >20 ml/100 g/min) (Taken from: El-Koussy et al., 2014).

3.2.5 Anoxic Depolarization

A typical consequence of a cerebral hypoxic/ischemic insult is the appearance of anoxic depolarization (AD), a rapid and regenerative wave of depolarization that propagates in the brain tissue (Figure 13). A similar response occurs in cerebral grey matter under normoxic conditions, because of neuronal hyper-excitability (for example during epileptic discharges) and is called spreading depression (SD). These two events are strictly correlated and present the same diagnostic criterion: an accelerating, regenerative, all-or-none type depolarization spreading out from a restricted *core* of grey matter into the surrounding tissue (Somjen, 2001).

Aristides Leão, described SD for the first time in 1944, by recording a cortical electrogram (ECoG) of epileptic discharges obtained by "tetanic" stimulation in anesthetized rabbits. Leão (1947) noticed that, immediately after the induced seizure, an unexpected silencing of the ongoing normal electrical activity occurred, and hypothesised that SD and propagation of focal seizures were related phenomena generated by the same cellular elements. These first observations, confirmed also by Harreveld and Stamm (1953), have been later supported by detailed investigations

demonstrating that SD is caused by a “negative slow voltage variation” in the cortical surface that is identical to the voltage shift recorded after a few min of blood flow deprivation in the cerebral cortex (Leão, 1947). Similarly, severe hypoxia or generally sudden energy failure induce an SD-like response, and “spontaneous” waves of SD emanate from the border of an ischemic *core* and propagate into the surrounding brain areas (the “penumbra”).

To date, SD and AD have been recorded in almost all brain regions, with high similarity and reproducibility among *in vivo* and *ex-vivo* experiments, but they are more readily provoked in some brain areas than in others. The CA1 region of the hippocampal formation is considered the most susceptible zone, closely followed by the neocortex (Somjen, 2001). Under normoxic conditions, a number of different stimuli can trigger SD. Among the chemical agents, noteworthy are K^+ ions, glutamate, and acetylcholine, because these molecules are normally present in the brain, and ouabain, because it reflects the reduced function of the membrane Na^+/K^+-ATPase during high energy demand or low energy supply and ultimately raises extracellular K^+ concentration, which is known to play an important role in this phenomenon. When ion-selective microelectrodes became available in the ‘60s, allowing to measure “real time” ion concentration changes in live tissues, it was reported an overflow of K^+ ions from the cortical surface during both SD (Brinley et al., 1960) and AD (Vyskocil et al., 1972). A precipitous drop in extracellular Cl^- and Na^+ levels and a passive release of organic anions also accompanies this K^+ overload from the cell cytoplasm (Davies, 1995). This sequence of events suggests that during AD or SD, intra- and extracellular ion concentrations equilibrate and the electrochemical gradient across the cell membrane, which is essential for living functions, is lost. This hypothesis is also supported by the nearly complete depolarization that accompanies these events (Collewijn and Harreveld, 1966), typical of cells exposed to high extracellular K^+ concentrations as during SD or AD. Indeed, as suggested by Grafstein in the “potassium hypothesis” of SD (Grafstein, 1956), K^+ released during intense neuronal firing, accumulates in the restricted interstitial space of brain tissue and depolarizes the different cells that released it, resulting in a vicious circle that leads to neuronal hyper-excitability.

Progresses in describing SD- and AD-related phenomena were made by using intracellular sharp microelectrodes to measure resting membrane potential before, during and after the passage of SD or AD waves (Collewijn and Harreveld, 1966; Müller and Somjen, 2000). The “all-or-none” nature of both events was confirmed by the observation that once started, the amplitude of membrane depolarization was independent of the severity of the triggering event (Müller and Somjen, 2000). When membrane potential recordings are made simultaneously in stratum radiatum and in stratum pyramidale of CA1 hippocampal slices, cell depolarization always begin earlier. Furthermore, it has a larger amplitude and a longer duration in the dendritic layer than in cell somata, suggesting that the triggering region is the dendritic layer from which the depolarization spreads to the cell bodies (Herreras and Somjen, 1993a). A detailed analysis of the time course of different AD-related events in the hippocampus demonstrates that the onset of voltage changes is usually preceded by increased neuronal excitability that produces a “shower of population spikes” reflecting synchronized firing of pyramidal neurons (Grafstein, 1956; Herreras and Somjen, 1993b). This neuronal hyperactivity, also recorded during *in vivo* experiments (Rosenblueth and García Ramos, 1966), is strictly related to glutamate-induced excitotoxicity. Indeed, the voltage changes produced during AD or SD are greatly reduced by NMDA receptor antagonists (Herreras and Somjen, 1993a), that are well known neuroprotective agents against brain ischemia-induced (Calabresi et al., 2000; Lee et al., 1999). In addition, the exogenous application of glutamate in the cortical surface is able to induce SD (van Harreveld and schade, 1959).

Evidence indicates that a very reduced but substantial ion gradient remains across the plasma membrane during AD (Müller and Somjen, 2000). The massive redistribution of ions between the intra- and extracellular compartment during AD and SD leads to significant changes in cell homeostasis reflected by the prominent cell swelling that accompanies the phenomena. Leão (1951) firstly described a transient increase in tissue electrical impedance accompanying SD and others authors (Hoffman et al., 1973; Ochs and Hunt, 1960) after confirmed this. The major effect was the increase in tissue resistance, and was confirmed by morphological studies to be caused by swelling of cells leading to shrinkage of the interstitial space (Kow and van Harreveld, 1972; van Harreveld and schade, 1959). Another parameter

usually measured to monitor *ex-vivo* SD or AD is the intrinsic optical signal (IOS). Cell swelling produces a marked change in intracellular volume that is reliably associated with a decrease in light scattering (Aitken et al., 1999), attributable to the dilution of scattering particles in the cytosol (Barer, 1953). This sequence of events leads to a pronounced increase in the IOS of brain tissue, as reported for the first time by Snow et al. (1983) in hippocampal slices during SD. This new approach allows a detailed real-time two-dimensional mapping (not reachable with microelectrodes) of SD and AD spread, simultaneously recorded with other parameters (for example membrane potential or extracellular K^+ concentration), as exemplarily reported by Obeidat and Andrew (Obeidat and Andrew, 1998). Moreover, in hippocampal slices, the IOS triggered by AD or SD is stronger and occurres more rapidly in the dendritic layer of stratum radiatum than in the somatic region of stratum pyramidale (Aitken et al., 1998; Müller and Somjen, 1999).

In spite of the many similarities between SD and AD events, some differences have also been described. For example, depolarization in SD is "self-limiting" and is followed by complete restoration of neuronal functions as soon as neurons recover their resting membrane potential, without any "irreversible side effects" at least after small SD events. On the contrary, after an AD, membrane potential and neuronal functions only recover if oxygen is restored soon after the onset of depolarization (Lipton, 1999) since oxidative energy (O_2 and ATP consumption) is required to restore ion gradients (Wang et al., 2003). If the ischemic insult persists during AD manifestation, neuronal damage will become irreversible and only a partial recovery of brain tissue functionality can be achieved. This seems to be mainly due to the deleterious role of a protracted intracellular Ca^{2+} increase, an event typically recorded during ischemia. It may be argued that, during normoxic SD, neurons gain as much Ca^{2+} as during AD but do not encounter such irreversible damage because membrane depolarization is self-limiting and only lasts for a few seconds. Indeed, it has been demonstrated that if neurons are forced to remain depolarized for extended periods after SD triggering, even in well-oxygenated tissues they do not regain function (Herreras and Somjen, 1993b). Accordingly, if Ca^{2+} is removed from the bathing solution before oxygen is withdrawn, neurons recover their functions following a period of hypoxia that otherwise have caused irreversible damage (Siesjö

and Bengtsson, 1989). So that cell damage to be irreversible it is necessary that the intracellular Ca^{2+} levels remain elevated for a critical length of time (Deshpande et al., 1987; Morley et al., 1994). It follows that any treatment that postpones the onset of AD should extend the time limit of cell recovery. This concept has been largely confirmed by both *in vivo* and *ex-vivo* studies and represents the basis of the present work to investigate the effect of different drugs acting on purinergic systems during ischemia. On this basis, we monitored AD appearance in hippocampal slices as an index of irreversible tissue damage induced by *ex-vivo* ischemia and we investigated the role of purinergic receptors during a severe insult.

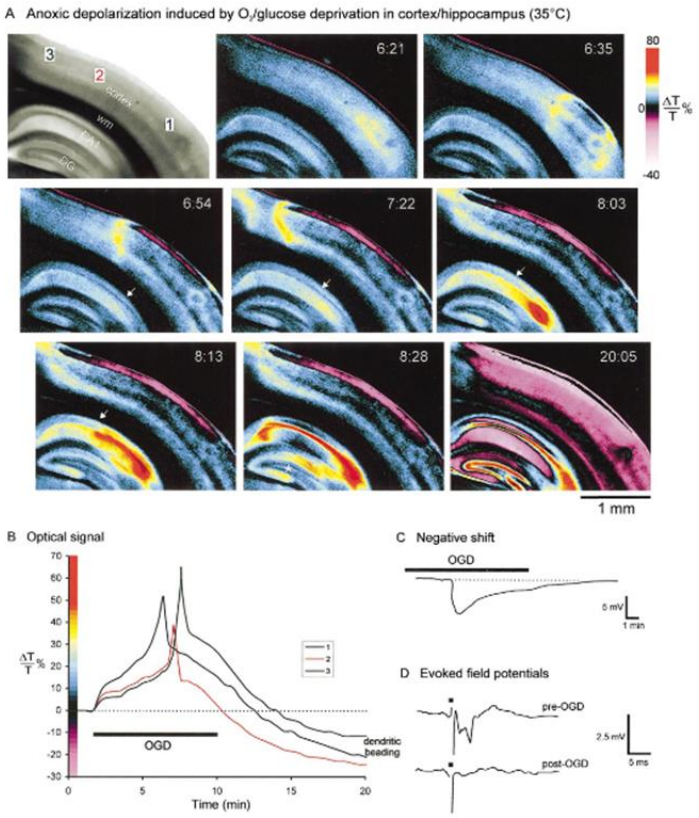


Figure 13. ***The anoxic depolarization (AD) induced by O2/glucose deprivation (OGD) for 8 min at 35°C a slice of the mouse neocortex/striatum***. A: the focal increase in light transmittance (LT) represents AD onset, first in layers II/III in the neocortex (5:55) and then in the striatum near anterior commissure (7:35). A wave of elevated LT (blue-yellow) propagates through the neocortex (5:55 to 6:45) and later through the striatum (7:35 to 8:25). Where the AD has passed, there is a delayed and irreversible decrease in LT (magenta pseudocoloring) representing dendritic beading. B: time course of LT changes in zones of interest in the neocortex (denoted as 1–3 in A). The initial LT peak represents the AD front passing through zone 1, followed by zones 2 and 3. By 12 min, all zones display an irreversible decrease in LT, representing damage. C: the evoked field potential, recorded prior to AD induced by OGD, is permanently lost following AD. D: extracellular field recordings in response to OGD. A negative shift recorded in the neocortex is followed by a second negative shift in the striatum 1.5 min later (Taken from: Joshi and Andrew, 2001).

3.3 Purines in cerebral ischemia

Many authors have indicated purines (ATP and adenosine) and purinergic receptors as significant targets for therapeutic implementation in the treatment of stroke. After an ischemic insult both extracellular ATP and adenosine are release in large quantities (Frenguelli et al., 2007). Juranyi et al. (1999) demonstrated for the first time the tritiated ATP release in rat hippocampus slices in response to short-term hypoxia and hypoglycemia. Moreover, the microdialysis method showed an increase in extracellular ATP from the striatum following *in vivo* ischemia (Melani et al., 2005). Concerning adenosine, an increase of its extracellular concentration during *in vivo* ischemia was demonstrated first by the cortical cup technique (Phillis et al., 1994a) and later on by the microdialysis technique (Dux et al., 1990; Hagberg et al., 1987; Matsumoto et al., 1992; Melani et al., 1999).

Therefore, in relation to the above-mentioned evidences and to numerous others, purinergic receptors are considered important targets in brain ischemia (Coppi et al., 2022; Khan et al., 2022; Zhang et al., 2022).

3.3.1 Adenosine receptors in cerebral ischemia

The extracellular adenosine concentration under physiological conditions, as mentioned above, is usually relatively constant in a range between 30-200 nM in brain tissue (Latini and Pedata, 2001). After ischemia, adenine nucleotides are released into the extracellular compartment and rapidly metabolized to adenosine by NTPDases and e5'-NTs ubiquitously expressed on the cell surface (Melani et al., 2012). Both *in vivo* and *ex-vivo* studies largely demonstrate that adenosine concentration dramatically increases during cerebral ischemia (Hagberg et al., 1987; Latini et al., 1999b; Melani et al., 2003, 1999; Pearson et al., 2006) reaching values in the order of micromolar (μM), able to activate all 4 subtypes of adenosine receptors. Initially, adenosine derives from extracellular ATP degradation (Melani et al., 2012); thereafter adenosine is released *di per sè* from cells. This is principally due to the rapid and massive depletion of intracellular ATP occurring under metabolic stress conditions, such as hypoxia or ischemia that leads to an accumulation of AMP, which in turn is degraded to adenosine. Thus, the rate of adenosine production exceeds its

deamination to inosine or its rephosphorylation to AMP (Deussen, 2000) leading to a concentration gradient from the intra- to the extracellular space that causes the neuromodulator release by the membrane transporter proteins (ENTs). For these reasons, extracellular adenosine concentrations greatly increase reaching micromolar values during hypoxic/ischemic conditions.

Different authors have long recognised a neuroprotective role of extracellular adenosine during cerebral ischemia. Indeed, it is largely known that adenosine-potentiating agents, which elevate endogenous adenosine by either inhibiting its metabolism (Lin and Phillis, 1992) or preventing its reuptake (Dux et al., 1990), offer protection against ischemic neuronal damage in different *in vivo* ischemic models. Furthermore, adenosine infusion into the ischemic striatum during MCAo significantly ameliorates the neurological outcome and reduces the infarct volume (Kitagawa et al., 2002).

Several protective effects of adenosine during cerebral ischemia are mainly due to A_1AR stimulation that causes a reduction of Ca^{2+} influx, thus inhibiting the presynaptic release of excitatory neurotransmitters. (Corradetti et al., 1984; Pedata et al., 1993; Zetterström and Fillenz, 1990). During this condition, the main NT released is glutamate, whose over-stimulation of NMDA receptors is one of the principal mechanisms of neuronal excitotoxicity (Choi, 1990). In addition, by directly increasing the K^+ and Cl^- ion conductances at postsynaptic level, adenosine stabilises the neuronal membrane potential and reduces the neuronal hyper-excitability caused by increased glutamate release during ischemia (Tominaga et al., 1992). The consequent reduction in cellular metabolism and energy consumption (Tominaga et al., 1992) and a moderate reduction of the body/brain temperature (Gourine et al., 2004) are protective events under ischemic conditions. Accordingly, *ex-vivo* models demonstrated that selective A_1AR stimulation reduces neuronal damage following hypoxia and/or glucose deprivation in primary cortical or hippocampal cell cultures (Daval and Nicolas, 1994) and brain slices (Dux et al., 1990; Marcoli et al., 2003; Mori et al., 1992; Newman et al., 1998). Although data converge in demonstrating a neuroprotective effect of adenosine through A_1AR during ischemia, the clinical utility of selective A_1AR agonists is hampered by side central and peripheral effects i.e. sedation, bradycardia, hypotension (White et al., 1996).

The few studies present in the literature concerning the role of A_3AR in cerebral ischemia are rather contradictory. The effects of A_3AR stimulation appear to depend on drug administration (acute vs chronic), dosage and timing of treatment with respect to the onset of the ischemic insult. It is demonstrated that selective antagonism of these receptors facilitates the recovery of synaptic activity in rat hippocampal slices (Pugliese et al., 2003). In particular, it has been observed that during oxygen and glucose deprivation (OGD) the block of A_3ARs abolishes or delays the AD and it is protective from the irreversible disruption of excitatory neurotransmission caused by a severe ischemic episode (Pugliese et al., 2006). A chronic pre-ischemic administration of A_3AR agonist protects against neuronal damage in ischemia (von Lubitz et al., 1994). This effect is dependent by the desensitization of A_3ARs; in human and rat there is a desensitization few min after agonist exposure (Palmer et al., 1995; Trincavelli et al., 2002). It is also demonstrated that in hypoxic conditions an activation of A_3ARs inhibits the excitatory neurotransmission: they have a neuroprotective action like ones of the A_1ARs (Hentschel et al., 2003). These opposite results can be reconciled: in the first phase of ischemia, A_3ARs play a protective role with adenosine A_1ARs, while after a severe ischemia transforms the A_3ARs effect to injurious (Pedata et al., 2007).

The role of A_{2A}AR and A_{2B}AR in cerebral ischemia has been recently studied and will be described in detail in the paragraphs below.

3.3.1.1 A_{2A}ARs in cerebral ischemia

It has been established that A_{2A}AR antagonists have a neuroprotective role in ischemia models. In 1994 it has been demonstrated, for the first time, that CGS15943, an A_{2A}AR antagonist, reduced cerebral ischemic injury in the gerbil hippocampus and neocortex (Gao and Phillis, 1994). Subsequently, many studies have confirmed this neuroprotective role in various animal models of ischemia. For example, SCH58261, another A_{2A}AR antagonist, reduced brain damage in neonatal and adult rat models of cerebral ischemia (Bona et al., 1997; Monopoli et al., 1998; Melani et al., 2003). These receptors are present not only in neurons but also on microglia and astrocytes (Fiebich et al., 1996; Saura et al., 2005), where they inhibit the glutamate uptake transporter and stimulate glutamate outflow (Nishizaki et al.,

2002). Hence, A_{2A}AR antagonists play a protective role in cerebral ischemia because they reduce glutamate outflow from neurons and glial cells and furthermore they inhibit the production of inflammatory molecules (Pedata et al., 2007). However, the agonism of A_{2A}ARs could result protective during ischemic conditons for its capacity to decrease, rather than increase, the GABA release, which may represent a compensatory mechanism to counteract the marked enhancement of GABA release induced by stroke (Rombo et al., 2016).

Moreover, as mentioned above, A_{2A}AR is up regulated during brain ischemia on neurons and microglia (Trincavelli et al., 2007) and accordingly to this, hippocampal injections of the A_{2A}AR antagonist ZM241385 reduce kainate-induced neuronal damage (Jones et al., 1998).

For the first time, our research group demonstrated that the selective block of A_{2A}AR by ZM241385 and SCH58261, is protective in a CA1 hippocampal model of OGD. These antagonists delayed AD appearance, reduced astrocyte activation and improved neuronal survival and recovery of synaptic activity under reperfusion with oxygenate glucose-containing artificial cerebrospinal fluid (aCSF) (Pugliese et al., 2009).

Of note, it is important to specify that differently to data that support that A_{2A}AR antagonists protect against central excitotoxicity, the protective effect of agonists appears attributable to different mechanisms. Indeed, in the traumatic brain injury (TBI) model, it was demonstrated that the administration of the selective A_{2A}AR agonist, CGS21680 (0.1 mg/kg), was protective 3 h after trauma when glutamate concentrations started to reduce. Furthermore, it was observed that higher dose of CGS21680, injected 5 min after a global ischemia in gerbil, presented a significant protection against neuronal loss, but was inefficacious at lower concentrations (Sheardown and Knutsen, 1996). Moreover, recent experiments have demonstrated that CGS21680, chronically administered (twice/day for 7 days) after transient MCAo, at doses of 0.01 and 0.1 mg/kg, protected from neurological deficit, weight loss, infarct volume, myelin disorganization, and glial activation at 7 days after ischemia (Melani et al., 2014). Two days after transient MCAo, CGS21680 has massively reduced blood cell infiltration in the ischemic areas (Melani et al., 2014). Unfortunately, the main problem to translate to the clinic the use of A_{2A}AR is their

cardiovascular effects. The stimulation of these receptors placed on blood vessels results in a vasodilatory action, which induces hypotension and raises heart.

3.3.1.2 A_{2B}ARs in cerebral ischemia

A_{2B}AR is the least studied and still remains the most enigmatic between adenosine receptors subtype, because of the relatively low potency of adenosine for this receptor (Fredholm et al., 2011) and because there are very few selective ligands that have been described so far. Most of the present knowledge on A_{2B}ARs originates from their peripheral role on the control of cardiac myocyte contractility, intestinal tone, asthma, inflammation, cancer and diabetes (Allard et al., 2017; Chandrasekera et al., 2010; Feoktistov et al., 1998; Kolachala et al., 2008). However, in the last years, the role of this receptor subtypes in the CNS and in particular, during pathological conditions such as brain ischemia has been started to study. Besides brain cells, A_{2B}AR are present on endothelial and blood immune cells and in most cases are coexpressed with A_{2A}ARS.

The inhibition of A_{2B}ARs may be neuroprotective as it prevents glutamate overload by maintaining the inhibitory action of A_1ARs on neurotransmission (Goncalves et al., 2015; Fusco et al., 2019; 2018). Indeed, this is the case demonstrated of our research group of an *ex-vivo* model of brain ischemia reproduced in rat hippocampus slices by OGD (Fusco et al., 2018, 2019).

Recently, we demonstrated that, hippocampal slices subjected to 7-min OGD and treated with prototypical A_{2B}AR antagonists PSB603 (50 nM) or MRS1754 (200 nM) presented a completely abrogation of AD, an unequivocal indicator of glutamate-induced excitotoxicity (Colotta et al., 2012), whereas slices subjected to 30-min OGD presented a considerable delay in AD appearance. These results were corroborated by immunohistochemical analysis, which demonstrated that the selective A_{2B}AR block also prevented the loss of neurons, observed 3 hours (h) after OGD insults in the CA1 stratum pyramidale and reduced astrogliosis in the CA1 stratum radiatum. Notably, neither of the A_{2B}AR antagonists tested protected CA1 neurons from the neurodegeneration due to the application of exogenous glutamate, indicating that the antagonistic effect occurred before glutamate is released. This conclusion is

consistent with data showing that A_{2B}AR have a presynaptic effect on glutamatergic terminals (Goncalves et al., 2015; Fusco et al., 2018, 2019).

Moreover, regarding the neuroprotection exerted by A_{2B}AR antagonists acting at the neuro-glial level, a short-term TNF-alpha treatment induces A_{2B}AR desensitization in human astroglial cells (Trincavelli et al., 2008). These results suggested that in the acute phase of brain ischemia that is characterized by both cytokine and adenosine high release, A_{2B}AR desensitization on astroglia might represents a cell defence mechanism (Trincavelli et al., 2008).

Few studies have investigated the role of A_{2B}AR in brain ischemia *in vivo*. In a recent paper, it was reported that a selective A_{2B}AR antagonist, MRS1754, reduced the ceramide production in astrocytes and attenuated inflammatory responses and neuronal damage after global cerebral ischemia (Gu et al., 2013). This effect was related to an early reduction of p38-MAPK activation. Indeed, A_{2B}AR plays a key role in the rapid activation of p38 and in the subsequent inflammatory process (Koscsó et al., 2011; Wei et al., 2013). All together, these experiments indicated that the antagonism of A_{2B}ARs might be protective in ischemic brain damage. The recent introduction of new pharmacological and genetic tools let to understand a role of A_{2B}AR in the regulation of inflammation, immunity and tissue repair (Crespo et al., 2013; Feoktistov and Biaggioni, 2011; Hinz et al., 2014; Ortore and Martinelli, 2010).

Our research team gave a significant contribution to the field by proving that chronic treatment with the selective A_{2B}AR agonist BAY60-6583, given intraperitoneally twice daily for seven days at a dose of 0.1 mg/kg, four h after focal ischemia caused by tMCAo and continuing until one day after ischemia, protected against neurological deficit (Dettori et al., 2020). It was interesting to note that the A_{2B}AR agonist increased IL-10 levels and reduced TNF- levels in the blood, 7 days after ischemia (Dettori et al., 2020). In particular, BAY60-6583 decrease TNF- expression in primary microglia cultures [78] and increased IL-10 production from murine microglial cells (Koscsó et al., 2011) with the resultant recovery of the resting state of microglia as one proposed mechanism mediating protective effects of A_{2B}AR agonists. Moreover, BAY60-6583 significantly reduced granulocyte infiltration in the cortex (Dettori et al., 2020) and supported the idea that A_{2B}AR activation on peripheral endothelial and blood cells is involved in counteracting inflammation of brain parenchyma.

Taken together, these data suggest that the stimulation of A_{2B}ARs plays a dual time-related role after ischemia. Indeed, in the first h after an ischemic adenosine released in large quantities is able to activate low affinity A_{2B}ARs in the brain and this could contribute to expand excitotoxicity. However, in the hours and days following the ischemic insult, A_{2B}ARs located on glial, vascular endothelial and blood cells exert a prevalent immunomodulatory role attenuating the neuroinflammation. Thus, it appears that A_{2B}AR, located on any cell type of the brain and on vascular and blood cells, could represent an important target for drugs with different therapeutic time-windows after stroke (Figure 14).

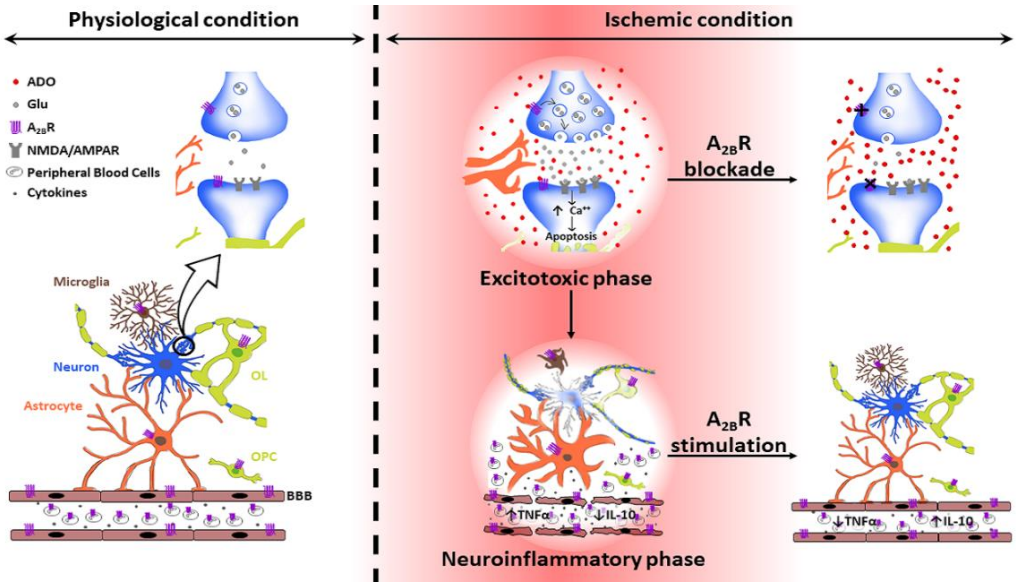


Figure 14. ***Effects of A2B adenosine receptors (A_{2B}R) during physiological or ischemic condition***. The block of this receptors subtypes results protective during the excitotoxic phase of an ischemic insult, while, during the neuroinflammatory phase is the activation of the A_{2B}Rs that exert neuroprotective effects. (Taken from: Coppi et al., 2020b).

A part of my Thesis was focused on the role of A_{2A}ARs and A_{2B}ARs during the application of a severe ischemic-insult in an *ex-vivo model* that mimics the principal causes responsible of the *in vivo* ischemic attack (embolism). Our pharmacological characterization was performed by using adenosinergic multi-target compounds. It has been described that after an *in vivo* ischemic insult follows a demyelination process linked to loss of OLs after the injuries (Cui et al., 2016; Guo et al., 2021). The better knowledge about the role of "A_2" receptors on de/re-myelination processes is important in order to identify new molecular targets under cerebral ischemia.

3.4 Stroke and demyelination

The ischemic insult results in white matter damage that is commonly observed in surviving stroke patients and is an important clinical aspect of cerebral ischemia (Wang et al. 2009; Yoshioka et al. 2011). The white matter injury is also clinical related with long-term functional outcame and cognitive deficit. Demyelination is the major pathological component of white matter damage that affects oligodendrocytes, the myelin-forming glial cells that wrap neuronal axons and ensure impulse transmission under normal conditions and that are involved in remyelination after axonal damage (see below). However, myelinating oligodendrocytes are highly vulnerable to ischemic or traumatic insults (Petito et al., 1998) and the loss of oligodendrocytes is known to be a significant factor underlying demyelination after injury (Caprariello et al., 2012). Therefore, in order to increase functional recovery following a stroke, novel approaches targeted at promoting endogenous myelin repair through the repopulation of myelin-forming oligodendrocytes provide intriguing therapeutic options. Moreover, agents or approaches improving recovery in stroke models, like permanent middle cerebral artery occlusion, are believed to prove efficacious also in other neurodegenerative conditions characterized by neuronal dysfunction and myelin deterioration associated to neurological and cognitive defects (Raffaele et al., 2021).

4 Oligodendrogliogenesis

Oligodendrocyte progenitor cells (OPCs) are a population of cycling cells in the developing and adult CNS that, under opportune stimuli, differentiate into mature myelinating oligodendrocytes (OLs). Before being able to produce myelin, oligodendroglial cells progress through a series of highly regulated steps of differentiation from OPCs to mature OLs (Barateiro and Fernandes, 2014; De Castro and Bribián, 2005). During embryonic development, OPCs are generated in restricted areas, such as the subventricular zone (SVZ), and present a significant migratory ability that allow them to spread and populate the brain and spinal cord (Emery, 2010). Their differentiation and maturation are postnatal processes characterized by

the loss of proliferative activity and the acquisition of an elaborate morphology with highly branched processes (De Castro and Bribián, 2005).

Oligodendrogliogenesis involves a sequence of distinct phases that can be identified by the expression of stage-specific surface antigens and by morphological changes (Gard and Pfeiffer, 1990; Jung et al., 1996; Levi et al., 1986; Warrington et al., 1992). On these bases, a classification into three stages of differentiation has been proposed: proliferating OPCs, post-mitotic pre-OLs and mature myelinating OLs (Barateiro and Fernandes, 2014; Coppi et al., 2015, 2013a; Szuchet et al., 2011). The initial stage of maturation presents a bipolar (or tripolar) morphology, typical of proliferating OPCs (Fumagalli et al., 2011). Several are the markers of precocious maturation stages, such as platelet-derived growth factor receptor a (PDGFa), nerve glial antigen 2 (NG2) or the OL transcription factor 2 (Olig2) (Ligon et al., 2006; Nishiyama et al., 2002; Pringle et al., 1992; Yu et al., 1994).

When OPCs start to differentiate in pre-OLs, secondary ramifications emerge from the soma and the expression of new molecular markers, typical of intermediate steps of maturation, is detected, such as O4 (Szuchet et al., 2011) and the recently deorphanized P2Y-like GPR17 receptor (Coppi et al., 2013b; Emery, 2010; Fumagalli et al., 2011; Lecca et al., 2008). During this phase, cells acquire the typical phenotype of postmitotic, but not yet myelinating, immature OLs characterized by a complex multipolar morphology (Back et al., 2001).

Finally, when OLs reach the fully mature, myelinating phase, they acquire a highly ramified profile and immunoreactivity for myelin specific structural proteins such as 2',3'-Cyclic-nucleotide-3'-phosphodiesterase (CNPase), myelin associated glycoprotein (MAG) and myelin basic protein (MBP) (Scolding et al., 1989; Zhang, 2001). Mature OLs synthesize large amounts of myelin, giving rise to multilamellar myelin sheaths that wrap and insulate neuronal axons, which allow electrical isolation and saltatory conduction of electric impulses (Cherchi et al., 2021b).

It is known that, during their maturation, oligodendroglial cells display different functional voltage-gated ion channels (Barres et al., 1990; Sontheimer and Kettenmann, 1988; Williamson et al., 1997) including either inward or outward rectifying K^+ channels, Na^+ currents and different subtypes of Ca^{2+} channels (Verkhratsky et al., 1990) and the density of channels differs within age and region

(Spitzer et al., 2019). Such a heterogeneity may therefore reflect different cellular states, where densities of ion channels define a particular cell function.

When OPCs first appear, i.e. at embryonic day 13 (E13) in the mouse, they have no detectable voltage-gated ion channels nor glutamate receptors and may be therefore considered in a naive state (Spitzer et al., 2019). The first ion channels detected are KV and glutamate AMPA and/or Kainate receptors (AMPARs/KARs), at E18. OPCs with these properties are considered migrating cells because of the strong expression of migratory genes at this time point. The fraction of OPCs with detectable NaV increases sharply around birth. It is conceivable that OPCs with high NaV and KV, and low AMPA/KAR, densities reflect a high proliferation state because (i) OPCs in S/G2/M phase have a higher density of NaV than OPCs in G0/G1 phase (Spitzer et al., 2019). OPCs expressing NaV, KV, AMPA/KARs, and NMDARs are typically found throughout oligodendrogliogenesis during development, when myelin gene expression starts (Marques et al., 2018; Spitzer et al., 2019), and during the beginning of the differentiation phase of myelin regeneration (Gautier et al., 2015) and so it might reflect a ‘‘primed’’ OPC state for differentiation. Either KV or AMPA/KAR channels are expressed in nearly all recorded postnatal OPCs, whereas, intriguingly, not all OPCs express NaV or NMDARs, as their density reaches a maximum after the first postnatal week, when myelination starts, and then declines when myelination decays. The last state of OPC maturation is distinguished by low NaV density, lack of NMDARs and high AMPA/KAR density and is observed at a time when OPC cell-cycle time lengthens, differentiation genes are downregulated and senescent molecular signature genes appear. In this phase, OPCs differentiation potential declines and thus it can be considered a ‘‘quiescent’’ OPC state (Cherchi et al., 2021a, 2021b).

Among K^+ currents, OPCs show outward currents conductances mainly composed by delayed rectifying K^+ currents (IK) (Sontheimer and Kettenmann, 1988) characterized by scarce time- and voltage-dependent inactivation and by a threshold for activation around -40 mV. They also express a transient outward K^+ current (IA), which is typically found in undifferentiated OPCs and presents a rapid time-dependent inactivation (approximately 50 ms) and a voltage-dependent inactivation at potentials from -40 and above (Gallo et al., 1996). A subpopulation (about 60%) of immature OPCs also express inward, tetrodotoxin-sensitive, Na^+ currents (INa)

typically found in neurons, with a rapid time-dependent inactivation (less than 1 ms) and a current peak amplitude at about -10 mV (Kettenmann et al., 1991). INa is never observed in mature oligodendroglial stages, as previously reported by my research group (Coppi et al., 2013b) and others (Sontheimer et al., 1989). Of note, a subpopulation fraction of electrically excitable, spiking, $NG2^+$ OPCs, able to generate full action potentials when stimulated by depolarizing current injection, have been described in brain slices, but the functional role of this "electrically excitable" OPC subpopulation is still unknown (Káradóttir et al., 2008). Of note, single action potentials have also been detected in a minority of cultured OPCs (Barres et al., 1990). During maturation, membrane outward K^+ conductances (both IK and IA) in OPC undergo a strong downregulation up to almost completely disappearance in mature OLs (Barres et al., 1990; Coppi et al., 2013a; Sontheimer and Kettenmann, 1988). In parallel with outward K^+ current downregulation, there is a gradual increase in the expression of inwardly rectifying K^+ currents (Kir), activated at potentials lower than -100 mV. Indeed, Kir currents are the main conductance observed in mature OLs (Knutson et al., 1997). Among the mentioned currents, IK are crucially linked to cell cycle regulation and hence to myelin formation (Chittajallu et al., 2005) because of the following: (i) a downregulation of IK occurs as OL lineage cells mature (Barres et al., 1990; Sontheimer and Kettenmann, 1988) and (2) pharmacological block of IK induced by tetra-ethyl-ammonium (TEA) in cultured OPCs is sufficient to inhibit their proliferation and differentiation (Chittajallu et al., 2005; Coppi et al., 2013b; Gallo et al., 1996; Knutson et al., 1997). Hence, treatments aimed at modulating these currents may affect OL proliferation and myelination. Steps and markers of oligondendroglial differentiation described above are observed not only in the brain but also in the spinal cord, where a significant fraction of OPCs also persists throughout adult life.

What is clear is that these changes in voltage-gated channels will have a profound effect on how OPCs sense neuronal activity and on the effect neuronal inputs will have on OPCs (Coppi et al., 2013a; Spitzer et al., 2019; Cherchi et al., 2021b). Therefore, neurotransmitters, cytokines and growth factors have been shown to regulate glutamate receptor expression in OPCs (Gallo et al., 1994; Lundgaard et al., 2013; Malerba et al., 2015; Spitzer et al., 2019; Stellwagen and Malenka, 2006).

Accordingly, a combination of G-protein coupled receptors, growth factors, and cytokines may modify K^+ current expression. This heterogeneity in physiological properties may cause differences in the myelination potential of OPCs and implicate distinct functions or cell states (Figure 15).

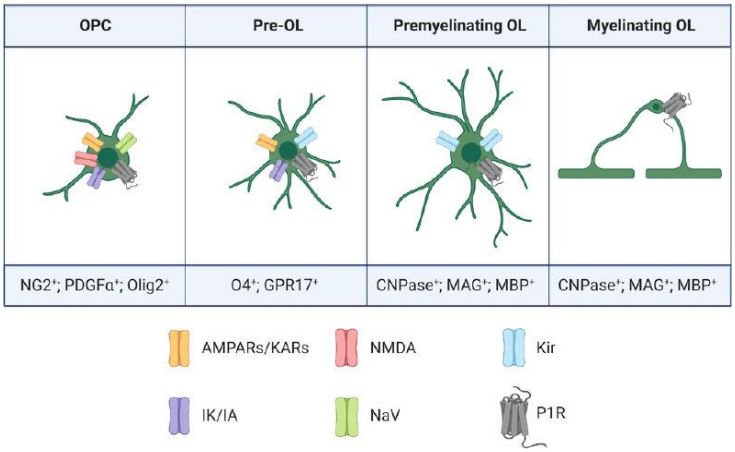


Figure 15. ***Schematic representation of morphological and antigen/channel expression changes during oligodendrogliogenesis***. A typical oligodendrocyte precursor cell (OPC) is positive to the antigens: nerve glial antigen 2 ($NG2^+$), platelet-derived growth factor alpha ($PDGF\alpha^+$) and to the transcription factor Olig2 ($Olig2^+$) and express glutamate AMPA and/or kainate receptors (AMPARs/KARs) and voltage-dependent Na^+ (Nav) and K^+ (IK/IA) channels. A typical pre-oligodendrocyte (Pre-OL) is positive to the markers: oligodendrocyte 4 ($O4^+$), the purinergic-like receptor GPR17 ($GPR17^+$) and express AMPARs/KARs, inward-rectifier potassium channels (Kir) and IK/IA channels. Premyelinating OLs and myelinating OLs are positive to the antigens: 2',3'-Cyclic-nucleotide-3'-phosphodiesterase ($CNPase^+$), myelin associated glycoprotein (MAG^+) and myelin basic protein (MBP^+) and express Kir channels. During oligodendrogenesis P1Rs are expressed at all maturation stages (Taken from: Cherchi et al., 2021b).

4.1 Adenosine receptors in oligodendrogliogenesis

All adenosine receptors are expressed at all maturational stages of oligodendroglial cells (Fields, 2004; Stevens et al., 2002) and exert a key role in cell development. Furthermore, the expression by OLs of the equilibrative nucleoside transporters ENT1 and ENT2, as well as adenosine degrading enzymes, such as adenosine deaminase and adenosine kinase, has been demonstrated (González-Fernández et al., 2014), supporting the notion that purinergic signalling exerts a prominent role in these cells (Burnstock et al., 2011). Indeed, it was demonstrated that adenosine can affect numerous OPC functions such as migration, proliferation and maturation (Coppi et

al., 2015, 2013a, 2013b; Fields, 2004; Fields and Burnstock, 2006; Stevens et al., 2002), with distinct effects mediated by different receptor subtypes, as described below.

One of the first work about adenosine receptor and OPCs dates back to 2002; Stevens and et al. (2002), following the addition of exogenous adenosine to OPCs cultured in the presence of the mitogen PDGF, observed a concentration-dependent reduction of cell proliferation and promotion of differentiation towards pre-myelinating oligodendrocytes, an effect that is mainly mediated by A_1AR. Furthermore, tonic electrical stimulation of co-cultures of OPCs with dorsal root ganglion neurons also promotes myelination by increasing the number of MBP^+ cells (Stevens et al., 2002), an effect blocked by a cocktail of A_1AR, A_{2A}AR and A_3AR antagonists, suggesting that endogenous adenosine released in response to impulse activity promotes oligodendrocyte development and myelination (Stevens et al., 2002). In addition, A_1AR agonists have been reported to stimulate OPC migration (Othman et al., 2003). On these bases, it was proposed that A_1ARs on OPCs prompt myelination thus offering new approaches for the treatment of demyelinating diseases of the CNS, such as multiple sclerosis (MS). In accordance, A_1AR -/- mice developed more severe experimental autoimmune encephalomyelitis (EAE) with worsened demyelination, axonal injury, and enhanced neuroinflammation and activation of microglia/macrophages (Tsutsui et al., 2004). Furthermore, A_1ARs promotes myelin repair by recruiting endogenous progenitor cells in an experimental model of optic nerve demyelination (Asghari et al., 2013) and, when activated on astrocytes, exert immunosuppressive properties (G. Liu et al., 2018a).

Such protective effects, however, are at variance from what has been described *in vivo* neonatal rats, where the treatment with A_1AR agonists reduces white and gray matter volume, induces ventriculomegaly (Turner et al., 2002) and decreases the expression of MBP, similarly to what observed in neonatal rats reared in hypoxia. Ventriculomegaly was also observed in mice lacking the enzyme adenosine deaminase, which degrades adenosine (Turner et al., 2003). Moreover, hypoxia-induced periventricular white matter injury (PWMI, a form of brain injury present in preterm infants) was prevented in A_1AR -/- mice (Turner et al., 2003). These data support the notion that adenosine, acting on A_1ARs, mediates hypoxia-induced brain

injury and ventriculomegaly during early postnatal development. Such an effect could be attributed to the fact that adenosine, which is released in huge amounts during hypoxic-ischemic conditions (Latini and Pedata, 2001), activates A_1ARs leading to premature differentiation and reduced proliferation of oligodendroglia precursors. Indeed, studies on OPCs and pre-OLs in hypoxic conditions, when increased glutamate outflow impairs neuronal functions (Rossi et al., 2000) and synaptic transmission (Colotta et al., 2012), revealed a reduced proliferation and an accelerated maturation, as demonstrated by the increased expression of the cell cycle regulatory proteins p27 (Kip1) and phospho-cdc2 (Akundi and Rivkees, 2009). This series of events would lead to a reduced number of OLs available for myelination, thus contributing to PWMI (Rivkees and Wendler, 2011). Therefore, strategies aimed at stimulating OPC proliferation in neonatal hypoxia/ischemia may be of value to prevent PWMI. Accordingly, Cao and co-workers (Cao et al., 2019) found that OLs pre-treated with 100 µM caffeine or the A_1AR antagonist DPCPX (100 nM) during hypoxia showed a significant reduction in A_1AR and Olig2 expression, at early stages, and a decreased CNPase expression, at later stages of hypoxia. In addition, they demonstrated that either hypoxia or adenosine treatment induced significant elevation in resting $[Ca^{2+}]i$, which was restored to normal levels when cells were treated with caffeine or DPCPX. During hypoxia, adenosine increase leads to A_1AR activation which resulted in excessive Ca^{2+} release from intracellular stores (Annunziato et al., 2013), a condition that is considered to initiate cell injury (Cao et al., 2019) (Figure 16).

No data are present in the literature about the effect of A_3ARs on oligodendrocyte differentiation. However, results obtained by Gonzalez- Fernandez et al. 2014 demonstrate that the A_3AR agonist 2-CI-IB-MECA induces apoptosis of cultured $O4^+$ OLs isolated from rat optic nerve through the activation of Bax and Puma proapoptotic proteins. Furthermore, incubation of *ex vivo* preparations of optic nerve with adenosine or 2-CI-IB-MECA induces OL damage and myelin loss, effects prevented by the A_3AR antagonist MRS220. Moreover, MRS220 also prevented OL damage and myelin loss in the optic nerve exposed to *ex-vivo* ischemic like conditions, i.e. oxygen–glucose deprivation (González-Fernández et al., 2014). Thus,

data suggest that adenosine, via activation of A_3ARs, triggers OL death and contributes to white matter ischemic damage (Figure 16).

The role of A_{2A}AR and A_{2B}AR in oligodendrocyte differentiation has been recently studied and will be described in detail in the paragraphs below.

4.1.1 A_{2A}ARs in oligodendrogliogenesis

Our group of research (Coppi et al., 2013a) has reported the first functional characterization of the adenosine A_{2A}AR subtype in OPCs. We demonstrated that the selective A_{2A}AR agonist CGS21680 inhibits IK currents in cultured OPCs and delays *ex-vivo* OPC differentiation since it increases the percentage of $NG2^+$ immature OPCs and reduces $O4^+$ pre-OLs and MAG^+ mature OLs along 12 days of cell culture, without affecting neither cell viability nor proliferation (Coppi et al., 2013a). These effects were completely prevented in the presence of the selective A_{2A}AR antagonist SCH58261 (Coppi et al., 2013a). Tetraethylammonium (TEA), at 3 mM concentration, which blocks sustained IK but not transient IA currents in cultured OPCs, mimics and occludes the effect of the A_{2A}AR agonist on membrane currents, confirming that this purinergic receptor subtype electively affects IK in cultured OPC (Coppi et al., 2013a). In keeping with data demonstrating that IK inhibition impairs proliferation and maturation of cultured OPCs (Attali et al., 1997; Coppi et al., 2013b; Gallo et al., 1996) and blocks myelin deposition in the embryonic spinal cord, it appears that A_{2A}AR stimulation inhibits OPC differentiation by reducing IK currents. The observation that selective activation GPR17, a Gicoupled P2Y-like receptor, enhances TEA-sensitive IK and improves OPC differentiation is in line with the above assumption (Coppi et al., 2013b). Recently, Fontenas et al. (2019) demonstrated that the A_{2A}AR antagonist SCH58261 induced ectopic OPC migration from motor exit point in transition zones in zebrafish larvae, an effect that is not shared by antagonists at the other adenosine receptor subtype. A pathological condition associated with defects in cell metabolism and OPC maturation is the Niemann-Pick type C 1 (NPC) disease, an autosomal recessive and progressive neurovisceral disorder characterized by intracellular cholesterol accumulation and myelin defects (Kodachi et al., 2017; Walterfang et al., 2010). De Nuccio et al. (2019) observed that in primary cultures of OPCs exposed to a cholesterol transport inhibitor (U18666a), used to induce the NPC1-like phenotype

ex-vivo, A_{2A}AR expression was significantly decreased whereas treatment with the A_{2A}AR agonist CGS21680 triggered a protective effect by reducing cholesterol accumulation and mitochondrial membrane potential (mMP) alterations in U18666a-treated OPCs. Consistent with data from Coppi et al. (2013b) the same study demonstrates that CGS21680 induced a decrease in the percentage of $O4^+$, $O1^+$ and MBP^+ in control OPCs (De Nuccio et al., 2019). In contrast, after 48 h of U18666a treatment, CGS21680 overcame the maturation arrest induced by the compound, even when A_{2A}AR stimulation occurred 24 h after U18666a exposure. Finally, the same study also demonstrated that PKA activation is responsible for the A_{2A}AR -dependent effect on cholesterol accumulation since the PKA inhibitor KT5720, but not the extracellular signal-regulated kinases 1/2 (ERK1/2) inhibitor PD98059, prevented the cholesterol redistribution induced by CGS21680 in NPC-OPC.

The dual effect of CGS21680 on OPC differentiation, arresting OLs maturation in control cultures and promoting differentiation in U18666a-treated cultures, is in keeping with differential effects by CGS21680 previously reported in a model of Huntington's disease (HD), where the compound induces opposite effects in the striatum of Huntington versus wild-type mice (Martire et al., 2007).

However, other intracellular pathways, in addition to IK block, could contribute to the A_{2A}AR -mediated inhibition of OPC differentiation. OPCs also express the tyrosine kinase fibroblast growth factor (FGF) receptor whose activation promotes cell proliferation and inhibits the expression of myelin components (Besnard et al., 1989). As an example, in PC12 cells (a cell line that was confirmed to express the A2AR and FGFRs), the simultaneous activation of both A_{2A}AR and FGF receptors by robust activation of the mitogen activated protein kinase (MAPK/ERK) pathway, brings to increased differentiation and neurite extension (Flajolet et al., 2008). It is possible that a crosstalk between A_{2A}ARs and FGF receptors regulates cell maturation also in OPCs.

Of note, upregulation of A_{2A}AR expression has been observed in cerebral white matter of patients with secondary progressive MS and a higher density of brain A_{2A}AR appeared to correlate with higher disability scale scores in MS patients (Rissanen et al., 2013). On these bases, it was hypothesized that A_{2A}AR upregulation on brain cells is associated with disease progression. In agreement, in a mouse model of MS,

EAE, A_{2A}AR antagonists protected from disease development (Mills et al., 2012), suggesting that activation of A_{2A}AR glial and neuronal cells is responsible for EAE development in mice. Moreover, in a rat model of focal brain ischemia (by middle cerebral artery occlusion: MCAo), systemic administration of A_{2A}AR antagonists after ischemia prevented the activation of JNK mitogen activated kinase (Melani et al., 2009) and subsequent activation cascade of caspase3 and the pro-apoptotic regulator DP5 (Yin et al., 2005), involved in OL death (Howe et al., 2004). Accordingly, selective A_{2A}AR antagonists also prevented myelin disorganization in the basal nuclei and striatum of MCAo rats (Melani et al., 2009). Therefore, it emerges from above data that A_{2A}AR activation is deleterious in demyelinating disorders. Moreover, in a rat model of focal brain ischemia, the A_{2A}AR antagonist SCH58261, that reduced the activation of JNK mitogen activated kinase in oligodendrocytes and subsequent activation of caspase3-mediated oligodendrocyte cell death (Melani et al., 2009), significantly prevents the myelin damage inflicted to the striatum by the ischemic insult.

In keeping with these data, it can be concluded that the activation of A_{2A}ARs by adenosine released during a demyelinating insult contributes to brain damage by hampering OPC maturation and myelin deposition. Such a role might appear in contrast with the observation that A_{2A}AR agonists proved protective in EAE models by decreasing immune cell infiltration and lymphocyte Th1 cell activation (Y. Liu et al., 2018b). Furthermore, genetic ablation of both central and peripheral A_{2A}AR exacerbates brain damage and neuroinflammation in EAE (Ingwersen et al., 2016; Yao et al., 2012). Indeed, A_{2A}ARs expressed on peripheral leucocytes are known to exert important anti-inflammatory effects, i.e. by reducing adhesion cell factor production and neutrophil activation (Sitkovsky et al., 2004). Thus, genetic ablation of adenosine A_{2A}ARs on blood cells exacerbates leucocyte infiltration, neuroinflammation and brain damage in a model of chronic inflammation such as EAE (Pedata et al., 2014). It appears that, beside disadvantageous central effects on OPC differentiation, A_{2A}AR stimulation may also alleviate neuroinflammation by peripheral mechanisms, thus complicating the role of this endogenous nucleoside in neurodegenerative diseases. Successive studies contributed to elucidate the multifaceted role played by A_{2A}ARs in EAE. Ingwersen et al. demonstrated that A_{2A}ARs were upregulated predominantly on

T cells and macrophages/microglia within the inflamed tissue and preventive EAE treatment with A_{2A}AR-specific agonist inhibited myelin-specific T cell proliferation ex vivo and ameliorated disease, while application of the same agonist after disease onset exacerbated non-remitting EAE progression and tissue damage (Ingwersen et al., 2016). Similarly, Chen and co-workers (Chen et al., 2019) demonstrated that the administration of the selective A_{2A}AR antagonist SCH58261 at 11-28 days post-immunization with MOG prevented neurological deficits and reduced local infiltration and demyelination. By contrast, the same treatment was ineffective when administered at the beginning of the onset of EAE (i.e., 1-10 after immunization). Therefore, it appears that, while providing anti-inflammatory effects on T cells and thus protection at early stages, A_{2A}AR seems to play a detrimental role during later stages of the disease and may thus contribute to sustained tissue damage within the inflamed CNS. Hence, the identification of the effective therapeutic window to optimize the beneficial effects of A_{2A}AR antagonists is of crucial importance to support SCH58261 as a candidate for the treatment of MS in human (for a review see: Rajasundaram, 2018) (Figure 16).

4.1.2 A_{2B}ARs in oligodendrogliogenesis

The A_{2B}ARs functional involvement in OLs is not yet fully clarify. Therefore, Wei and co-workers demonstrate that the pharmacological blockade of A_{2B}AR with selective antagonists or receptor knock out in a rodent model of EAE protects from myelin disruption and neurological impairment due to this pathological condition (Wei et al., 2013). However, unless the lack of preclinical studies where A_{2B}AR agonists are administered in EAE mice up to date, it cannot be ignored that the A_{2B}AR subtype shares with the A_{2A}AR the anti-inflammatory impact in many different pathologies (Dettori et al., 2020; Eckle et al., 2008; Pedata et al., 2016; Yang et al., 2006). Therefore, possible side effects could arise in MS patients treated with A_{2B}AR blockers. Of note, the above results demonstrating a deleterious role of A_{2B}ARs in demyelinating diseases are in agreement with our *ex-vivo* data demonstrating that A_{2B}AR blockade (Coppi et al., 2020a), as well as A_{2A}AR antagonism (Coppi et al., 2013a), facilitates OPC differentiation. Indeed, our research group recently discovered that selective A_{2B}AR agonists, BAY60-6583 and its recently synthesized analog P453 (Betti et al., 2018), inhibited *ex-vivo* OPC differentiation, as shown by the decreased expression of myelin-related proteins like MBP or MAG in primary purified OPC cultures. This finding suggests that A_{2B}ARs are essential for OPC maturation (Coppi et al., 2020a). Moreover, we demonstrated that the TEA- sensitive (sustained I_K), and 4-amynopyridine- (4-AP) sensitive (transient I_A) conductances were inhibited by the selective activation of A_{2B}AR. Since I_K are involved in OPC maturation (Gallo et al., 1996), this could be one of the mechanisms by which A_{2B}ARs inhibited myelin production. In accordance with previous data (Soliven et al., 1988), the adenylyl cyclase activator forskolin mimicked BAY60-6583-mediated action by reducing I_K currents. A second administration of BAY60-6583 in the presence of forskolin had no impact, which is significant since it showed that A_{2B}AR activation suppresses I_K through raising intracellular cAMP levels (Coppi et al., 2020a) (Figure 16).

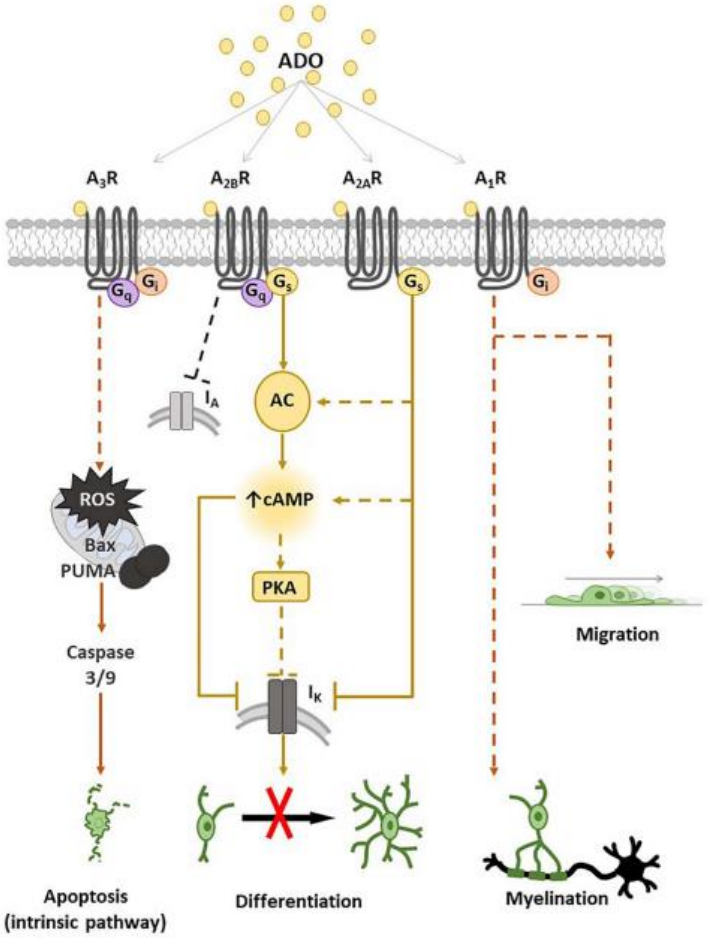


Figure 16. ***Adenosine (ADO) receptor expression and main transduction pathways activated in oligodendrocyte progenitor cells (OPCs) and oligodendrocytes (OLs)***. Schematic representation of ADO A_1, A_{2A}, A_{2B} and A_3 receptor effects on OPCs and main intracellular pathways involved. The activation of A_1AR by ADO or other selective receptor agonists promotes myelination and migration. The stimulation of Gs-coupled A_{2A}AR and/or A_{2B}AR leads to adenylyl cyclase (AC) activation with a consequent increase in intracellular cyclic adenosine monophosphate (cAMP), which closes I_K channels and inhibits OPC differentiation, probably by a mechanism involving protein kinase A (PKA). A_3AR stimulation induces OPC apoptosis by the activation of an intrinsic pathway, i.e., through reactive oxygen species (ROS) production and activation of Bcl-2-associated X (Bax), p53-upregulated modulator of apoptosis (PUMA) and caspase 3/9. Dotted lines are used when the intracellular pathway/s have not been described. (Taken from: Coppi et al., 2021a).

5 Adenosine receptors on Dorsal Root ganglion neurons

The Dorsal Root Ganglion (DRG) contains cell bodies of the primary sensory neurons responsible for modulation and transduction of sensory information to the spinal cord. The primary sensory neuron is a pseudounipolar neuron with one branch extending to the peripheral receptive field and the other entering the spinal cord (Aldskogius et al., 1986). In humans, there are 31 pairs of “mixed” spinal nerves carrying sensorimotor information between the spinal cord and the periphery. These

spinal nerves are formed by dorsal afferent sensory axons (dorsal rootlets) and ventral efferent motor axons (ventral rootlets). As the dorsal sensory root fibres travel laterally, their processes connect via a T-junction with their cell bodies, which form the DRG. The T-junction of the DRG neurons can act as an impediment to electrical impulses traveling from the peripheral nociceptor to the dorsal root entry zone of the spinal cord, can participate in the propagation of the electrical pulse, or can act as a low-pass filter to electrical information from the periphery (Gemes et al., 2013). The cell bodies of the DRG neurons are separated from each other by an envelope of satellite glial cells (SGCs) that respond to peripheral and central processes including nociception, peripheral afferent fiber injury, and inflammation.

As most studies of the DRG are performed in rats, it should be stated that human and rat differ with respect to the number of spinal segments and thus primary sensory nerves (Gelderd and Chopin, 1977). In 1985, Harper and Lawson classified rat DRG neurons into small- (20-27 µm), medium- (33-38 µm), and large- (45-51 µm) sized, depending on cell bodies diameter (Harper and Lawson, 1985). These neurons give rise to C (less than 1.4 m/s), Aδ (2.2-8 m/s), and Aα/β (more than 14 m/s) fibres, respectively (Harper and Lawson, 1985). Because of its important roles in the modulation of sensory processing, and the development of neuropathic pain (Hasegawa et al., 1993, 1996), the DRG is an excellent clinical target for pain control.

Of note, in the last decades the isolation of DRG neurons and their co-culture with OPCs was became a widely used experimental model to study the myelination processes. In particular, this method resulted useful for many different investigations such as the study of signals that are involved in the myelin sheath, the impact of mechanical stimulation on oligodendrocyte biology and the effects of neural activity on myelination. The advantage of using pure DRG-OPC co-cultures for studying myelination is that they remove any potential confounding factors from other cell types, including astrocytes, which may facilitate the process. Moreover, both neurons and oligodendrocytes can be manipulated independently of each other prior to setting up the co-culture (Marangon et al., 2021).

On the contrary, the principal limit of this co-culture is the nature of DRG neurons themselves. Indeed, they project their axons connecting to both central and peripheral neurons, despite having neuronal cell bodies that are external to the CNS.

Because of this, these neurons might not accurately recreate CNS myelination. However, it has been discovered that oligodendrocytes produce myelin sheaths on DRG neurons that are comparable to those produced *in vivo* (Marangon et al., 2021). Several early electrophysiological studies reported the effects of adenosine and its receptors on isolated rat DRG neurons (Dolphin et al., 1986; Gross et al., 1989; MacDonald et al., 1986). However, adenosine receptors subtypes were initially poorly characterized on these cells, with only A_1AR and a "generic" A_2 subtype being described based on their ability to modulate intracellular cAMP accumulation. It has been demonstrated that A_{2A}ARs are expressed on DRG neurons and the presynaptic terminals of sensory afferents (Kaelin-Lang et al., 1998) and that their activation in peripheral afferents contributes to mechanical hyperalgesia. Then, it was demonstrated the A_3ARs expression on DRG neurons, with species-specific differences (Usoskin et al., 2015). Zheng et al. (2014) demonstrated, by using confocal microscopy, that A_1ARs and A_3ARs mediated adenosine-induced intracellular calcium release in the DRG neurons. Concerning the A_{2B}ARs, their expression was demonstrated on ST36 DRG neurons by Li et al. (2022).

AIMS of the THESIS

AIMS SECTION I – Pharmacological and functional characterization of adenosine "A_2" receptors on synaptic plasticity and during an ischemic-like insult: an *ex-vivo* study in the CA1 region of rat hippocampal slices

i) Evaluation of novel multi-target adenosine A_{2A}-A_{2B} receptor agonist and/or antagonist on CA1 synaptic plasticity and during oxygen-glucose deprivation

It is known that the selective activation A_{2A}AR and A_{2B}AR by CGS21680 and BAY60-6583, respectively, inhibited hippocampal paired-pulse facilitation, an electrophysiological paradigm whose reduction reflects an increase in presynaptic glutamate release. Furthermore, the selective antagonism of A_{2A}AR or A_{2B}AR protected from the irreversible synaptic failure induced by severe oxygen and glucose deprivation in the CA1 hippocampus and prevented or delayed anoxic depolarization appearance, an unequivocal sign of neuronal injury. As mentioned above, a multi-target pharmacological approach has become an increasingly pursued strategy for the cure of complex pathologies, included cerebral ischemia. Recently, a new mixed A_{2A}-A_{2B}AR agonist, MRS3997, and a new mixed A_{2A}-A_{2B}AR antagonist, P626, were synthetized. Moreover, in the last five years concurring evidences demonstrated the presence of an A_{2A}-A_{2B}AR heterodimer at least in engineered CHO cells. Hence, the use of multi-target compounds could be advantageous for the treatment of cerebral ischemia in order to block both A_{2A}ARs and A_{2B}ARs simultaneously.
Therefore, the first aim of this Thesis was to investigate the synergic activation or blockade of A_{2A}ARs and A_{2B}ARs (by MRS39997 or P626, respectively) on CA1 synaptic plasticity under basal conditions or during oxygen and glucose deprivation.

ii) Characterization of new adenosine A_{2A} receptors antagonists conjugated to antioxidant compounds in an *ex-vivo model* of cerebral ischemia

In the last years, the A_{2A}AR subtype emerged as a potential therapeutic attractive target in ischemia. Evidence suggests that A_{2A}AR has dual role: in a first phase of ischemia, it potentiates excitotoxicity, while hours and days after ischemia, A_{2A}ARs

on immune blood cells potentiate peripheral blood cell adhesion mechanisms and infiltration in the ischemic parenchyma. Noteworthy, A_{2A}AR antagonists resulted neuroprotective in preventing or delaying anoxic depolarization appearance, a clear sign of neuronal injury, in an *ex-vivo* model of cerebral ischemia. Moreover, it has been demonstrated that antioxidant compounds, such as edavarone and L-2-oxothiazolidine-4-carboxylic acid (OTC), were neuroprotective during an ischemic insult. Indeed, during brain ischemia, the formation of free radicals triggers several pathways involved in the development of brain edema.

For these reasons, new multi-target ligands, composed by an A_{2A} antagonistic portion and an antioxidant molecule, were synthetized. These mixed A_{2A}AR ligands may result more efficacious in neuroprotection during cerebral ischemia, since oxidative processes and ROS formation concur in combination to excitotoxicity during neuronal damage that derives from the ischemic attack. On this basis, the second aim of this Thesis was to investigate the effects of these new multi-target compounds and a selective A_{2A}AR antagonist in CA1 region of rat hippocampus during severe oxygen and glucose deprivation.

iii) Effects of carbonic anhydrase inhibitors in an *ex-vivo model* of cerebral ischemia in rat hippocampal slices

Evidences that hypoxic microenvironments elicit the expression of specific isoforms of CA (in particular CA IX and CA XII) through the HIF, allow us to hypothesize a possible relevant role of CA in ischemia. Recently it has been demonstrated that CA inhibitors (CAIs), i.e. sulfonamide or coumarin, were able to improve neurological functionalities after cerebral ischemic insult in the rats.

On these bases, another aim of my Thesis was to investigate if the non-selective CAI acetazolamide (ACTZ) and new generation, subtype-specific, sulphonamide CAIs, such as AN11-740 and AN6-277, provided protection in an *ex-vivo* model of acute rat hippocampal slices that underwent to severe, 30 min long, oxygen and glucose deprivation episodes.

AIM SECTION II – Pharmacological and functional characterization of adenosine "A_2" receptors in oligodendrogliogenesis and myelination processes: an *ex-vivo* study

Differentiation of oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes is a key event for axonal myelination in the brain; this process fails during demyelinating pathologies, such as multiple sclerosis or after cerebral ischemia. Extracellular adenosine increases during ischemia or inflammation, suggesting adenosine receptors as valid therapeutic targets in these pathological conditions. Our research group previously demonstrated that the selective activation of A_{2A}ARs and A_{2B}ARs decreased *ex-vivo* OPC maturation by inhibiting potassium currents necessary to their differentiation. Therefore, the last, but not least, aim of my Thesis was to elucidate the role of "A_2" receptors on potassium currents in primary OPC cultures by using electrophysiological patch-clamp recordings. Moreover, the role of these receptors subtypes was investigated on myelination processes by: i) co-culturing OPCs with dorsal root ganglion neurons; ii) using immunocytochemical analysis. To this purpose, we used the endogenous ligand adenosine, selective A_{2B}AR agonist and the new multi-target A_{2A}-A_{2B}ARs antagonist, P626.

MATERIALS and METHODS

1 Animals

All animal procedures were conducted according to the Italian Guidelines for Animal Care, DL 116⁄92, application of the European Communities Council Directive (86／609／EEC) and approved by the Committee for Animal Care and Experimental Use of the University of Florence. Male Wistar rats and pregnant female Wistar rats were obtained from Envigo, Italy.

2 Cell culture preparation

2.1 OPC cultures

Purified cortical OPC cultures were prepared as described elsewhere (Coppi et al., 2013b; Malerba et al., 2015). Wistar rat pups (postnatal day 1–2) were sacrificed and cortices removed, mechanically and enzymatically dissociated, suspended in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 4 mM L-glutamine, 1 mM Na-pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin (all products are from EuroClone, Milan, Italy), and plated in poly-D-lysin coated T75 flasks (1 flask per animal). After 2–3 days in culture, OPCs growing on top of a confluent monolayer of astrocytes were detached by 5 h of horizontal shaking. Contaminating microglial cells were eliminated by a 1 h pre-shake and by further plating detached cells on plastic culture dishes for 1 h. OPCs, which do not attach to plastic, were collected by gently washing the dishes and plated onto poly-DL-ornithine-coated (final concentration: 50 μg/ml, Sigma- Aldrich, Milan, Italy) 13 mm-diameter glass coverslips laid in 24 multiwell chambers ($l0^4$ cells/well) for electrophysiological and immunocytochemical experiments. OPC cultures were maintained in Neurobasal medium (NB) (Thermo Fisher Scientific, Waltham, MA USA) containing 2% B27, 4 mM L-glutamine, 1 mM Na-pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin, 10 ng/ml platelet derived growth factor-BB (PDGF-BB) and 10 ng/ml basic fibroblast growth factor (bFGF; both mitogens were from PeproTech EC Ltd, London, UK) to promote cell proliferation.

2.2 DRG neuronal cultures

Wistar male rats (3-4 weeks old, Envigo, Udine, Italy) were housed in a temperature and humidity-controlled vivarium (12-hour dark/ light cycle, free access to food and

water) and were sacrificed with a guillotine under anesthesia with isoflurane (Baxter, Rome, Italy). Primary DRG neurons were isolated and cultured as described (Nassini et al., 2015; Coppi et al., 2019). Briefly, ganglia were bilaterally excised and enzymatically digested using 2 mg/mL of collagenase type 1A and 1 mg/mL of trypsin (both compounds from Sigma- Aldrich, Milan, Italy) in Hank's balanced salt solution (25-35 min at 37°C). Cells were then pelleted and resuspended in DMEM supplemented with 20% heat inactivated fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2-mM L-glutamine for mechanical digestion. After centrifugation (1200g, 5 min), neurons were suspended in the above mentioned medium, enriched with 100 ng/mL of mouse nerve growth factor (NGF; Serotec, Oxford, UK; 100 ng/mL), to promote neuron survival, and then plated on 13- mm glass coverslips coated by poly-L-lysine (8.3 mM) and laminin (5mM). To remove contaminating fibroblasts and glial cells, the cultures were treated ARA-C (Sigma-Aldrich, Milan Italy; 10 µM). Dorsal root ganglion neurons were cultured for 1 to 2 days before being used for experiments. In a set of experiments, DRG cultures were maintained in the absence of nerve growth factor. However, no difference was found in any of the effects tested in the present research, and data were pooled.

2.3 OPC-DRG co-cultures

DRGs were dissected from Wistar rat pups (postnatal day 5–6) and were dissociated with 2 mg/mL of collagenase type 1A and 1 mg/mL of trypsin (both compounds from Sigma- Aldrich, Milan, Italy) at 37ºC for 30 min. The dissociated cells were plated onto 13 mm coverslips coated with poly-L-lysine and laminin (Sigma-Aldrich, Milan Italy; 10 mg/ml) at a density of $1.5X10^4$ cells/coverslip. Neurons were grown for one week in Neurobasal medium (Thermo Fisher Scientific) with 2% B27 (Gibco), in the presence of NGF (Serotec, Oxford, UK; 100 ng/mL). To remove contaminating fibroblasts and glial cells, the cultures were treated ARA-C (Sigma-Aldrich, Milan Italy; 10 µM) for two days each time. After one week, OPCs were prepared, as explained above, from postnatal day 1-2 rats. OPCs were plated onto coverslips with purified DRGs at a density of $5X10^4$ cells/coverslip in NB medium (Figure 17). At this point, NGF was removed from the medium, while ARA-C was removed 2 days before. Co-cultures were maintained for 14 days, with fresh medium added every two days

together with the pro-myelinating hormone T3 (50 nM, Sigma-Aldrich, Milan Italy) was added every two days, because it has been demonstrated that promotes myelination (Marangon et al., 2021). The day at which OPCs were added onto DRGs was indicated as the time 0 (t0). After 3 days from t0, drugs or their corresponding vehicles were added every two days for two weeks. Experiments were performed in triplicate from at least three different OPC-DRG isolations.

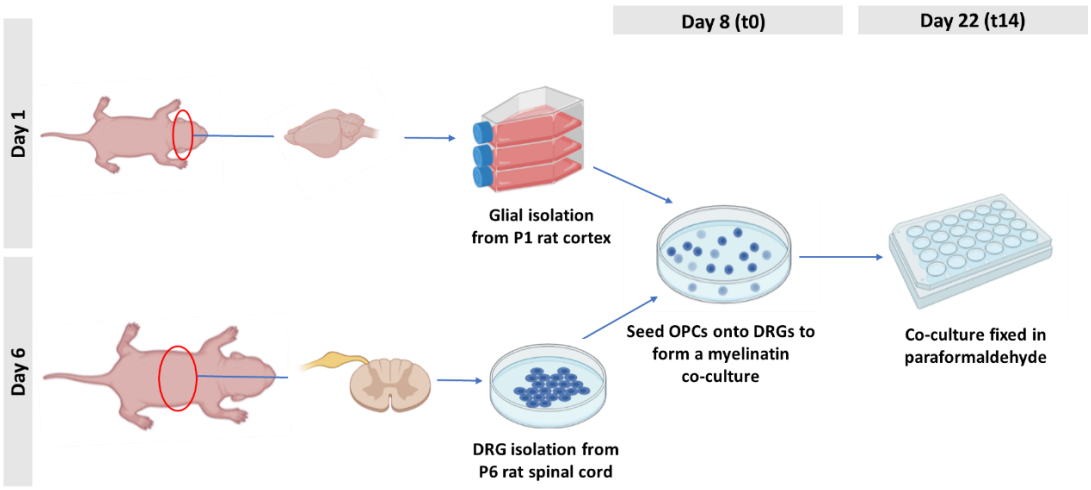


Figure 17. ***Co-culture preparation*****.** Schematic representation of oligodendrocyte precursor cells (OPCs) and dorsal root ganglia (DRGs) co-culture preparation. Cortical glial cells were isolated postnatal day 1 rats, while DRG neurons were isolated from the spinal cord of postnatal day 6 rats. The OPCs were then purified from mixed glial cell cultures and were seeded onto DRGs to start the co-culture at the time point 0 (t0), which corresponds with the day 8 from their isolation. After 14 days (t14) the co-cultures were fixed in paraformaldehyde.

3 Electrophysiological recordings

3.1 Extracellular recordings

3.1.1 Acute rat hippocampal slice preparation

Experiments were carried out on acute hippocampal slices (Pugliese *et al.,* 2006), prepared from Male Wistar rats (100-200 g; Envigo, Udine, Italy). Animals were sacrificed with a guillotine under anesthesia with isoflurane (Baxter, Rome, Italy) and hippocampi were rapidly removed and placed in ice-cold oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 125, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, $NaHCO_3$ 25, and D-glucose 10. Slices (400 µM nominal thickness) were cut using a McIlwain Tissue Chopper (Mickle Laboratory

Engineering Co. Ltd., Gomshall, United Kingdom) and kept in oxygenated aCSF for at least 1 h at room temperature. A single slice was then placed on a nylon mesh, completely submerged in a small chamber (0.8 ml) and superfused with oxygenated aCSF (31–32 °C) at a constant flow rate of 2 ml/min (Figure 18). The treated solutions reached the preparation in 60 s and this delay was taken into account in our calculations.

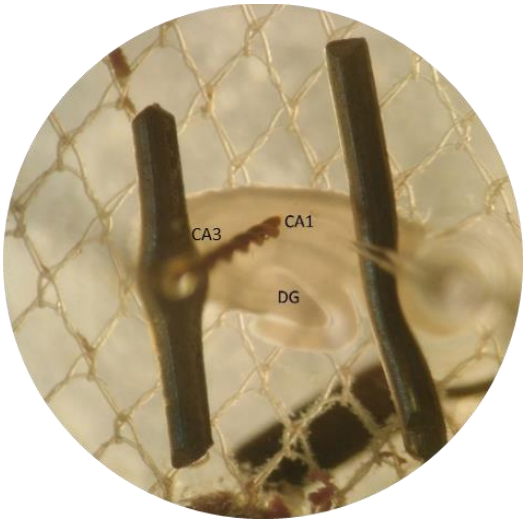


Figure 18. ***Rat hippocampal slice located on a nylon net in the perfusion chamber observed by an optical microscope (20X)***. The dentate gyrus (DG), the CA3 and the CA1 regions are easily recognizable in the image. The electrodes positioned in the CA1 region are identifiable: on the left the bipolar nichrome stimulation one and on the right the recording one.

3.1.2 Experimental procedure

Test pulses (80 μs, 0.066 Hz) were delivered through a bipolar nichrome electrode positioned in the stratum radiatum of the CA1 region of the hippocampal slice to stimulate the Schaffer collateral-commissural pathway (Figure 19). Evoked field excitatory post-synaptic potentials (fEPSPs) were extracellularly recorded with glass microelectrodes (2–10 MΩ, Harvard Apparatus LTD, United Kingdom) filled with 150 mM NaCl. The recording electrode was placed at the dendritic level of the CA1 region to record fEPSPs (Figure 19A). Responses were amplified (200, BM 622, Mangoni, Pisa, Italy), digitized (sample rate, 33.33 kHz), and stored for later analysis with LTP (version 2.30D) program (Anderson and Collingridge, 2001). The amplitude of fEPSP was measured as the difference between the negative peak following the afferent fiber volley and the baseline value preceding the stimulus artifact. In some experiments both the amplitude and the initial slope of fEPSP were quantified, but

since no appreciable difference between these two parameters was observed under control conditions, in the presence of drugs or during *ex-vivo* ischemia, only the measure of the amplitude was expressed in the figures. When a stable baseline of evoked responses was reached, fEPSP amplitudes were routinely measured and expressed as the percentage of the mean value recorded 5 min before the application of any treatment (in particular pre-OGD). Stimulus-response curves were obtained by gradual increase in stimulus strength at the beginning of each experiment. The test stimulus strength was then adjusted to produce a response whose amplitude was 40% of the maximum and was kept constant throughout the experiment.

3.1.2.1 Paired-pulse facilitation protocol

To elicit paired-pulse facilitation (PPF) of fEPSP, we stimulated the Schaffer collateral-commissural fibers twice with a 40 ms inter-pulse interval (Figure 19A, C). When a stable baseline of evoked responses was obtained by stimulating once every 15 s the Schaffer collateral fibers (basal synaptic neurotransmission: BSN), the double stimulation protocol (PPF) was applied, still once every 15 s, for 5 min either before or after 20 min application of the A_{2A}AR or/and A_{2B}AR agonists tested (Figure 19B). The synaptic facilitation was quantified as the ratio (P2/P1) between the slope of the fEPSP elicited by the second (P2) and the first (P1) stimuli. In some experiments the above protocol was carried out in combination with the application of a new mixed A_{2A}AR-A_{2B}AR antagonist, P626. Responses were quantified as the initial slope (between 20 and 80% of maximal amplitude) of the fEPSP trace. Input-output curves were obtained by gradual increase in stimulus strength at the beginning of each experiment.

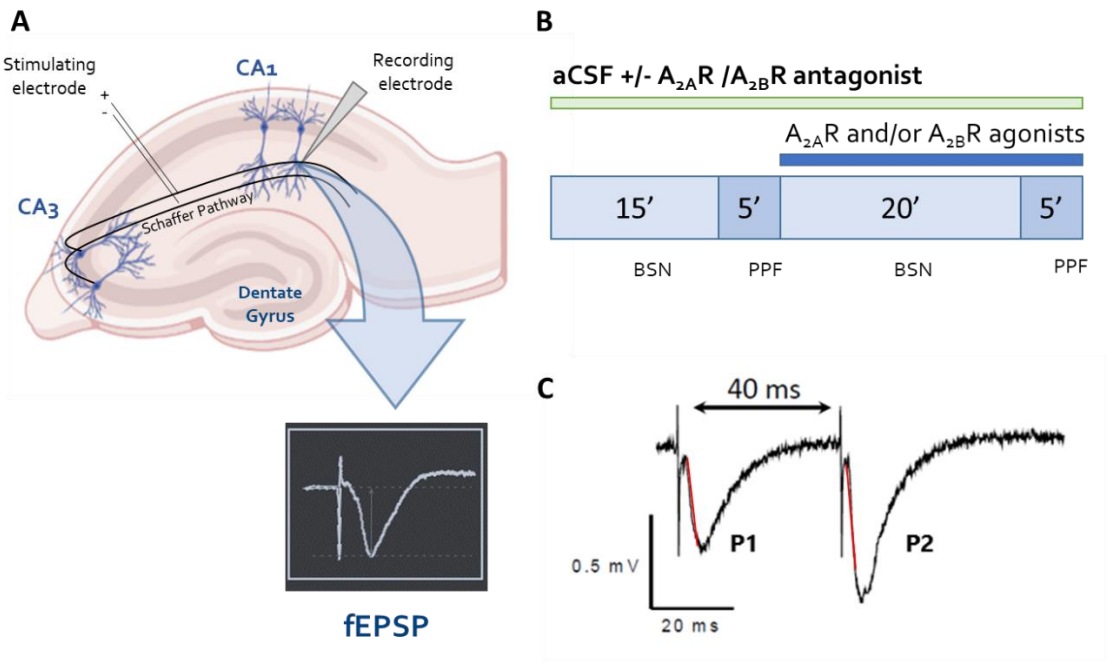


Figure 19. ***Experimental procedure*****.** (A) Schematic representation of an acute hippocampal slice showing the tri-synaptic circuit and the localization of the stimulating and recording electrodes. (B) Schematic synthesis of the experimental protocol used to apply A_{2B}AR and/or A_{2A}AR agonists alone or in the presence of the A_{2A}AR-A_{2B}AR antagonist. BSN: basal synaptic neurotransmission. (C) Representative trace of fEPSP responses evoked by a paired pulse facilitation (PPF) protocol (40-ms interval) in a typical hippocampal slice.

3.1.2.2 Oxygen-glucose deprivation protocol

Conditions of oxygen-glucose deprivation (OGD) were obtained by superfusing the slice with aCSF without glucose and gassed with nitrogen (95% N_2–5% CO_2) (Pedata *et al.,* 1993). This caused a drop in pO_2 in the recording chamber from ~500 mmHg (normoxia) to a range of 35–75 mmHg (after 7 min OGD) (Pugliese *et al.,* 2003). At the end of the ischemic period, the slice was again superfused with normal, glucose-containing, oxygenated aCSF. Throughout this Thesis, the terms "OGD slices" or "treated OGD slices" refer to hippocampal slices in which OGD episodes of different duration were applied in the absence or in the presence of drugs, respectively. Control slices were not subjected to OGD or drug treatment, but were superfused in oxygenated aCSF for identical time intervals.

The experimental method is shown in Figure 20. All the conjugated A_{2A}AR antagonists or the mixed A_{2A}AR-A_{2B}AR antagonist were applied 15 min before, during and 5 min after OGD. In a typical experimental day, first a control slice was subjected to severe

OGD. If the recovery of fEPSP amplitude after 60 min of reperfusion with glucose containing and normally oxygenated aCSF was 15% of the pre-OGD value, and AD developed into 30 min OGD, a second slice from the same rat was subjected to an OGD insult in the presence of the antagonist under investigation. To confirm the result obtained in the treated group, a third slice was taken from the same rat and another 30 min OGD was performed under control conditions to verify that no difference between slices was caused by the time gap between the experiments.

The same experimental protocol was use to tested Acetazolamide (ACTZ) and the CAIs, AN11-740 and AN6-277, that were applied 20 min before and during OGD application.

Simultaneously to fEPSP amplitude, AD was recorded as negative extracellular direct current (d.c.) shifts induced by OGD (Figure 20B). The d.c. potential is an extracellular recording considered to provide an index of the polarization of cells surrounding the tip of the glass electrode (Farkas et al., 2008). AD latency, expressed in min, was calculated from the beginning of OGD; AD amplitude, expressed in mV, was calculated at the maximal negativity peak. In the text and bar graphs, AD amplitude values were expressed as positive values. The terms “irreversible synaptic failure” or “irreversible loss of synaptic transmission” used in the present work refer to the maximal time window of cell viability in our experimental model (acutely isolated hippocampal slice preparation) which, according to our previous results, is 24 h (Pugliese et al., 2009).

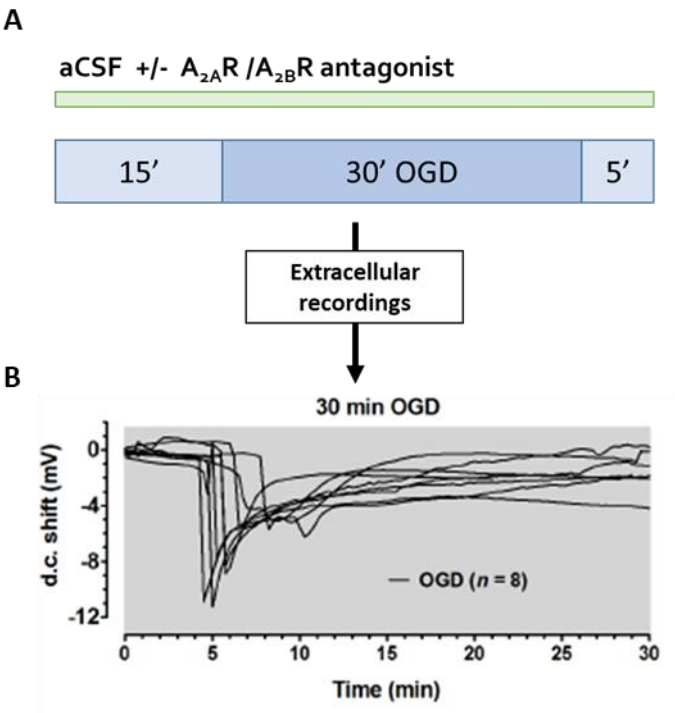


Figure 20. ***Experimental procedure***. (A) Schematic synthesis of the experimental protocol used to apply A_{2A}AR- A_{2B}AR antagonist during 30 min oxygen-glucose deprivation (OGD. (B) Representative direct current (d.c.) traces during 30 min OGD in untreated OGD hippocampal slices.

3.2 Patch-clamp recordings

3.2.1 Experimental procedure

For these studies patch clamp experiments in whole-cell configuration have been performed. To this purpose, cells were transferred to a recording chamber (1 ml volume) mounted on the platform of an inverted microscope (Olympus CKX41, Milan, Italy) and superfused at a flow rate of 1.5 ml/min with a standard extracellular solution, depending on the cell investigated (Table 1), by a three-way perfusion valve controller (Harvard Apparatus). Borosilicate glass electrodes Harvard Apparatus, Holliston, Massachusetts USA) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance suitable for each cell investigated (4÷7MΩ and 1.5÷3MΩ for OPCs and DRGs, respectively). Then, data were acquired with an Axopatch 200B amplifier (Axon Instruments, CA, USA), low pass filtered at 10 kHz, stored, and analysed with pClamp 9.2 software (Axon Instruments, CA, USA). All the experiments were carried out at room temperature (RT: 20–22°C). DRGs were voltage-clamped at -60 mV as previously described (Coppi et al., 2012), while OPCs at -70 mV, and input resistance (Rin) and membrane capacitance (Cm) were routinely measured by fast

hyperpolarizing voltage pulses (from -60 to -70 mV, 40 ms duration). Only cells showing a stable Cm and Rs before, during, and after drug application were included in the analysis. In some experiments, cell resting membrane potential (Vm) was determined immediately after seal breaking-through by switching the amplifier to the current-clamp mode. Current amplitude (measured as pA) was normalized to respective cell capacitance (Cm, measured in pF) and expressed as current density (pA/pF) in averaged results. All drugs were dissolved in extracellular solution and were applied by superfusion with a three-way perfusion valve controller (Harvard Apparatus, Holliston, MA USA) after a stable baseline was obtained. A complete exchange of bath solution in the recording chamber was achieved within 28 s.

Standard extracellular solution (mM)		
	OPCs	**DRGs**
Hepes	5	10
Glucosio	10	10
NaCl	140	147
KCl	5.4	4
$MgCl_2$	1.2	1
$CaCl_2$	1.8	2
pH	7.3 with NaOH 1M	7.4 with NaOH 1M

Standard intracellular solution (mM)	
	OPCs / DRGs
Hepes	10
K-gluconato	130
NaCl	4.8
EGTA	3
$MgCl_2$	2
$CaCl_2$	1
NaGTP	0.3
Na_2ATP	2
pH	7.4 with KOH 1M

Table 1. ***Electrophysiological solutions***. 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES); ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA).

3.2.1.1 Electrophysiological protocols used in OPCs

In all electrophysiological experiments, the following adenosine receptor antagonists were added to the extracellular solution in order to prevent nonspecific adenosine receptor activation upon the superfusion with various A_{2B}AR and A_{2A}AR agonists: DPCPX and MRS1523, all at 100 nM concentration, in order to block A_1AR and A_3AR respectively. A voltage ramp protocol (800 ms depolarization from −120 to +80 mV) was recorded every 15 s to evoke a wide range of overall voltage-dependent membrane currents before, during and after drug treatments. Variations in membrane potential (Vm) induced by drug treatments were measured by calculating the reversal potential (the "zero current" potential) of ramp evoked currents before, during and after drug application. Outward K^+ currents were evoked by two different depolarizing voltage-step protocols, in order to separate delayed rectifier outward K^+ currents (I_K) from transient outward (I_A) conductance. A first protocol consisted in 10 mV depolarizing voltage steps from −40 to +80 mV (200 ms each, 1 s inter-step interval) preceded by a 100 ms pre-step potential (Vpre) at −80 mV. This protocol activates a mixture of outward I_K and I_A currents in cultured OPCs. Since transient I_A currents present a voltage-dependent inactivation at potential positive to −80 mV, a second protocol was applied in the same cell with a pre step at −40 mV, to selectively inactivate I_A thus leaving the I_K component unchanged (I_K protocol). Net I_A current was then obtained in each cell by digital subtraction of the two current traces. Current-to-voltage relationships (I-V plots) of I_K or I_A currents were obtained by measuring current amplitude at the steady state (200–250 ms after step onset) of the I_K protocol or at the peak of subtracted trace (1–20 ms after step onset), respectively.

3.2.1.2 Electrophysiological protocols used in DRG neurons

Cell capacitance was used to estimate neuronal diameter by assuming an approximated spherical cell shape according to the calculated Cm for all biological membranes of 1 µF/cm^2 and to the equation of the sphere surface: $A = 4\pi r^2$ (Jain, 2000). Whole-cell patch-clamp recordings were performed on small-medium sized (Cm < 25 pF) DRG neurons. Current-clamp recordings were performed as described (Coppi et al., 2012; Coppi et al., 2019) in the standard extracellular solution, by lowering $CaCl_2$ (2 mM). A current step protocol lasting 1200 ms, from -10 pA to +40

pA, with current increments of 10 pA at each episode was applied. With this protocol it is possible to record the neuronal *firing*, i.e. the number of action potentials (APs) that are generated following a given depolarizing step. This protocol was applied before, during and after the drug application. To monitor the trend of Vm and the number of APs over time, a protocol consisting of a depolarizing step of 10 pA and lasting 10 ms, repeated every 30 s, was also applied. Current-clamp recordings were filtered at 10 kHz and digitized at 1 kHz.

4 Immunofluorescence analysis

DRG and OPC-DRG cultures grown on 13-mm diameter coverslips were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, Pan-Biotech, Milan, Italy) for 10 min at RT. Cells were washed twice with PBS and incubated in PBS solution containing 0.25% Triton X-100 (Sigma-Aldrich, Milan Italy) (PBST). After three washes in PBS, to block unspecific sites, cells were incubated with 10% goat serum (Sigma-Aldrich, Milan Italy) in PBST (PBST-GS) for 30 min.

DRG were then incubated for 2.5 h at RT in a solution of rabbit anti-β3-Tubulin (1:400; Cell Signaling, Massachusetts, USA) plus mouse anti- A_{2B}AR antibodies (1:200; Alomone, Jerusalem, Israel) in PBST-GS. Cells were then washed three times with PBS and incubated 1 h at RT with specific secondary antibodies AlexaFluor488 anti-mouse and AlexaFluor555 anti-rabbit (AbCam, UK, Cambridge), both diluted 1:500 in PBST-GS.

For OPCs-DRG, rabbit anti- β3-Tubulin (1:400; Cell Signaling, Massachusetts, USA) plus mouse anti-MBP primary antibodies (1:500; Merck Millipore, USA) were diluited in PBST-GS. Cells were then washed three times with PBS and incubated 1 h at RT with specific secondary antibodies (mentioned above). Coverslip were mounted with Fluoroshield (Sigma-Aldrich, Milan Italy) containing 4',6-diamidino- 2-phenylindole (DAPI) to stain cell nuclei. Immunocytochemical images were captured by a SP8 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany), using a 63X oil-immersion objective for DRG and a 20X for OPC-DRG. The collected images were analyzed with an open-source software (ImageJ, version 1.49v National Institutes of Health, Bethesda, MD, USA). Control experiments were performed by incubating fixed cells only with the secondary antibodies and DAPI to exclude

nonspecific binding. In the OPC-DRG cultures, MBP immunofluorescence was detected from the number of positive pixels above a threshold level in each confocal microscopy z-projections (Gerace et al., 2021) with the threshold tool in 3 random fields per coverslip. In the same images was evaluated the Manders' coefficients (M1) that estimate the co-occurrence fraction of a fluorescent signal on one channel (555 that corresponding to MBP) that overlaps the fluorescent signal of another channel (488 that corresponding to β3-Tubulin) in each region of interest (ROI) (Igado et al., 2020).

5 Drugs

The new mixed A_{2A}AR antagonists**, NPD168**, **NPD149**, **NPD133**, were synthetized by the pharmaceutical chemists of our team at the University of Florence, as mentioned in the introduction (Falsini et al., 2019). Their chemical structures are shown in Figure 22, 23 and 24. In addition, were tested **OTC**, **ALA**, **EDA** (Figure 20) and triazolopyrazine **NPD151** (Figure 21), a potent and selective A_{2A}AR antagonist (A_{2A}AR Ki = 8.1 nM; A_1AR, A_3AR, A_{2B}AR Ki> 30000), as reference molecules. The concentration of NPD 151, NPD 149, NPD 133, and NPD 168 was chosen according to binding experiments. The concentration of ALA, OTC and EDA was chosen in accordance with those used for the mixed compound (NPD).

Adenosine was purchased from Sigma/Merck Life Science S.r.l. (Milan, Italy).

The prototypical A_{2B}AR agonist 2-[[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide (**BAY60-6583**; Tocris, Bristol, United Kingdom) was used as a reference compound for the functional effects of A_{2B}AR activation.

The prototypical A_{2A}AR agonist [2-p-(2-carbossietil) fenetilammino-5'-N-etilcarbossiammide adenosine cloridrate idrate] (**CGS21680**; Tocris, Bristol, United Kingdom) was used as a reference compound for the functional effects of A_{2A}AR activation.

The prototypical A_1AR antagonist 8-cyclopentyl-1,3- dipropylxanthine (**DPCPX**; Sigma-Aldrich, Milan, Italy), the A_{2B}AR antagonist 8-(4-(4-(4-Chlorophenyl) piperazide-1-sulfonyl) phenyl)-1-propylxanthine (**PSB603**; Sigma-Aldrich, Milan, Italy)

and the A_3AR antagonist 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate (**MRS1523**; Sigma-Aldrich, Milan Italy) were used.

The new mixed A_{2A}AR-A_{2B}AR antagonist 7-ammino-2-(2-furanil) tiazolo[5,4-d] pirimidina derivate (**P626**, Figure 25) was synthesized by the research group of Pharmaceutical Chemists of Professor Varano at the University of Florence (Varano et al., 2020).

The new mixed A_{2A}AR-A_{2B}AR agonist 2-(2-(6-bromo-indol-3-yl)ethyloxy)adenosine (**MRS3997**) was synthesized by the research group of Pharmaceutical Chemists of Professor Jacobson of NIH (Figure 26).

CAIs 5-acetamido-1,3,4-thiadiazole-2-sulphonamide (**ACTZ**), the more lipid soluble compound, **AN11-740**, and **AN6-277** were used in the OGD experiments (Figure 27).

The concentration of each compound was chosen based on the affinity values for the receptors. Adenosine was dissolved in water. All drugs were dissolved in dimethyl sulphoxide (DMSO). Stock solutions, of 1000–10,000 times the desired final concentration, were stored at −20◦C. The final concentration of DMSO (0.05% in aCSF and 0.1% in extracellular solution) used in our experiments did not alter electrophysiological properties or drugs effects in hippocampal slices, OPCs and DRG neurons.

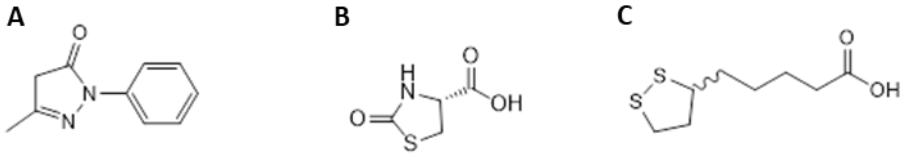


Figure 20. Antioxidant molecules. (A) Edaravon **(EDA)**; (B) 2-oxothiazolidin-4-carboxylic acid **(OTC)**; (C) α-Lipoic acid **(ALA)**.

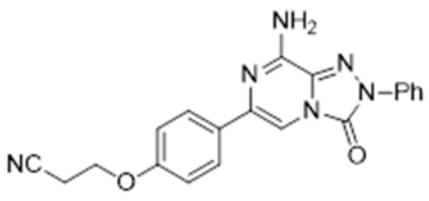


Figure 21. Ammino-6-(4-amminofenil)-2-fenil-[1,2,4] triazolo[4,3-a]pirazin-3(2H)-one **(NPD151)**. Selective A_{2A} Adenosine receptor antagonist.

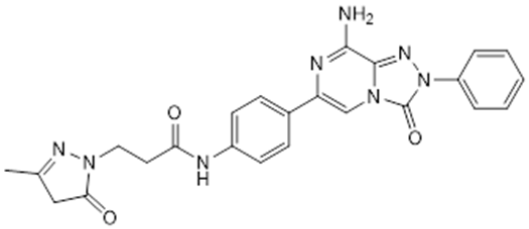


Figure 22. N-(4-(8-ammino-3-oxo-2-fenil-2,3-diidro-1,2,4-triazolo[4,3-a] pirazin-6-il)fenill)-3-(3-metil-5-oxo-2,5-diidro-1H-pirazol-1-il) propenammide **(NPD168)**. Mixed A_{2A} Adenosine receptor antagonist coniugate with EDA.

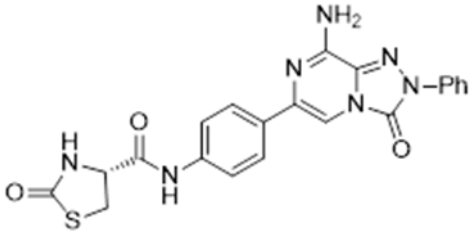


Figure 23. (NPD149). Mixed A_{2A} Adenosine receptor antagonist coniugate with OTC.

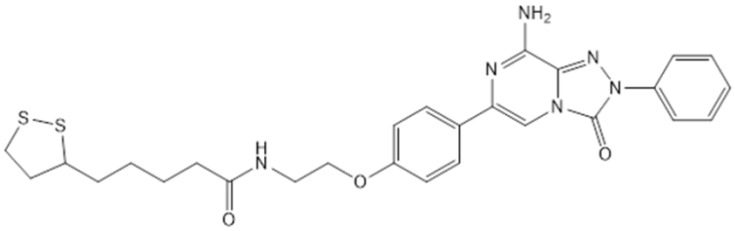


Figure 24. N-(2-(4-(8-amino-3-oxo-2-fenil-2,3 diidro -[1,2,4]triazolo[4,3-a]pirazin-6-il)fenossi)etil)-5-(1,2ditiolan-3-il)pentammide **(NPD133)**. Mixed A_{2A} Adenosine receptor antagonist coniugate with ALA.

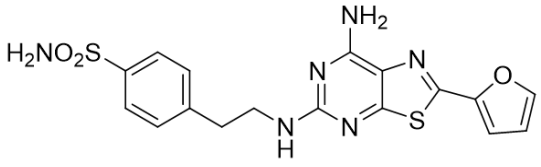


Figure 25. 7-ammino-2-(2-furanil) tiazolo[5,4-d] pirimidina derivate **(P626)**. Mixed A_{2A}- A_{2B} Adenosine receptor antagonist.

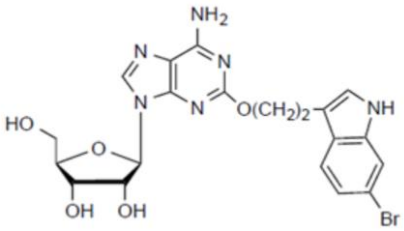


Figure 26. 2-(2-(6-bromo-indol-3-yl)ethyloxy)adenosine **(MRS3997)**. Mixed A_{2A}- A_{2B} Adenosine receptor agonist.

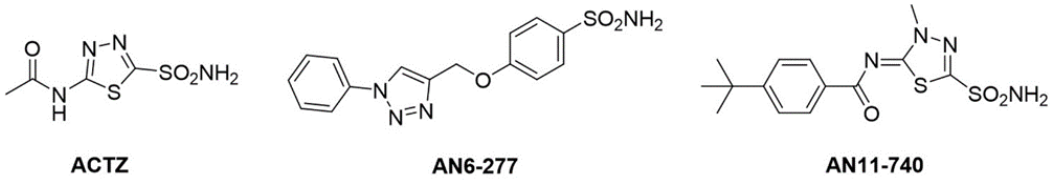


Figure 27. Chemical structure of the investigated CAIs: 5-acetamido-1,3,4-thiadiazole-2-sulphonamide (acetazolamide, **ACTZ**) and newly reported sulphonamide CAIs.

6 Statistical analysis

Data were expressed as mean ± SEM (standard error of the mean). Normality distribution of data was checked by Shapiro-Wilk test. Two-tailed Student's paired or unpaired *t*-tests or one-way ANOVA followed by Bonferroni post-test analysis were performed, as appropriated, in order to determine statistical significance (set at $P < 0.05$) between groups. Data were analyzed using "GraphPad Prism" (GraphPad Software, San Diego, CA, USA) software.

RESULTS and DISCUSSIONS

SECTION I - Pharmacological and functional characterization of A_{2A}/A_{2B}ARs on synaptic plasticity and during an ischemic-like insult: an ex-vivo study in the CA1 region of rat hippocampal slices

The role of adenosine "A_2" receptors was evaluated using extracellular electrophysiological recordings of fEPSPs in 91 slices of CA1 rat hippocampus, a brain area involved in synaptic plasticity phenomena and particularly susceptible to hypoxic-ischemic injuries. We monitored synaptic changes under different experimental conditions, as follow described, in the presence of selective agonists of these adenosinergic receptor subtypes or of new multi-target compounds (agonist and antagonist), able to simultaneously affect A_{2A}AR and A_{2B}AR.

1 Effects of BAY60-6583 and CGS21680, selective A_{2A}AR and A_{2B}AR agonists, respectively, on basal synaptic transmission

In a first series of experiments, we evaluated the effects of BAY60-6583, a selective A_{2B}AR agonist, on basal synaptic transmission in the CA1 region of rat hippocampus. As shown in Figure 28, BAY60-6583 (200 nM) did not significantly modify fEPSP slope during basal Schaffer collateral fiber stimulation (once every 15 s) in this region (from 0.37 ± 0.04 mV/ms before to 0.39 ± 0.05 mV/ms after 20 min of the application of the agonist; Figure 28A, n=10).

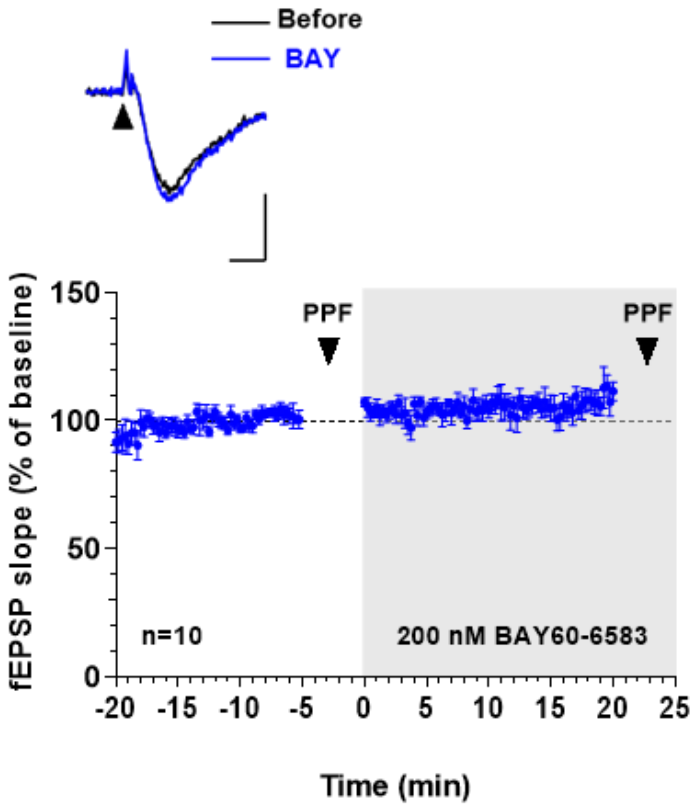


Figure 28. The selective A_{2B}AR agonist, BAY60-6583, did not modify basal synaptic transmission in the CA1 region of rat hippocampus. (A) Averaged time courses of fEPSPs slopes (mean ± SEM) evoked by Schaffer collaterals stimulation before and during BAY60-6583 application (BAY, 200 nM, n=10). The arrowheads indicate the time in which paired-pulse facilitation (PPF) protocol was applied. Insert: original fEPSP traces recorded in a typical experiment in control condition (before, black trace) or at the end of BAY application (blue trace). Calibration: 10 ms, 1 mV.

Conversely, the selective A_{2A}AR agonist, CGS21680 (50 nM, n=6), induced a modest, but significant, increase in fEPSPs slope (from 0.49 ± 0.03 mV/ms before (Ctrl) to 0.53 ± 0.03 mV/ms in the presence of the compound, Figure 29A). This result confirmed the involvement of A_{2A}ARs in the CA1 basal synaptic transmission in accord to Lopes et al. (2002). The enhancement in synaptic transmission was prevented by the new A_{2A}-A_{2B}ARs antagonist, P626 (200 nM, n=5). In particular, the slope values were 0.49 ± 0.04 mV/ms in P626 alone and 0.49 ± 0.04 mV/ms in combination with CGS21680 (Figure 29B). P626 did not modify *per sè* the basal synaptic transmission (see Table II).

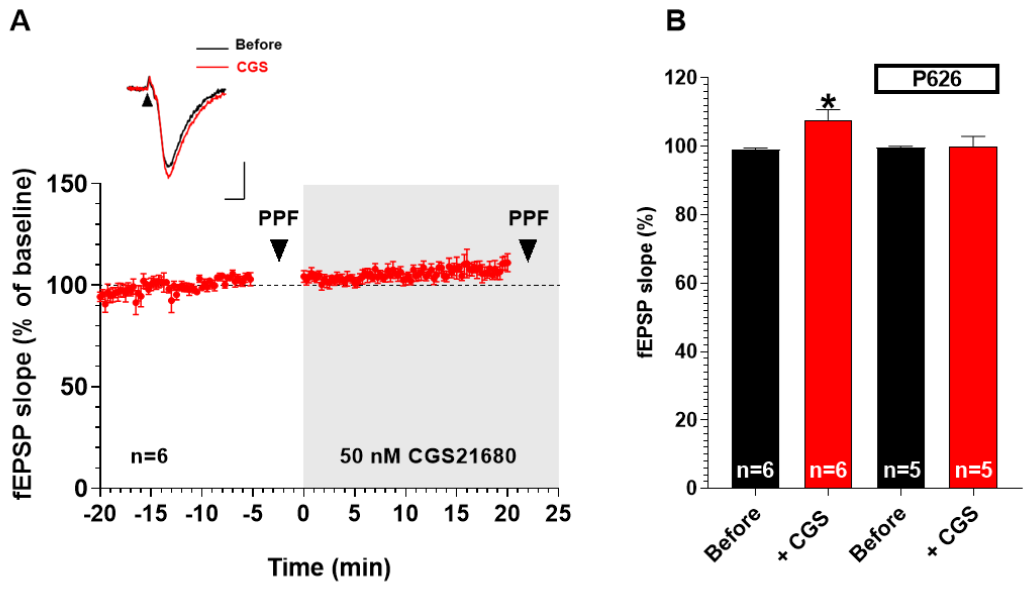


Figure 29. The new mixed "A_2" antagonist, P626, prevented the increase of synaptic transmission induced by CGS21680 in the CA1 region of rat hippocampus. (A) Averaged time courses of fEPSP slope measured during Schaffer collaterals stimulation under basal synaptic transmission before and during CGS21680 application (CGS, 50 nM, n=6). The arrowheads indicate the time of paired-pulse facilitation (PPF) application. Insert: original fEPSP traces recorded in a typical experiment in control condition (before, black trace) or at the end of 20 min CGS application (red trace). Calibration: 10 ms, 1 mV. (B) Pooled data of fEPSP slope (mean ± SEM) expressed as percentage of respective baseline calculated before PPF application. Note that CGS significantly enhanced basal synaptic transmission and that this effect was not evident in the presence of P626 (n=5). **P* < 0.05 Ctrl, paired Student's t-test. The number of slices (n) is reported in the columns.

1.1 The selective stimulation of A_{2A}AR or A_{2B}AR reduces paired-pulse facilitation (PPF), an effect prevented by the multi-target antagonist, P626

The previous series of experiments shows that only A_{2B}ARs are involved in basal synaptic transmission in the CA1 region of rat hippocampal slices. To investigate if "A_2" receptors are involved in synaptic plasticity, we studied the effects of their selective stimulation on paired-pulse facilitation. When a synapse is stimulated in rapid succession, the second post-synaptic response can be larger than the first and termed PPF. It has been reported that the paired-pulse ratio (P2/P1), which is the ratio of the slope of the second response to that of the first, depends on the probability of vesicular release at the synapse, and this parameter has been used as an easy measure of the neurotransmitter release probability. PPF is widely considered to be of pre-synaptic origin (Zucker and Regehr, 2002). Direct evidence

for the inverse relation of the PPF ratio to the release probability was shown by Dobrunz and Stevens (1997) using the minimal stimulation protocol. Low-strength paired stimuli delivered at an interval of 40 msec evoked two EPSPs. In accordance with previous results (Goncalves et al., 2015; Fusco et al., 2019; Lopes et al., 2002) we confirmed that the selective A_{2B}AR agonist BAY60-6583 and the selective A_{2A}AR agonist CGS21680, applied separately, significantly decreased P2/P1 of fEPSPs slope values measured at the end of a 20 min application *versus* respective pre-drug baseline in rat hippocampal slices (Figure 30, 31). Indeed, the P2/P1 ratio was reduced from 1.62 ± 0.06 in control (Ctrl) to 1.54 ± 0.05 in the presence of 200 nM BAY60-6583 (Figure 30A, n=10). Regarding the A_{2A}AR agonist, P2/P1 ratio was from 1.72 ± 0.01 in Ctrl to 1.67 ± 0.02 in the presence of 50 nM CGS21680 (Figure 31A, n=6). An action of "A_2" receptors ligands at presynaptic level could underlie an increase or a decrease in neurotransmitter release, and it will also be likely to greatly affect the paired-pulse facilitation (Manabe et al., 1993). Indeed, the facilitation of the second response is believed to be due to increase glutamate release consequent to a rise in Ca^{2+} concentration caused by the conditioning response (Wu and Saggau, 1994).

In addition, we demonstrated for the first time that the application of 200 nM P626 completely prevented the effect of BAY60-6583 (Figure 30B, n=5) or CGS21680 (Figure 31B, n=5) in reducing paired- pulse facilitation.

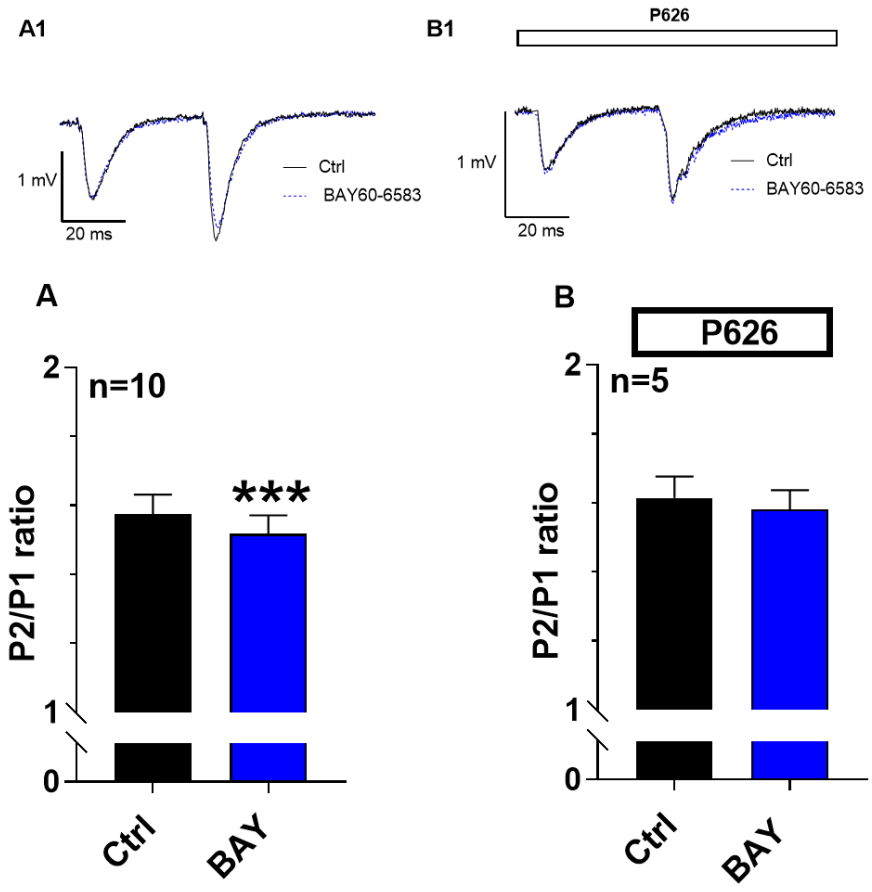


Figure 30. Effects of the newly synthesized multi-target antagonist, P626, on the inhibition of paired-pulse facilitation (PPF) induced by BAY60-6583. (A-B) Each graph shows pooled data of PPF quantified as the ratio (P2/P1) between the slopes of the fEPSPs elicited by the second (P2) and the first (P1) stimuli. It is evaluated before (control: Ctrl) or after BAY60-6583 (BAY, 200 nM) application in the absence (A, n=10) or in the presence of P626 (200 nM, n=5, B). (A1-B1) Representative traces of fEPSP responses evoked by a PPF protocol in a typical slice recorded in control conditions (Ctrl, black trace), in the presence of BAY applied alone (A1, blue trace) or with P626 (B1). ***P < 0.001 vs Ctrl, paired Student's t-test.

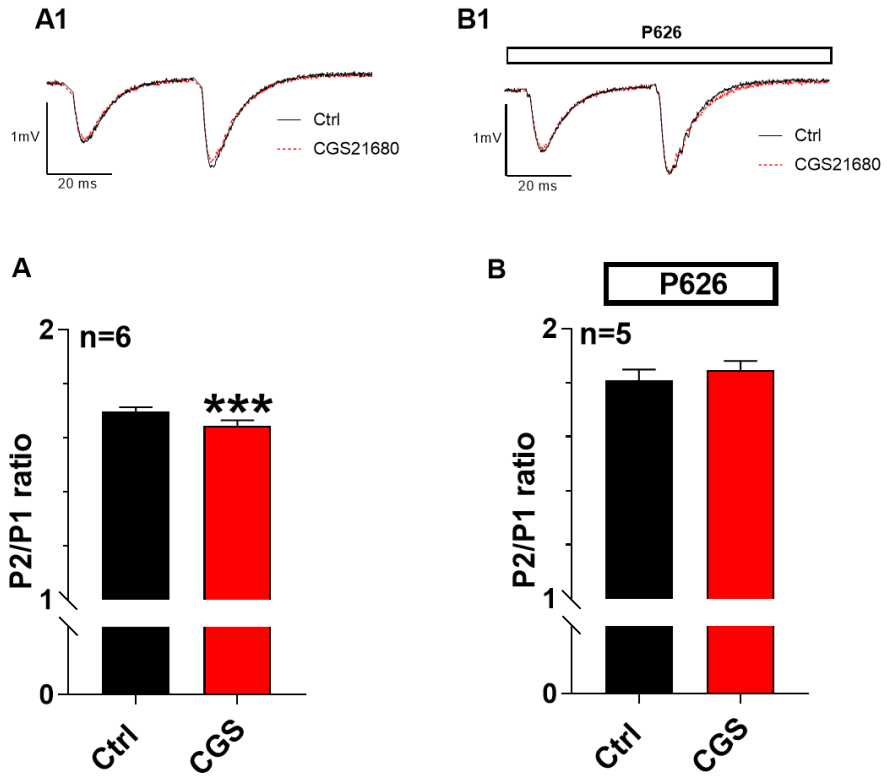


Figure 31. Effects of the newly synthesized multi-target antagonist, P626, on the inhibition of paired-pulse facilitation (PPF) induced by CGS21680. (A-B) Each graph shows pooled data of PPF quantified as the ratio (P2/P1) between the slopes of the fEPSPs elicited by the second (P2) and the first (P1) stimuli. It is evaluated before (control: ctrl) or after CGS21680 (CGS, 200 nM) application in the absence (C, n=6) or in the presence of P626 (200 nM, n=5). (A1-B1) Representative traces of fEPSP responses evoked by a PPF protocol in a typical slice recorded in control conditions (Ctrl, black trace), in the presence of CGS applied alone (A1, red trace) or with P626 (B1). ***$P < 0.001$ vs Ctrl, paired Student's t-test.

1.2 Effects of the simultaneous stimulation of A_{2A}AR and A_{2B}AR on CA1 neurotransmission under basal conditions and during PPF

In next experiments, we decided to apply BAY60-6583 and CGS21680 in combination to evaluate a possible additive effect on hippocampal synaptic transmission compared to that obtained in the presence of the drug alone. The combined use of BAY60-6583 and CGS21680 (applied at the same concentrations of previous experiments) had no effects on basal synaptic transmission (Figure 32A). In particular, when the compounds were applied together the increase in fEPSP induced by the selective A_{2A}AR agonist alone (See Figure 29) was never recorded in the 13 slices analyzed. Moreover, we tested in the same experimental protocol, a new synthetized A_{2A}-A_{2B}ARs agonist, MRS3997. Again, MRS3997 did not modify the basal

neurotransmission (Figure 32B) similarly to what observed with the co-application of the A_{2B}AR and A_{2A}AR agonists (see Table II).

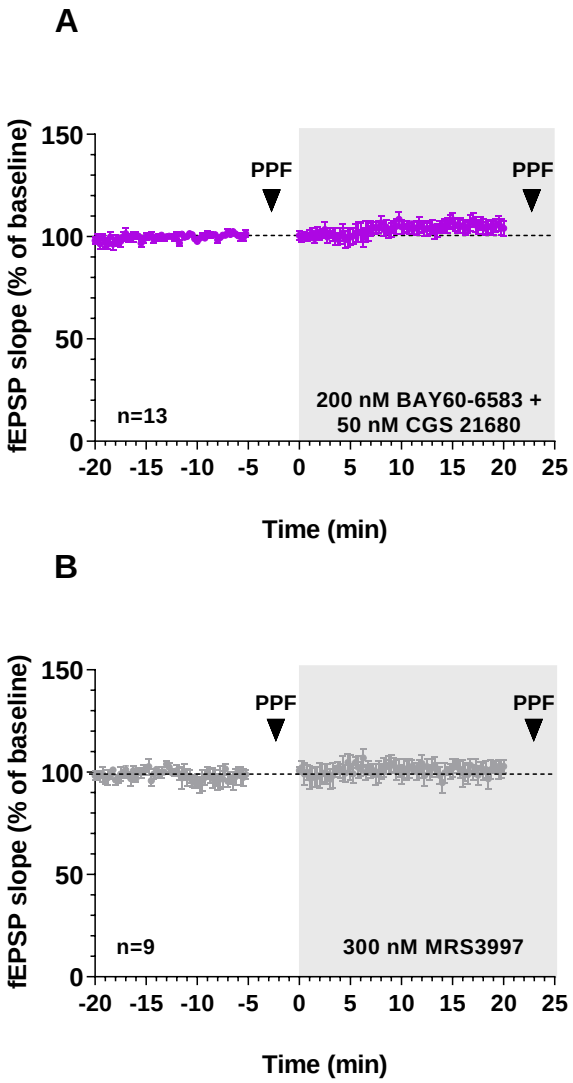


Figure 32. The simultaneously activation of A_{2A}AR and A_{2B}AR did not modify the basal synaptic transmission in the CA1 region of rat hippocampus. (A-B) Averaged time courses of fEPSP slope measured during Schaffer collaterals stimulation in conditions of basal synaptic neurotransmission, evoked once every 15 s, before and during CGS21680 (CGS, 50 nM) + BAY60-6583 (BAY, 200 nM) (n=13, A) or MRS3997 (300 nM, n=9, B) application. The arrowheads indicate the paired-pulse facilitation (PPF) duration.

Treatment	n	Before (mV/ms)	After 20' application (mV/mS)
BAY60-6583	10	0.37 ± 0.04	0.39 ± 0.05
CGS21680	6	0.49 ± 0.03	0.53 ± 0.03 *
BAY60-6583 + CGS21680	13	0.55 ± 0.03	0.57 ± 0.04
MRS3997	9	0.53 ± 0.10	0.54 ± 0.11
P626	4	0.62 ± 0.10	0.65 ± 0.12

Table II. Effects of the A_{2A}AR or/and A_{2B}AR ligands on fEPSP slope under basal synaptic transmission. Note that only the selective A_{2A}AR agonist, CGS21680, significantly affected basal synaptic transmission. *P < 0.05 vs Ctrl, paired Student's t-test.

In addition, we observed that the co-application of BAY60-6583 and CGS21680 did not reduce PPF ratio (Figure 33A, n=13) while, the application of the multi-target agonist, MRS3997, reduced P2/P1 ratio similarly to BAY or CGS. In particular, the P2/P1 ratio was from 1.68 ± 0.05 in Ctrl to 1.65 ± 0.05 in the presence of MRS3997 (Figure 33C, n=9,). This effect was blocked by the new mixed antagonist, P626 (Figure 33D).

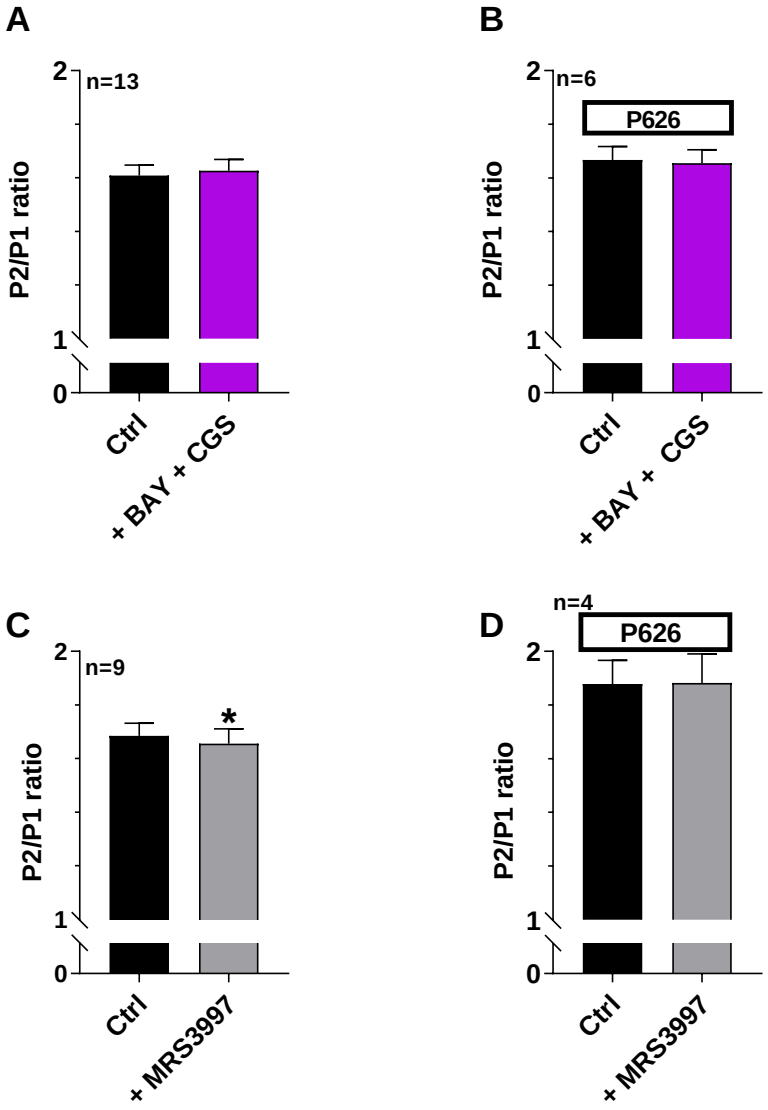


Figure 33. The mixed A_{2A}-A_{2B}ARs agonist reduced Paired-Pulse Facilitation (PPF) in the CA1 region of rat hippocampus; an effect that was not mimicked by the co-application of the selective A_{2A}AR and A_{2B}AR agonists. (A-B) Each graph shows pooled data of PPF quantified as the ratio (P2/P1) between the slope of the second fEPSP (P2) and the first fEPSP (P1) before (control: Ctrl) or after BAY60-6583 (BAY, 200 nM) + CGS21680 (CGS, 50 nM) application in the absence (A, n=13) or in the presence (B, n=6) of P626 (200 nM). (C-D) Each graph shows pooled data of PPF before (control: Ctrl) or after MRS3997 (200 nM) application in the absence (C, n=9) or in the presence of P626 (200 nM, n=4). Note that no significant difference was found in the P2/P1 measured in the absence or presence of the A_{2B}AR agonist + A_{2A}AR agonist applied alone or with the new mixed antagonist, P626 (A-B), while the new mixed agonist significantly reduced PPF and the effect was prevented by P626. *P < 0.05 vs Ctrl, paired Student's t-test. The number of slices (n) is reported in the columns.

1.3 Effects of the new multi-target A_{2A}-A_{2B}ARs antagonist during oxygen-glucose deprivation in the CA1 region of rat hippocampus

Finally, we monitored the neurotransmission before and after the application of a severe, 30 min-long, OGD in which the appearance of AD, an unequivocal sign of tissue damage, was always recorded (Pugliese et al., 2009). The experiments were conducted in the absence or in the presence of the new multi-target compound, P626, applied at different concentrations. As illustrated in Figure 34, an OGD of this duration always elicited the appearance of AD in untreated OGD slices (Figure 34A), with a mean latency of 6.23 ± 0.30 min and a mean peak amplitude of 7.16 ± 0.47 mV (n=13). When 30 min OGD was applied in the presence of 400 nM P626, the d.c. shift was always delayed and the AD latency values were postponed to 7.84 ± 0.21 min (n=8, Figure 34D and Figure 35A). The "A_2" receptors antagonist P626 was ineffective on AD latency induced by severe OGD at a concentration of 10 nM or 100 nM (Figure 35A). Finally, no difference in AD amplitude among all experimental groups was found (Figure 35B).

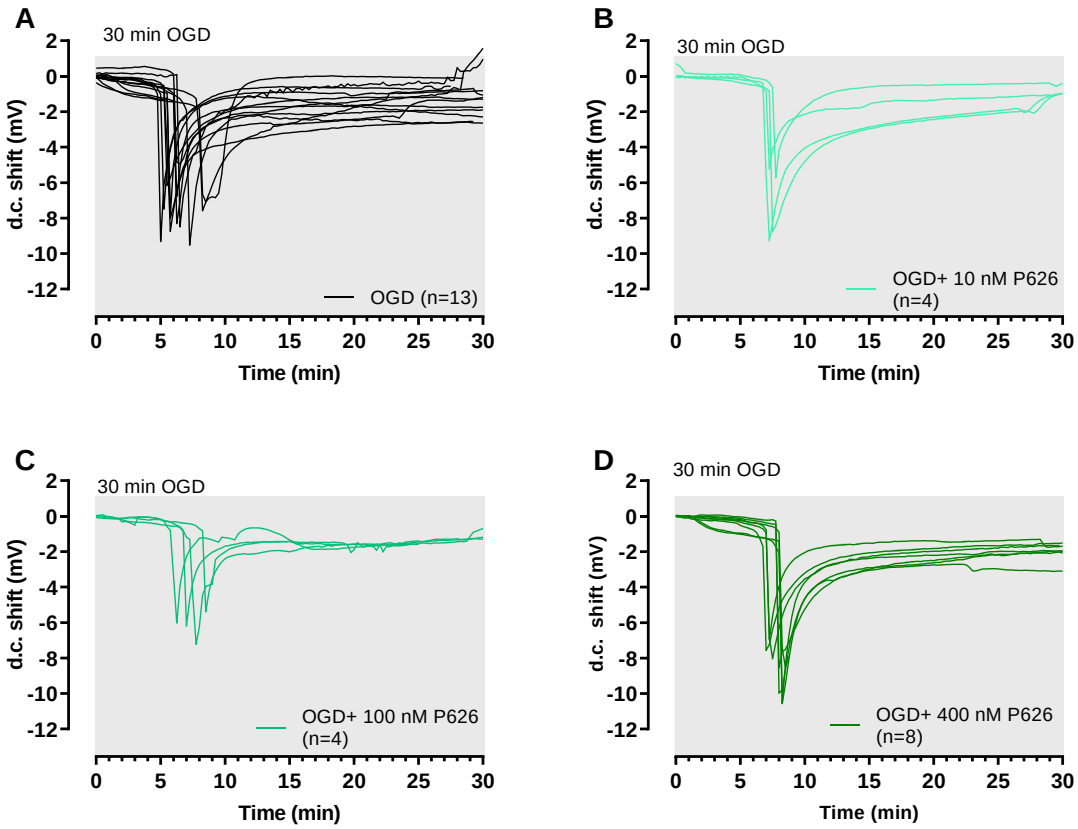


Figure 34. The new synthesized A_{2A}-A_{2B}ARs antagonist, P626, delayed the appearance of Anoxic Depolarization (AD) induced by 30 min OGD in rat hippocampal slices. (A–D) The graphs show the d.c. shift traces during 30 min OGD in untreated OGD slices (A, n = 13), in the presence of 10 nM P626 (B, n=4), 100 nM P626 (C, n=4), or 400 nM P626 (D, n=8).

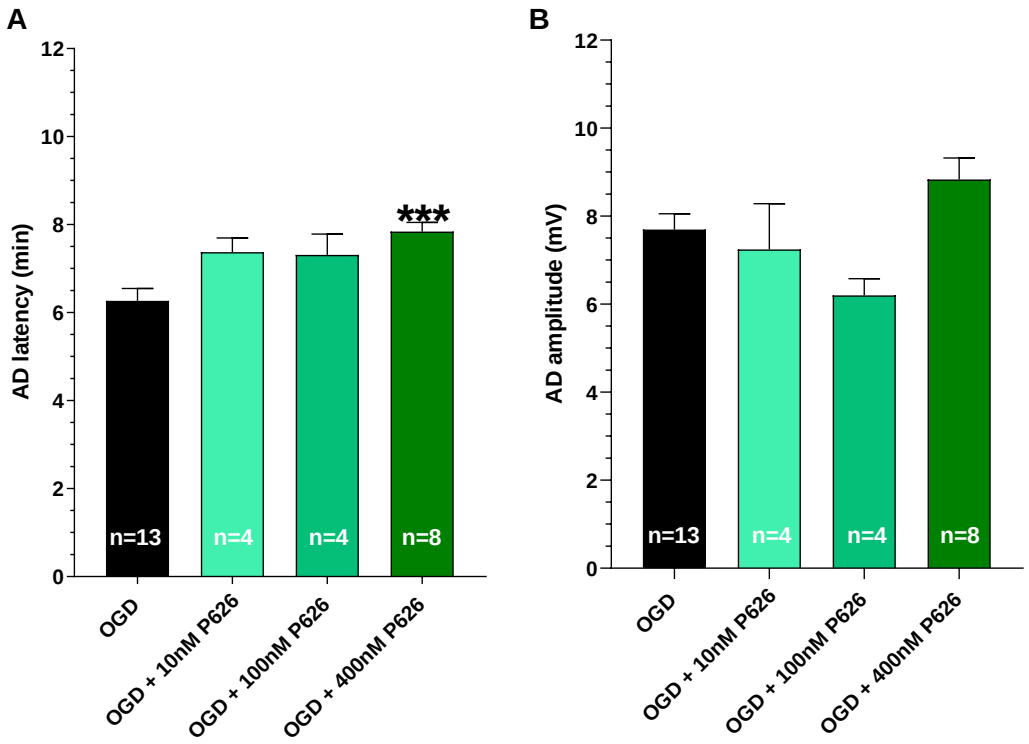


Figure 35. P626 delayed the appearance of Anoxic Depolarization (AD) induced by 30 min OGD in rat hippocampal slices without significantly affect AD amplitude. (A) Each column represents the mean ± SEM of AD latency recorded in hippocampal slices during 30 min OGD in different experimental groups. AD was measured from the beginning of OGD insult. Note that 400 nM P626 significantly delayed AD development. ***P < 0.001 vs. OGD, One-way ANOVA followed by Bonferroni multiple comparison test. (B) Each column represents the mean ± SEM of AD amplitude recorded in the CA1 during 30 min OGD. The number of slices (n) is reported in the columns.

1.4 DISCUSSION

The role of "A_2" receptor subtypes on synaptic plasticity and during oxygen-glucose deprivation in the CA1 hippocampus was studied by the application of selective A_{2A}AR and A_{2B}AR agonists and, for the first time, of the multi-target A_{2A}-A_{2B}AR agonist (MRS3997) and antagonist (P626). Multi-target compounds are designed to activate more than one cellular target simultaneously. Therefore, these molecules offer the possibility of allowing better pharmacokinetic and symptomatologic control in various pathological conditions by reducing side effects due to the administration of two different molecules.

The hippocampus is a brain region extremely used to study the effects of drugs on neurotransmission, synaptic plasticity phenomena and cerebral ischemia, as it plays an important role in learning and memory and it is known to be particularly susceptible to hypoxic-ischemic insults (Bird and Burgess, 2008). The A_{2B}AR has the lowest affinity for adenosine in comparison to other adenosine receptors, suggesting a relevant role of this receptor subtype under hypoxic injuries or all stress conditions, where adenosine concentration is significantly increased (A_{2B}AR Ki = 15,000 nM vs A_{2A}AR Ki = 310 nM, A_3AR Ki = 290 nM, and A_1AR Ki = 100 nM) (Erices et al., 2022). Up to now, it is known that A_{2B}ARs are localized on presynaptic glutamatergic terminals of the hippocampus (Goncalves et al., 2015), while A_{2A}ARs are expressed both presynaptically and postsynaptically.

In this Thesis, we firstly evaluated the effects of the selective A_{2A}AR and A_{2B}AR agonists, CGS21680 and BAY60-6583 respectively, on hippocampal neurotransmission either in basal condition or during PPF stimulation at Schaffer collateral-CA1 synapses. In particular, we confirmed that CGS21680 significantly increased basal synaptic transmission according to what described by Lopes et al. (2002) and Fusco et al. (2019), respectively. On the other hand, the A_{2B}AR agonist BAY60-6583 did not significantly modify basal synaptic transmission. The different expression level between A_{2A}ARs and A_{2B}ARs may explain the sole involvement of A_{2A}AR in basal synaptic transmission. The increase in fEPSP slope in the presence of CGS21680 is associated to a presynaptic increase in glutamate release (see our results on PPF) and a contemporary presynaptic attenuation of A_1AR inhibitory response (Lopes et al., 2002). The effect of CGS21680 was blocked by the new multi-target A_{2A}-A_{2B}ARs antagonist, P626, applied for the first time in this experimental paradigm. This compound, synthetized by the group of Professor Varano (2020), shows a high affinity for both "A_2" receptor subtypes.

The facilitation of neurotransmission induced by A_{2A}AR activation was not observed in the presence of MRS3997, the multi-target "A_2" receptors agonist, nor during co-application of the two A_{2A}AR and A_{2B}AR selective agonists, CGS21680 and BAY60-6584, respectively. We suppose that the lack of A_{2A}AR-mediated effects of basal neurotransmission when the "cognate" A_{2B}AR is activated may be linked to the presence of an A_{2A}AR-A_{2B}AR heterodimer in the CA1 hippocampal region, as already

observed by Hinz et al. (2018) and by Lillo et al. (2022). Hinz and colleagues (2018) further demonstrate an altered pharmacology of the A_{2A}AR when co-expressed with the A_{2B}AR in CHO cells, as well as in native cells. Indeed, they observe that increasing expression of the A_{2B}AR in CHO cells results in a decrease of A_{2A}AR-mediated signalling. We can conclude that the selective and simultaneous stimulation of both "A_2" receptors brings out to a loss of A_{2A}AR response, leaving the sole A_{2B}AR-mediated effect. This event could be relevant when extracellular ADO concentrations are increased to micromolar levels (stress conditions) or during particular short-term synaptic plasticity events, such as PPF. To deepen our investigation, we tested the "A_2" receptor agonists, alone or in combination, on PPF. This electrophysiological protocol is considered a clear index of neurotransmitter release probability. PPF is well characterized in the CA1 region of hippocampus, where it is known that the activation of A_1AR increased PPF ratio, due to a reduction of glutamate release, while the "A_2" stimulation reduced the PPF, by facilitating glutamate release (Fernandez-Fernandez et al., 2015; Lopes et al., 2002). In this Thesis, we confirmed that the selective application of A_{2A}ARs or A_{2B}ARs agonists decreased PPF (Lopes et al., 2002; Goncalves et al., 2015; Fusco et al., 2019). These inhibitory effects were antagonized by the new multi-target compound, P626. Surprisingly, the effect of the selective A_{2A}AR or A_{2B}AR agonist on PPF was mimicked only by the new mixed A_{2A}-A_{2B}AR agonist, MRS3997, and not by the co-application of CGS21680 and BAY60-6583. We do not have an unequivocal explanation for these contradictory results. However, if we assume again the hypothesis of A_{2A}AR-A_{2B}AR dimerization in CA1 hippocampal neurons as stated above, we can hypothesize that the efficacy of BAY60-6583 and that of MRS3997 in activating the A_{2B}AR "counterpart" of the heterodimer is not equivalent. This divergence could be due, as an example, to the fact that the binding site of BAY60-6583, but not that of MRS3997, to the A_{2B}AR is hindered within the dimer *core*. Furthermore, functional selectivity in the form of biased agonism could be another possible explanation because, as demonstrated in the literature, GPCRs are particularly prone to this phenomenon, especially if the receptor is part of a heteromer (Lillo et al., 2022).

Another unexpected finding, concerning MRS3997-mediated PPF inhibition, is the lack of additive effects of this compound in comparison to PPF inhibition obtained by

each selective agonist, CGS21680 and BAY60-6583, applied alone. Traditional interpretation of these pharmacological data would be the convergence of both receptor signalling on the same intracellular pathway, which is reasonable for two Gs-coupled receptors as the "A_2". However, in line with the interpretation of previous data on basal neurotransmission, we can confirm the hypothesis of an A_{2A}AR-A_{2B}R heterodimer where the A_{2B}AR "counterpart" inhibits the A_{2A}AR-mediated response. . Globally, our results on PPF could confirm that A_{2A}AR or A_{2B}AR signalling is context-dependent, i.e. heteromer structure and/or allosteric interactions with G-proteins and scaffolds. In addition, the chemical structure of MRS3997 can be responsible of the persistence of its inhibitory effect on PPF; however, differential selectivity depending on the context may also be the answer (Lillo et al., 2022). We consider that different concentrations of these compounds should be applied and further experiments are necessary to better explain these responses.

We can conclude that stimulation of A_{2A}AR or A_{2B}AR induced an increase in glutamate release in the CA1 hippocampal slices. This glutamate increase plays different roles under physiological condition, facilitating neuronal excitability, synaptic plasticity and, coordination of neural networks. However, under pathological conditions (such as cerebral ischemia), an increase in glutamate release contributes to excitotoxic damage (Dirnagl et al., 1999). The early phases of a hypoxic-ischemic insult are characterized by a significant increase in extracellular glutamate levels, which triggers a hyper-activation of glutamate receptors, production of reactive oxygen species, pathological increase in intracellular Ca^{2+}, rapid decrease in ATP reserves, and activation of various proteolytic enzymes (Káradóttir et al., 2005; Al-Majed et al., 2006; Kovacs et al., 2006). Contemporarily to the glutamate-increase, also the extracellular concentration of adenosine significantly raised, as demonstrated by both *in vivo* and *ex-vivo* experiments (Latini and Pedata, 2001).

OGD, which is an experimental condition that mimics the most common consequences of cerebral ischemia, although with the limits of the *ex-vivo* methodology, enable us to obtain highly valuable information in terms of the time-course of the electrophysiological events, changes in membrane potential (i.e. AD), and in synaptic transmission. As mentioned above, the hippocampus, and particularly CA1 stratum pyramidale, is one of the most vulnerable brain regions to an ischemic

damage. *Ex-vivo* model of acute rat hippocampal slices allows measurements of synaptic transmission with good spatial and temporal resolution during an ischemic-like insult. It has been demonstrated that the synaptic potentials are depressed after a moderate or severe OGD in the CA1 hippocampal region (Latini et al., 1999; Pugliese et al., 2003). However, depending on the OGD duration, fEPSPs can restore their amplitude (Pugliese et al., 2003; Pearson et al., 2006; Frenguelli et al., 2007) when reperfused with oxygenated aCSF. After short OGD (2–5 min) periods, which never elicit the appearance of AD, synaptic transmission fully recovers (Fowler, 1992; Latini et al., 1999; Pugliese et al., 2003). On the other hand, a prolonged OGD (at least 7 min) causes a fully disappearance of synaptic activity and development of AD (Pugliese et al., 2009). It is well established that sustained activation of N-methyl-d-aspartate (NMDA)-type glutamate receptors is essential to AD initiation and propagation leading to excitotoxic neuronal death in stroke. Therefore, it is well accepted that a pharmacological treatment that postpones the onset of a perinfarct depolarization helps to protect brain tissue after ischemia (Somjen, 2001). It is known from the literature, that the selective A_{2A}AR or A_{2B}AR antagonists, ZM241385 and PSB603, respectively, prevent or delay the onset of AD and reduce the synaptic damage induced by severe OGD in the CA1 region of rat hippocampus (Pugliese et al., 2009; Fusco et al., 2019). Therefore, we tested the multi-target antagonist P626 during 30 min OGD in this hippocampal region. In agreement with data present in the literature (Latini et al., 1999; Pugliese et al., 2003), we confirmed that 30-min OGD always induces the appearance of AD (Somjen, 2001), detectable as a negative deflection of membrane potentials (d.c shift), in the CA1 rat hippocampal slices. Furthermore, according to previous results obtained in the same *ex-vivo* preparation (Pugliese et al., 2006 and 2007; Frenguelli et al., 2007), after the onset of AD, no recovery of neurotransmission is observed under control conditions (data not shown). We demonstrated for the first time that the multi-target antagonist, P626 is able to delay the AD onset during 30-min OGD in the CA1 rat hippocampus. The entity of the effect of P626 on AD latency was similar to those observed in the presence of the selective A_{2A}AR or A_{2B}AR antagonists applied alone, indicating, again, no additive effects of P626. We supposed that it could be due to different temporal activation of the adenosine receptor subtypes. Indeed, during the first minutes (2-3-min) after

an ischemic insult the concentration of adenosine gradually increased activating principally the higher affinity A_1AR, A_{2A}AR and, A_3AR subtypes. Then (after ~ 4 min, see in Latini et al., 1998, 1999), when the adenosine concentration reaches micromolar levels, it is able to activate also the A_{2B}AR subtype. This hypothesis agrees with the results of Fusco et al. (2019), which demonstrated that during the first two min of an OGD, the A_{2B}AR antagonist PSB603 is unable to modify OGD-induced fEPSP depression. This is consistent with the extracellular adenosine levels measured over such period, that is about 5 µM (Latini et al., 1998, 1999b; Frenguelli et al., 2007; Zur Nedden et al., 2014), which are not sufficient to activate the A_{2B}AR subtype (Fredholm et al., 2011). Therefore, the new multi-target antagonist, P626, could explain its action in delaying AD only on the A_{2A}AR subtype during the first minutes of an OGD. On the other hand, in the min following the OGD, when the adenosine reaches micromolar concentration, P626 could predominantly antagonize the A_{2B}AR subtype. Thus, on the bases of the experiments previously described on PPF, we hypothesized the presence of an A_{2A}AR-A_{2B}AR heterodimer in the CA1 hippocampus able to functionally reduce or occlude the effects of at least one of the two "A_2" receptors. However, this hypothesis has no relevance during oxygen and glucose deprivation if the sequentially and not simultaneously "A_2" receptors activation by endogenous adenosine is confirmed.

In conclusion, the novel dual A_{2A}-A_{2B}AR antagonist, P626, prevented the decrease of PPF induced by the selective stimulation of A_{2A}ARs or A_{2B}ARs, indicating that the compound counteracted the increase of glutamate release induced by the selective agonists. In line with this effect, P626 delayed the appearance of AD induced by a severe OGD, indicating a neuroprotective role of the drug. Hence, P626 could represent a favorable strategy to achieve neuroprotection by a simultaneous block of "A_2" receptors subtypes during an acute ischemic insult. Meanwhile, the contemporary activation of "A_2" receptors subtypes during synaptic plasticity needs further investigations, in order to also highlight the activity of the A_{2A}AR-A_{2B}AR heterodimer.

2 Functional characterization of new selective and multi-target A_{2A}ARs antagonists during an ischemic-like insult: an *ex-vivo* study in the CA1 rat hippocampus

The experiments were performed on 82 slices in order to test the role of new selective and multi-target A_{2A}AR antagonists during severe OGD in the CA1 rat hippocampus. The role of these compound on A_{2A}ARs was evaluated as mentioned in the previously results.

2.1 NPD149 and NPD133, two A_{2A}AR antagonists conjugated with OTC and Lipoic Acid, respectively, prevent the appearance of AD and the irreversible loss of neurotransmission induced by 7 min OGD in CA1 hippocampus

In agreement with our previous results (Pugliese et al., 2006; 2007; 2009), a d.c. shift was always present when the OGD was 7 min long. The d.c. shift presented a mean latency of 6.24 ± 0.2 min (calculated from the beginning of OGD) and a mean peak amplitude of -7.87 ± 0.9 mV (n=7) (Figure 36A). In addition, 7 min OGD exposure induced a rapid and irreversible depression of fEPSPs amplitude evoked by Schaffer-collateral stimulation, since synaptic potentials did not recover their amplitude after return to oxygenated aCSF (OGD, n=7, Figure 36D). OGD was then applied in the presence of the new mixed compounds NPD149 and NPD133, administered 15 min before, during and 5 min after OGD. The two mixed antagonists did not modify basal synaptic transmission measured before OGD. Nevertheless, NPD149 and NPD133 were able to prevent or delay the appearance of AD and to permit synaptic recovery after OGD only in the slices in which AD was absent during OGD (Figure 36B-E).

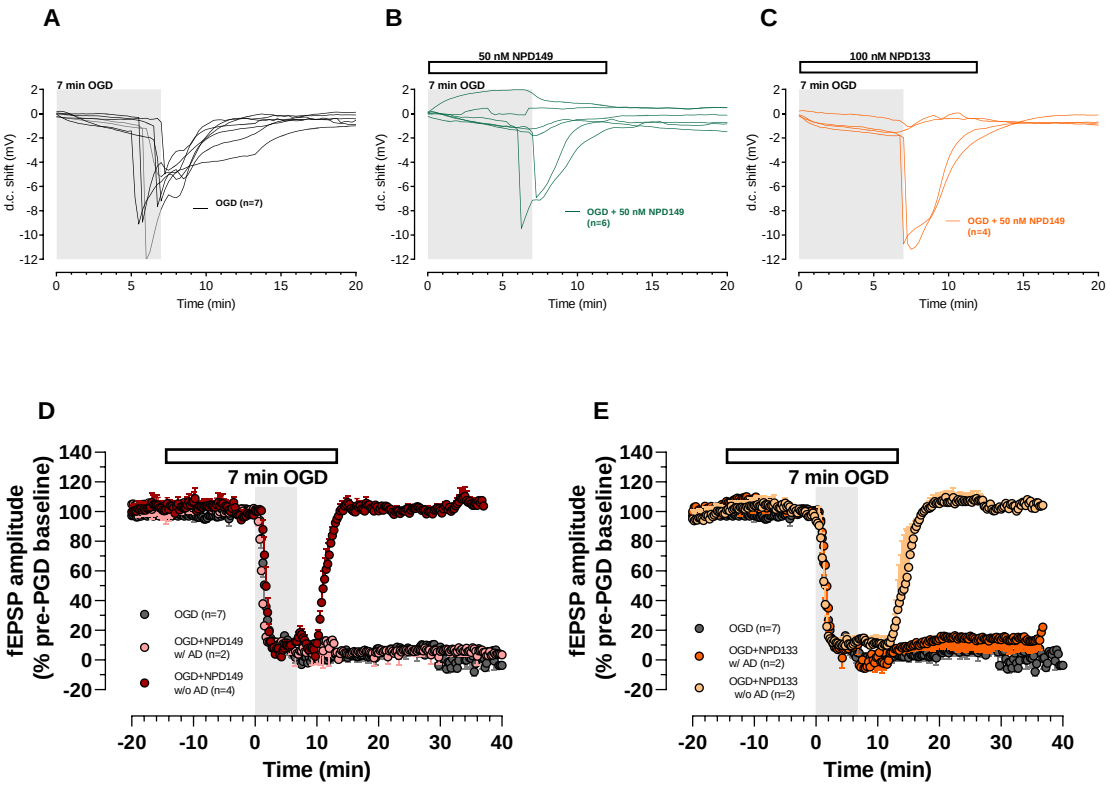


Figure 36. Effects of NPD149 and NPD133 on synaptic failure induced by 7 min OGD in rat hippocampal slices. (A) The graph shows the d.c. shift traces during 7 min OGD in untreated slices (n=7), during 7 min OGD carried out in the presence of 50 nM NPD149 (B, n=6) or of 100 nM NPD133 (C, n=4). AD was measured from the beginning of OGD insult. (D) Graph shows the time course of 7 min OGD effects on fEPSP amplitude in untreated (mean ± SEM, n=7) OGD slices and in 50 nM NPD 149 (mean ± SEM, n=6) treated OGD slices. Amplitude of fEPSPs is expressed as percentage of baseline (pre-OGD). Note that, after reperfusion in normal oxygenated standard solution, a significant recovery of fEPSP was found only in treated OGD slices in which AD was absent. (E) Graph shows the time course of 7 min OGD effects on fEPSP amplitude in untreated (mean ± SEM, n=7) OGD slices and in 100 nM NPD 133 (mean ± SEM, n=4) treated OGD slices. Amplitude of fEPSPs is expressed as percentage of baseline (pre-OGD). Note that, after reperfusion in normal oxygenated standard solution, a significant recovery of fEPSP was found only in treated OGD slices in which AD was absent.

2.2 Effects of new mixed compounds on AD development induced by 30 min OGD

In order to characterize the possible neuroprotective role of different A_{2A}AR antagonists, alone or conjugated with antioxidant molecules, we investigated their effects on AD development induced by severe OGD. Therefore, we prolonged the duration of the OGD from 7 to 30 min; this longer duration of OGD is invariably associated with the appearance of AD, tissue damage and the complete loss of

neurotransmission (Pearson et al., 2006; Pugliese et al., 2006). We compared the time of the appearance and the magnitude of depolarizing d.c. shift, in the absence or in the presence of different compounds. As illustrated in Figure 35, 30 min OGD elicited the appearance of AD in untreated OGD slices, with a mean latency of 6.34 ± 0.2 min (n=22, Figure 37C) and a mean peak amplitude of -6.6 ± 0.4 mV (n=22, Figure 37D). In a first series of experiment OGD was applied in the presence of the new selective A_{2A}AR antagonist 100 nM NPD151 that significantly delayed the AD time to 8.95 ± 0.9 min (n=5, Figure 37C), although the AD amplitude was not changed (-5.64 ± 0.9 mV, Figure 37D).

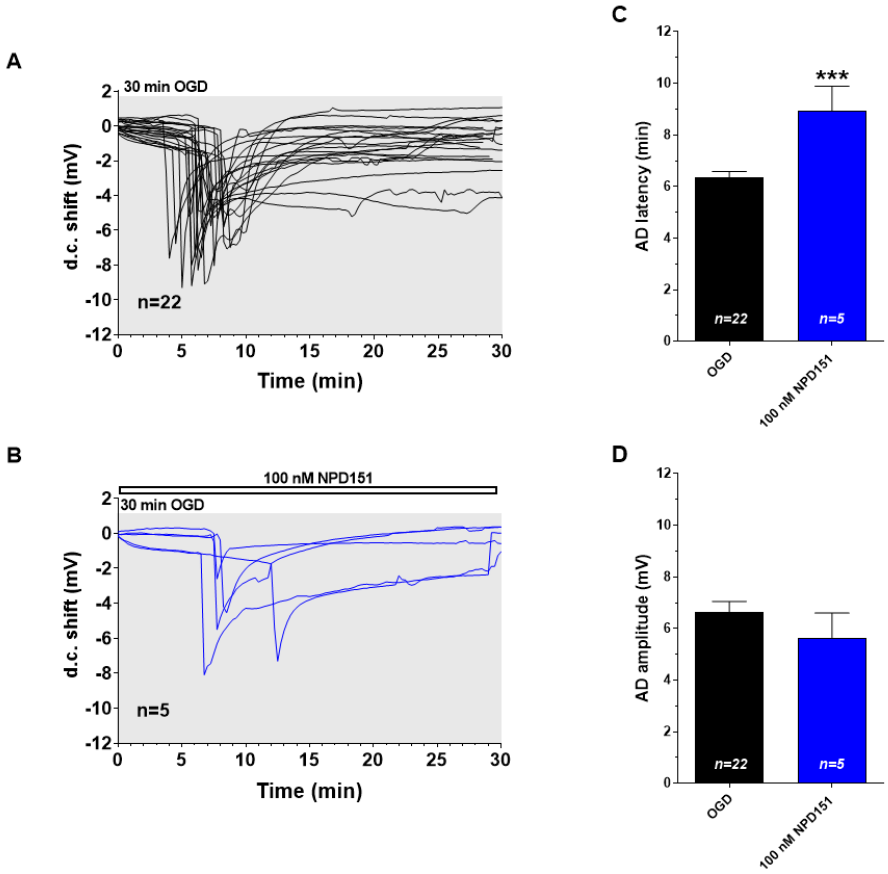


Figure 37. The selective antagonism of A_{2A}AR delayed the appearance of anoxic depolarization (AD) induced by 30 min OGD in rat hippocampal slices. (A-B) In each graph the d.c. shift traces during 30 min OGD are showed in different experimental conditions: in OGD-untreated slices (A, n=22); in the presence of 100 nM NPD151 (B, n=5). NPD151, a selective adenosine A_{2A} receptor antagonist, delayed the appearance of anoxic depolarization (AD) induced by 30 min OGD in CA1 rat hippocampal slices. (C) Each column represents the mean ± SEM of AD latency recorded during 30 min OGD in the absence or in the presence of A_{2A}AR antagonist. AD latency was measured from the beginning of ischemic insult. ***P < 0.001, One Way-ANOVA followed by Bonferroni test, in comparison to untreated OGD slices. (D) Each column represents the mean ± SEM of AD amplitude recorded in the CA1 region during 30 min OGD. The number (n) of slices tested was reported inside columns.

Subsequently, the effects induced by the derivative NPD168 and its conjugated EDA on AD appearance were analyzed (Figure 38). When OGD was applied in the presence of 200 nM NPD168, the d.c. shift was significantly delayed from 6.34 ± 0.2 min (Figure 38A, D; n=22) in OGD-untreated slices to 8.45 ± 0.5 min (Figure 38B, D; n=5). Similarly, when OGD was applied in the presence of 200 nM EDA, the d.c. shift was significantly delayed to 8.25 ± 0.3 min (Figures 38C, D; n=6). Both NPD168 and EDA did not significantly modify AD amplitude (Figure 38E).

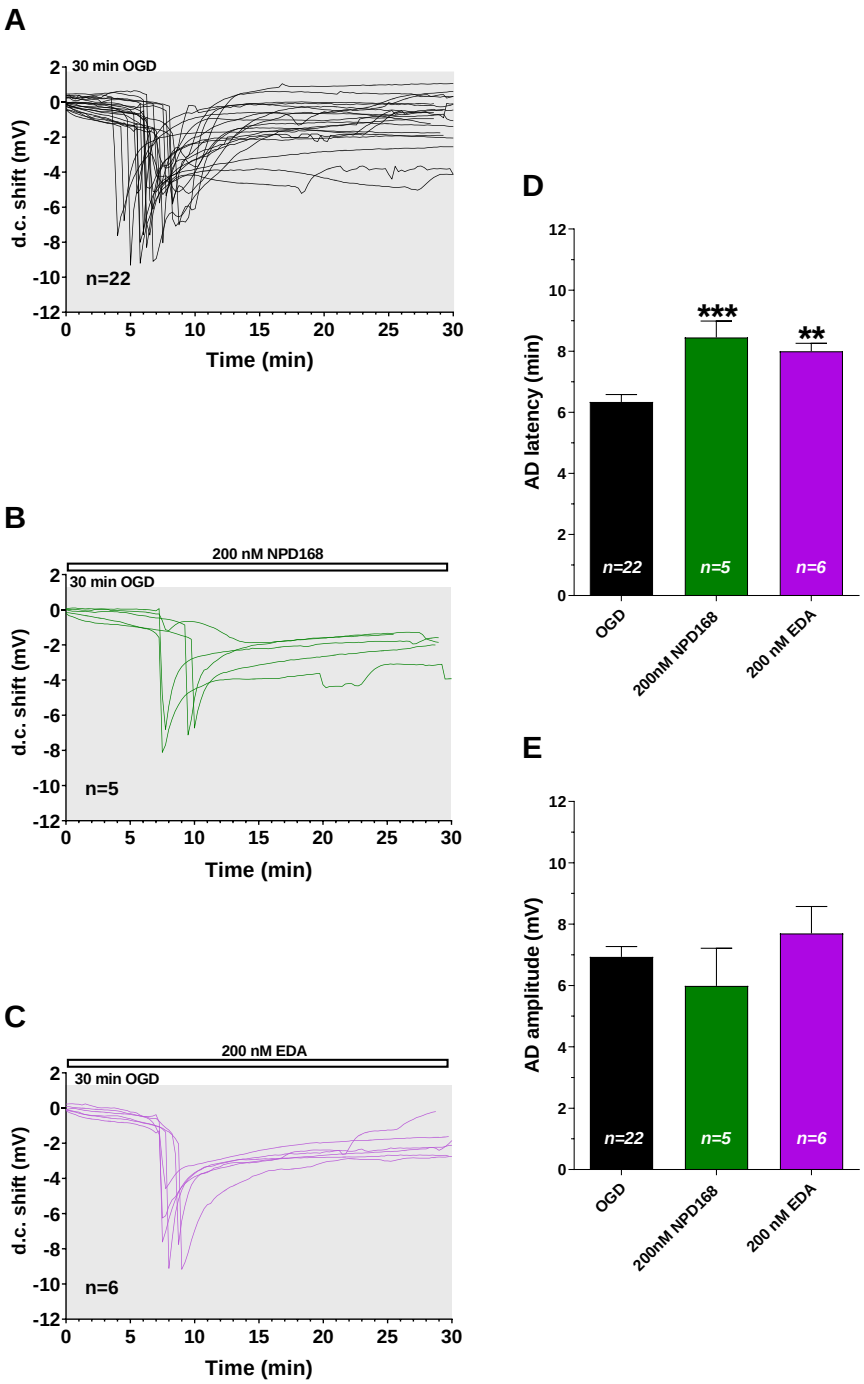


Figure 38. The mixed multi-target compound, NPD168, and the antioxidant compound Edaravon (EDA) delayed the appearance of anoxic depolarization (AD) induced by 30 min OGD in rat hippocampal slices. (A-C) The graphs show the d.c shift traces during 30 min OGD in untreated OGD slices (A, n=22), in the presence of 200 nM NPD168 (B, n=5) or 200 nM EDA alone (C, n=6). (D) Each column represents the mean ± SEM of AD latency recorded during 30 min OGD in the absence or in the presence of NPD168 or EDA. AD latency was measured from the beginning of the ischemic insult. $^{**}P < 0.01$, $^{***}P < 0.001$, One Way-ANOVA followed by Bonferroni test, *vs* untreated OGD slices. (E) Each column represents the mean ± SEM of AD amplitude recorded in the CA1 region during 30 min OGD. The number (n) of slices tested was reported inside columns.

In next experiments we applied 30 min OGD in hippocampal slices in the absence or in the presence of NPD133 or its conjugated lipoic acid alone (Figure 39). Only the derivative NPD133 was able to significantly delay the AD latency from 6.34 ± 0.2 min (Figures 39A, D; n=22) in OGD-untreated slices to 9.25 ± 0.7 min (Figure 39B, D; n=7), without effect on AD amplitude (Figure 39E).

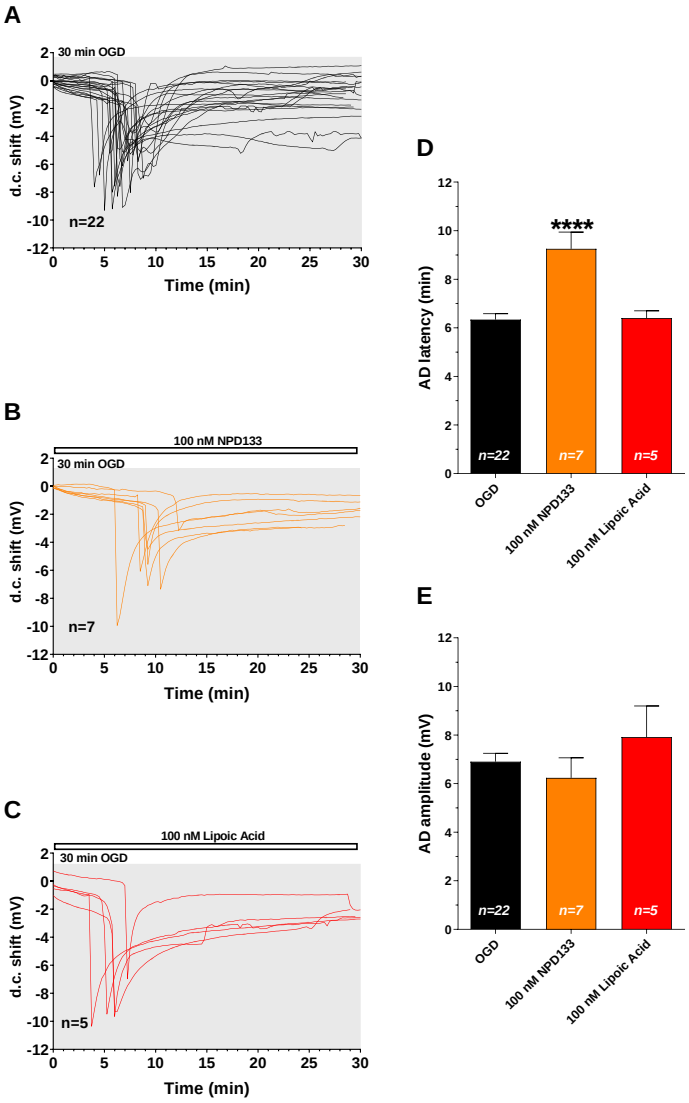


Figure 39. Only NPD133, a mixed multi-target compounds formed by an A_{2A} antagonistic component conjugated to an antioxidant molecule, Lipoic Acid, delayed the appearance of anoxic depolarization (AD) induced by 30 min OGD. (A-C) The graphs show the d.c shift traces during 30 min OGD in untreated slices (A, n=22), in the presence of 100 nM NPD133 (B, n=7) or 100 nM Lipoic Acid (C, n=5). Note that lipoic acid alone did not affect AD latency. (D) Each column represents the mean ± SEM of AD latency recorded during 30 min OGD in the absence or in the presence of NPD133 or Lipoic Acid. AD latency was measured from the beginning of the ischemic insult. ****$P < 0.001$, One Way-ANOVA followed by Bonferroni test, in comparison to untreated OGD slices. (E) Each column represents the mean ± SEM of AD amplitude recorded in the CA1 region during 30 min OGD. The number (n) of slices tested was reported inside columns.

Finally, the derivative NPD149 and its conjugated OTC were tested in the same experimental model (Figure 40). Only the derivative NPD149 (50 nM) was able to significantly delay the AD latency from 6.34 ± 0.2 min (Figures 40A, D; n=22) in untreated slices to 8.42 ± 0.5 min in the presence of the compound (Figure 40B, D; n=10), whereas AD amplitude (5.67 ± 0.5 mV) was unchanged (Figure 40E) in all experimental conditions. OTC (50 nM) did not significantly modify both AD latency and AD amplitude (Figure 40D, E; n=5).

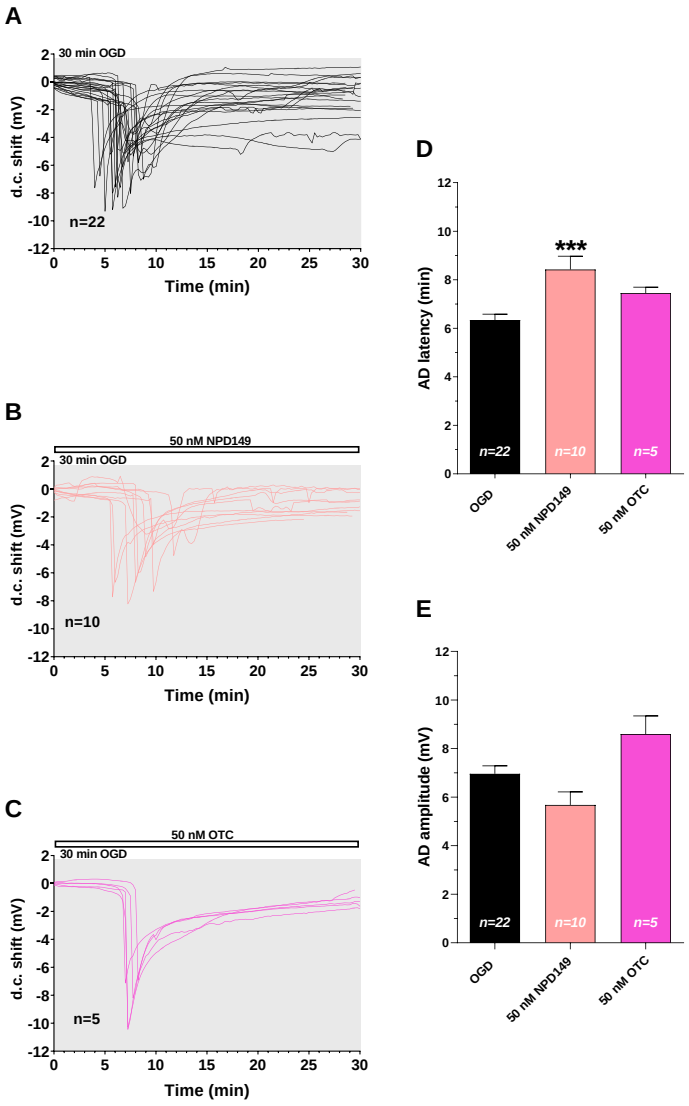


Figure 40. NPD149, a mixed multi-target compounds formed by an A_{2A} antagonistic component conjugated to an antioxidant molecule, OTC, delayed the appearance of anoxic depolarization (AD) induced by 30 min OGD. (A-C) The graphs show the d.c shift traces during 30 min OGD in untreated slices (A, n=22), in the presence of 50 nM NPD149 (B, n=10) or 50 nM OTC (C, n=5). Note that OTC alone did not affect AD latency (D) Each column represents the mean ± SEM of AD latency recorded during 30 min OGD in the absence or in the presence of NPD149 or OTC. AD latency was measured from the beginning of the ischemic insult. ***$P < 0.001$, One Way-ANOVA followed by Bonferroni test,

in comparison to untreated OGD slices. (E) Each column represents the mean ± SEM of AD amplitude recorded in the CA1 region during 30 min OGD. The number (n) of slices tested was reported inside columns.

2.3 DISCUSSION

The putative protective role of A_{2A}AR in cerebral ischemia was studied in the CA1 rat hippocampal slices during severe OGD, which is an experimental condition that mimics the most common causes of cerebral ischemia, such as vessel occlusion.

As mentioned during this Thesis, the A_{2A}AR are metabotropic receptors expressed in different brain area and are coupled to Gs proteins, leading to an increase of cAMP (Fredholm et al., 2011). A_{2A}ARs show a high affinity for the endogenous ligand and are activated by nanomolar concentration of adenosine (Fredholm et al., 2001). It is known that the selective A_{2A}AR antagonist, ZM241385, protected the CA1 hippocampal slices from a severe OGD by preventing or delaying AD appearance. Interestingly, molecules with antioxidant activity (i.e., OTC, EDA and Lipoic acid) also appeared to be protective in brain ischemia (Isahaya et al., 2012; Liu et al., 2020). In this work, we used new mixed A_{2A}AR antagonists with antioxidant properties to test their possible neuroprotective effect on brain ischemia. These mixed compounds may result more effective in neuroprotection because oxidative process and the ROS formation concur in the damage caused by the ischemic attack. Therefore, multi-target compounds with these pharmacological properties could have a synergic effect in reducing the ischemic damage, due to excitotoxic and oxidative stress.

Our data showed that the mixed A_{2A}AR antagonists conjugated with antioxidant molecules (OTC or Lipoic Acid), delayed AD appearance in the CA1 hippocampus, obtained by 7 min OGD, as demonstrated by the significant recovery of the neurotransmission. We also recorded a delay in AD development induced by 30 min OGD carried out in the presence of NPD151, NPD149, NPD133, and NPD168.

When the antioxidant molecules OTC, EDA, and Acid Lipoic were tested alone during 30 min OGD, only EDA resulted efficacious in delaying AD appearance without affecting its amplitude. This result was in line with other studies that demonstrated the protective effect of this antioxidant molecule in *in vivo* model of cerebral ischemia (e.g., Zhang et al., 2005). In particular, Zhang et al. (2005) demonstrated that EDA,

currently used in Japan in acute ischemic stroke, was important for the expansion of the therapeutic time window in stroke patients (Zhang et al., 2005).

The knowledge about the adenosine receptors and its involvement in the cerebral ischemia has been established and would support research and the discovery of new strategies, including the development of adenosine receptor antagonists and of dual antagonists, both for the A_{2A}AR and A_{2B}AR, given their functional interconnection as neuroprotective agents. Therefore, considering the promising effect of adenosine receptor antagonists, they could represent alternative treatments stroke by using them alone or in combination with tPA to improve better clinical outcome.

3 Role of carbonic anhydrase inhibitors (CAIs) during an ischemic-like insult in the CA1 rat hippocampal slices

The experiments were performed on 49 slices to test the role of CAIs during 30-min long OGD, a time-duration that is invariably harmful for the tissue (Pugliese et al, 2006). The role of these compound was evaluated as mentioned above.

3.1 The application of selective CAIs significantly delays the AD appearance induced by 30 min OGD

The effects of two new selective CAIs, AN6-277 and AN11-740, on the time of the AD appearance and amplitude, were evaluated (Figure 41) and compared to those obtained in the presence of the prototypical CAI, ACTZ. As illustrated in Figure 41 A, E) 30-min OGD elicited the appearance of AD in untreated OGD slices, which presented a mean latency of 6.2 ± 0.3 min (calculated from the beginning of OGD; n=20) and a mean peak amplitude of -6.3 ± 0.5 mV (n=20, Figure 41A, F). When OGD was applied in the presence of CAIs, the d.c. shifts were always delayed (Figure 41B-E). Indeed, the latency of AD was postponed to 8.65 ± 0.5 min in the presence of 20 µM ACTZ, (n=5, Figure 41B, E), to 8.8 ± 0.6 min in the presence of 3 µM AN11-740 (n=8, Figure 41C, E) and to 9.04 ± 0.7 min in the presence of 5 µM AN6-277 (n=7, Figure 41D, E). AN6-277 affected AD latency also when tested at lower concentrations (Figure 41E). All compounds tested did not significantly change AD amplitude in comparison to that found in OGD untreated slices (Figure 41F).

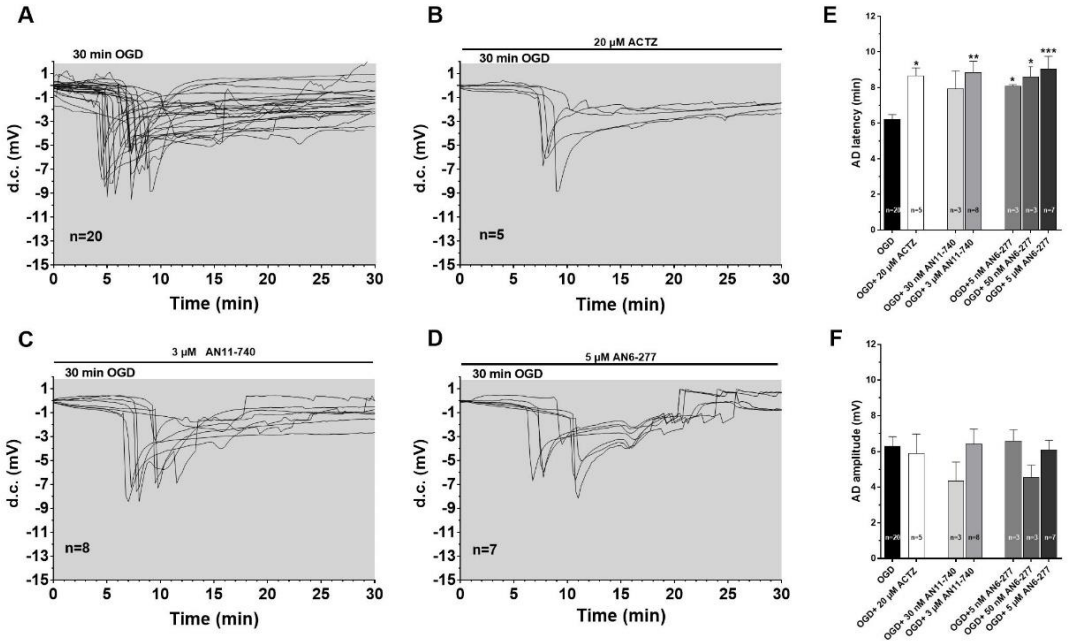


Figure 41. Effects of different CA inhibitors on AD development during 30 min OGD in the CA1 region. (A-D) The graphs show the d.c. shift traces during 30 min OGD in untreated OGD slices (A, n=20), in the presence of 20 µM ACTZ (B, n=5), 3 µM AN11-740 (C, n=8) and 5 µM AN6-277 (D, n=7). Each inhibitor was applied at least 20 min before OGD and maintained for all the insult. (E) Each column represents the mean ± SEM of AD latency recorded in hippocampal slices during 30 min OGD in different experimental groups. AD was measured from the beginning of OGD insult. **P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 vs. OGD, One-way ANOVA followed by Bonferroni post hoc test. (F) Each column represents the mean ± SEM of AD amplitude recorded in the CA1 region during 30 min OGD. The number (n) of slices is reported in the columns.

3.2 DISCUSSION

In this Thesis it was observed that ACTZ and two different sulfonamide CAIs, AN11-740 and AN6-277, protected from a strong neuron depolarization induced by an ischemic-like insult in hippocampal slices. We stated that 30 min OGD induced the appearance of the electrophysiological phenomenon of AD recorded in all the hippocampal slices. As stated above, AD is clear sign of neuronal injury and treatments that postpones its appearance protect brain tissue after an ischemic insult (Somjen, 2001).

The maintenance of pH homeostasis in the CNS is pivotal for neurotransmission mechanisms and variations from this homeostasis are crucial for processes underlying a spectrum of pathological conditions including ischemia (Obara et al., 2008). Indeed, under ischemia, the loss of oxygen caused by hypoxia leads to a switch from aerobic to anaerobic glucose metabolism, and subsequently increased

production of lactic acid and lowered intracellular pH (Neri and Supuran, 2011; Swietach et al., 2007) in neurons and glial cells (Obara et al., 2008).

Neurons are particularly sensitive to the pH decrease (Obara et al., 2008) and acidosis augments the vulnerability of glia to injury induced by OGD (Giffard et al., 1990). Indeed, changes in the intracellular pH may affect neurotransmitters release. Lowering of pH results in increased release of dopamine (Cannizzaro et al., 2003; Pittaluga et al., 2005), noradrenaline and serotonin from rat brain synaptosomes (Pittaluga et al., 2005). Glial acidosis has been shown to trigger glial glutamate release and neuronal cell death (Beppu et al., 2014).

The CA isoforms IV, IX and XII, all extracellular and membrane-bound enzymes, are highly expressed in glial cells (Tong et al., 2000; Svichar et al., 2006) and contribute to pH homeostasis, both in physiological and pathological conditions. Astrocytes have a key role in pH regulation in the brain Bélanger and Magistretti, 2009). Glial CA converts neuron derived CO_2 to bicarbonate and protons which are extruded of the glial cell by a $Na^+/HCO3^-$ cotransporter and monocarboxylate transporters. Extracellularly CA is pivotal in buffering extracellular pH by recycling CO_2 in bicarbonate and protons (Tong et al., 2000). The evidence that under hypoxic conditions, the two CA isoforms IX and XII increase (Wykoff et al., 2000; Supuran, 2017) supports the possibility that during OGD, the sulfonamides ACTZ, AN6-277, and AN11-740, by reducing the activity of CA isoforms, reduce the concentration of hydrogen ions, the excitatory amino acid efflux and therefore the participation of glutamate in triggering the AD.

SECTION II - Pharmacological and functional characterization of adenosine "A_2" receptors in oligodendrogliogenesis and myelination processes: an ex-vivo study

In this part of the Thesis, we studied the effects of the endogenous ligand, adenosine, which has different affinity for A_{2A}AR (nM) and A_{2B}AR (μM), in the modulation of outward-K^+ currents in cultured OPCs. Patch clamp recordings were performed on 95 OPCs taken from 16 rat pups. The OPCs showed, on average, a Vm of -68.6 ± 1.9 mV, a Cm of 9.7 ± 0.3 pF and a Rm of 902.9 ± 64.9 MΩ. All electrophysiological experiments were performed in the continuous presence of the A_1AR and A_3AR antagonists DPCPX and MRS1523, respectively (500 nM each), in order to isolate A_{2A}AR and A_{2B}AR mediated responses. In addition, we characterized the role of these adenosinergic receptors subtypes on *ex-vivo* myelination process in OPC-DRG co-cultures by immunofluorescence technique.

4 Effects of the endogenous ligand, adenosine, on outward-K^+ currents in cultured OPCs

As shown in Figure 42A, we applied a voltage ramp protocol (from −120 to +80 mV, 800 ms duration: inset of Fig. 42A) in cultured OPCs in the absence or presence of adenosine (50 μM) and we found that the agonist, in the continuous presence of A_1AR and A_3AR antagonists, inhibited ramp-evoked outward currents (Figure 42A). Adenosine-inhibited current, obtained by subtraction of the ramp recorded in the presence of adenosine from the control ramp, was a voltage-dependent outward conductance consistent with the activation of voltage-dependent outward rectifying K^+ channels (Figure 42B). In line with this observation, ramp-evoked outward currents, as well as the effect of adenosine, were absent when extra- and intra-cellular K^+ ions were replaced by equimolar Cs^+, demonstrating that the current involved in adenosine-effect is a voltage-dependent K^+ current (data not shown). The inhibitory effect of adenosine on ramp-evoked currents is maximal after about 5 min of application and it is statistically significant in 18 cells investigated, as evident in Figure 42C.

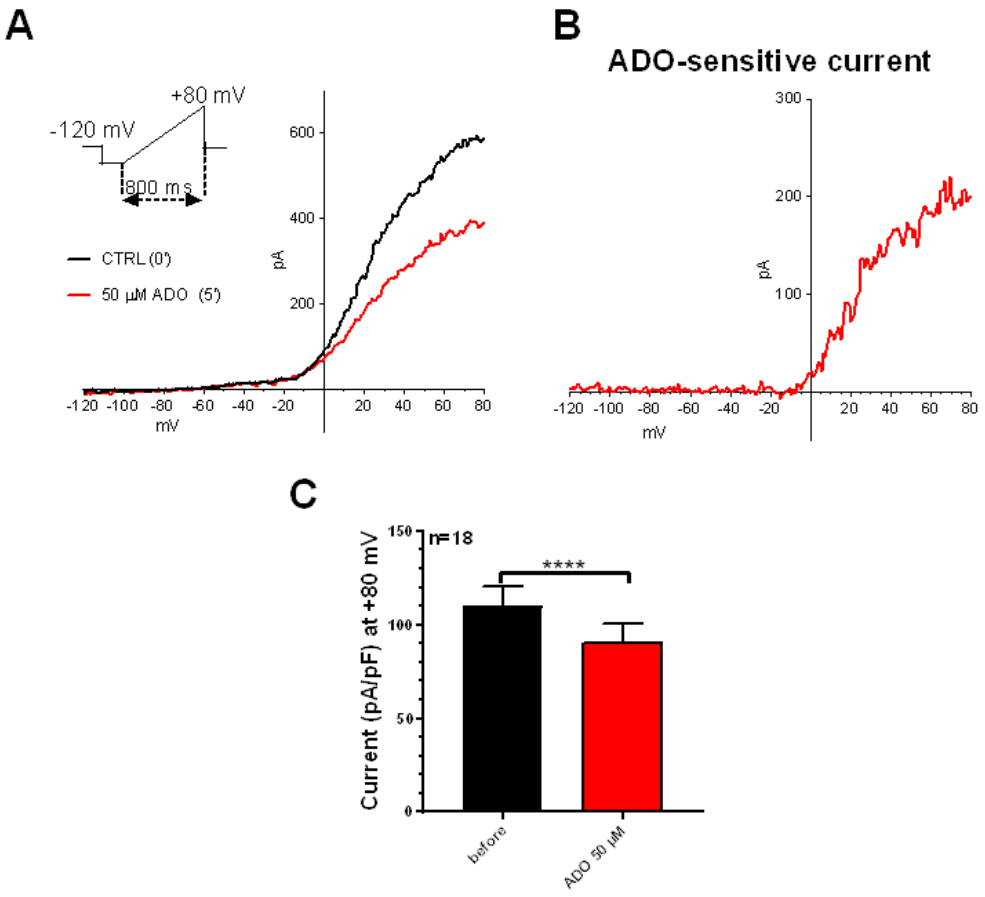


Figure 42. Adenosine (ADO) reduces outward K^+ currents evoked by a voltage ramp protocol in OPC cultures. (A) Original current traces recorded in a typical OPC by whole-cell patch clamp technique. A wide range of voltage-dependent outward K^+ currents were evoked by a ramp protocol (from -120 to +80 mV, 800 ms: inset) before (Ctrl), during and after ADO (50 µM) application. B) Net ADO-sensitive current, obtained by subtraction of the trace recorded in ADO from the CTRL ramp, in the same cell. C) Pooled data recorded at +80 mV (mean ± SEM), in the absence (before) or presence of 50 µM ADO (n=18). ****$P < 0.0001$, paired Student's t-test. In order to evidence the ADO effect on A_{2A}AR and A_{2B}AR, all experiments were conducted in the presence of the selective A_1AR and A_3AR antagonists, DPCPX and MRS1523, respectively (both at 500 nM).

In the next experimental series, we tested the effects of different concentrations of adenosine: 0.1 µM on 7 cells (n=7) and 20 µM on 12 cells (n=12). The inhibitory effect of adenosine was evident at all tested concentrations (Figure 43A-C). In particular, the current amplitude was: 142.4 ± 32.52 pF/pA before and 126.6 ± 30.73 pA/pF after 0.1 µM adenosine application, 98.65 ± 9.01 pA/pF before and 83.92 ± 8.88 pA/pF after 20 µM adenosine and 110.2 ± 10.63 pA/pF before and 91.17 ± 9.26 pA/pF after 50 µM adenosine application. In all the experiments, the effect of adenosine was measured 5 min after its application, when the steady-state level was reached at all

concentrations. Of note, the current values at +80 mV showed that adenosine-mediated inhibitory effect on outward-K^+ currents was concentration-independent.

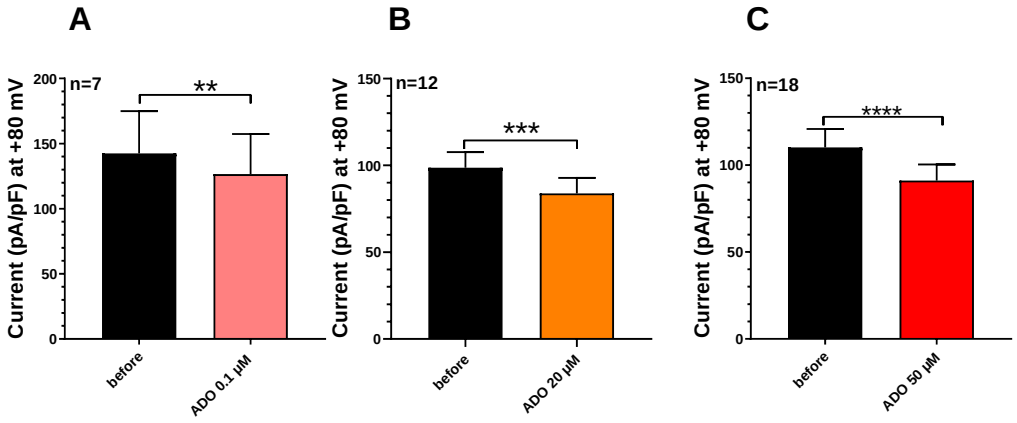


Figure 43. Effects of different adenosine (ADO) concentrations on outward K^+ currents in OPC cultures. (A-C) Pooled data (mean ± SEM) of ramp current amplitude (pA/pF) at +80 mV recorded in the absence (before, black column) or presence (coloured column) of different ADO concentration (ADO 0.1-20-50 µM) in 7, 12, 18 cells, respectively. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, paired Student's t-test. In order to evidence the ADO effect on A_{2A}AR and A_{2B}AR, all experiments were conducted in the presence of the selective A_1AR and A_3AR antagonists, DPCPX and MRS1523, respectively (both at 500 nM).

The adenosine concentration-independent effect was also detected by the isolation of adenosine-sensitive currents (Figure 44). These currents were obtained by averaging the ramp obtained by subtraction of the ramp recorded in the presence of adenosine from the control ramp, in each cell. The averaged adenosine-sensitive current showed an activation at -10 mV reaching the maximal amplitude at +80 mV. The inhibited currents at +80mV were: +15.37 ± 2.43 pA/pF in the presence of 0.1 µM adenosine; +18.57 ± 2.67 pA/pF in the presence of 20 µM adenosine; +19.24 ± 3.14 pA/pF in the presence of 50 µM adenosine. Hence, no differences in adenosine sensitive currents at all concentration tested were found.

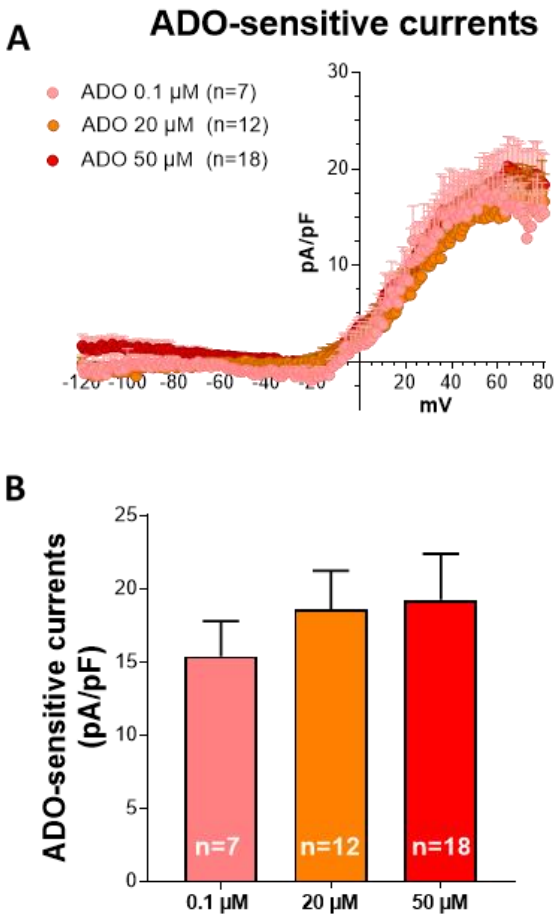


Figure 44. Adenosine (ADO) reduces outward currents in a concentration-independent manner in OPC cultures. (A) ADO -sensitive currents, obtained by subtraction of the trace recorded in ADO from the CTRL ramp, in the same cell, in the presence of different ADO concentrations: ADO 0. 1 µM (n=7), ADO 20 µM (n=12) and 50 µM (n=18). (B) Pooled data (mean ± SEM) of ADO-sensitive currents (pA/pF) measured at +80 mV in the presence of increased ADO concentrations (0.1-50 µM) in OPCs cultures. In order to evidence the ADO effect on A_{2A}AR and A_{2B}AR, all experiments were conducted in the presence of the selective A_1AR and A_3AR antagonists, DPCPX and MRS1523, respectively (both at 500 nM).

Data in literature reported that the most present K^+ currents in OPCs are the delayed rectifier I_K currents (Sontheimer & Kettenmann, 1988), but they also express a transient K^+ current (I_A) which is usually present in undifferentiated states. In order to dissect the nature of adenosine-inhibited potassium currents, two step voltage protocols were applied (Vh = -70 mV, 60 ms pre-step to -80 mV, steps from -40 to +80 mV, 200 ms, interval 10 mV and a second identical protocol but with a pre-step at -40 mV) capable of discriminate between I_K and I_A currents in the absence or presence of 0.1 µM adenosine (Figure 45A-B). At this concentration, adenosine significantly reduced only the amplitude of I_K without effects on I_A currents (n=4; Figure 45D, E).

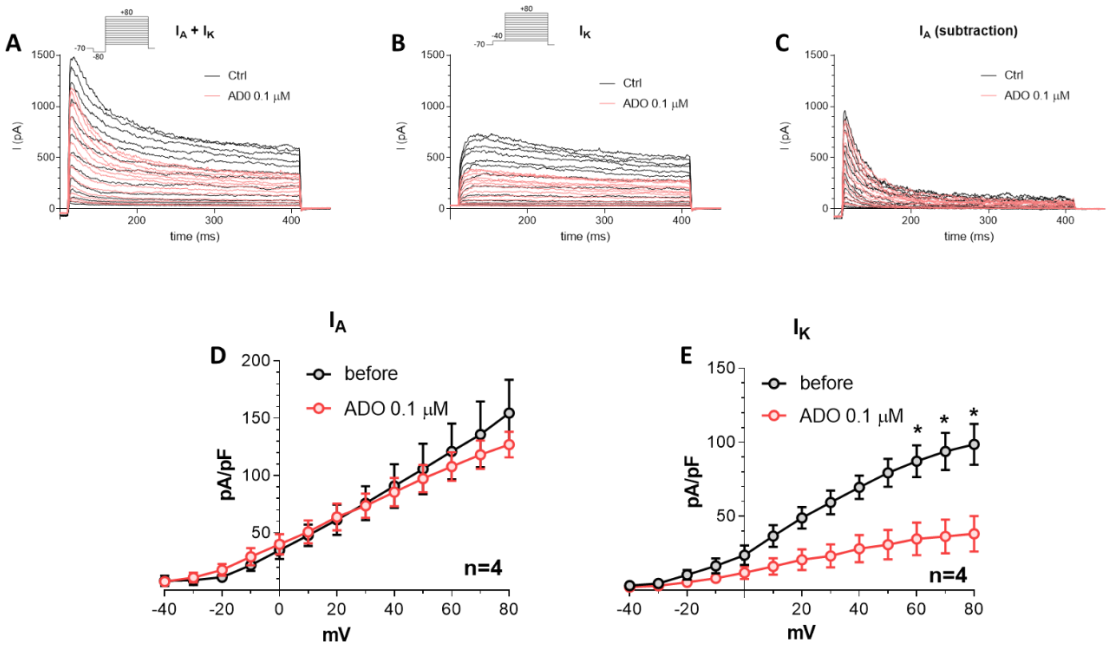


Figure 45. Low concentration of adenosine (ADO) inhibits I_K currents but not I_A, consistent with A_{2A}AR activation only. (A-B) Original current traces evoked by two different voltage-step protocols (from −40 to +80 mV, Vpre = -80 mV; 200 ms: inset in A; or from −40 to +80 mV, Vpre = -40 mV; 200 ms: inset in B), in order to evoke I_A and I_K currents or I_K alone, in a representative OPC before (ctrl: black traces) or after the application of ADO (0.1 μM, 5 min; light pink traces). (C) Net I_A current in the same OPC obtained by subtraction of traces reported in A and B. (D-E) Averaged current-to-voltage relationships (I-V plot) of steady-state, sustained, I_K currents (E) or peak, transient, I_A currents (D) recorded in the absence (bsl: black circles) or in the presence (light pink circles) of 0.1 μM ADO in 4 cells investigated. **P* < 0.05; paired Student's t-test. In order to evidence the ADO effect on A_{2A}AR and A_{2B}AR, all experiments were conducted in the presence of the selective A_1AR and A_3AR antagonists, DPCPX and MRS1523, respectively (both at 500 nM).

At difference, the application of the same protocol, in the absence or in the presence of 50 μM adenosine (Figure 46), showed that the endogenous ligand, applied at highest concentration, was able to inhibit both I_A and I_K (n=8; Figure 46D, E).

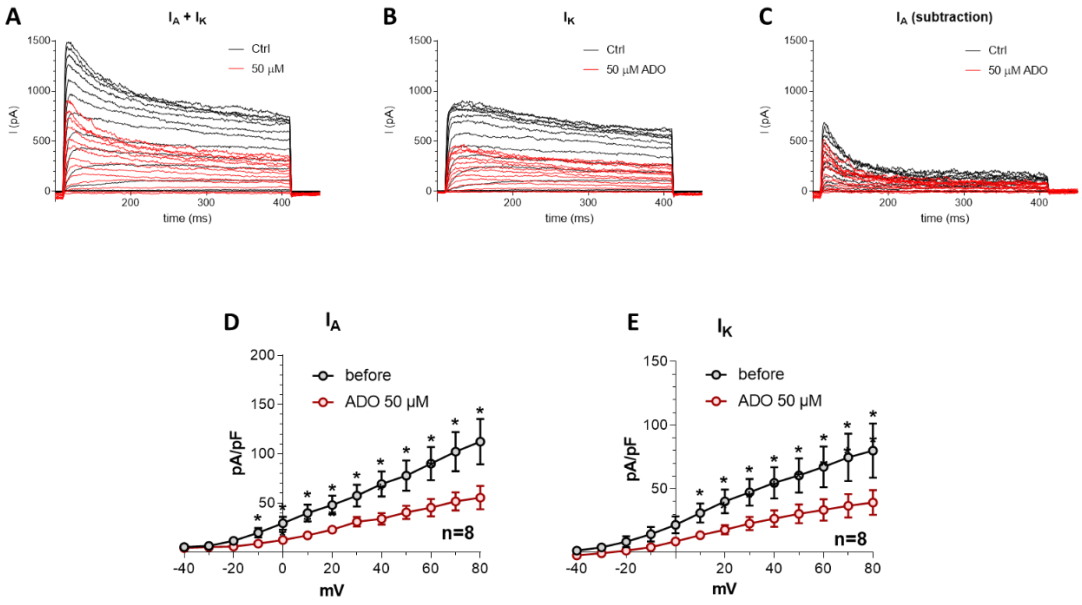


Figure 46. High concentration of adenosine (ADO) inhibits both I_K and I_A currents, consistent with A_{2B}AR and A_{2A}AR activation. (A-B) Original current traces evoked by two different voltage-step protocols (from −40 to +80 mV, Vpre = -80 mV; 200 ms: inset in A; or from −40 to +80 mV, Vpre = -40 mV; 200 ms: inset in B), in order to evoke I_A and I_K currents or I_K alone, respectively, in a representative OPC before (ctrl: black traces) or after the application of ADO (50 µM, 5 min; red traces). (C) Net I_A current in the same OPC were obtained by subtraction of traces reported in A and B. (D-E) Averaged current-to-voltage relationships (I-V plot) of steady-state, sustained, I_K currents (E) or peak, transient, I_A currents (D) recorded in the absence (bsl: black circles) or in the presence (red circles) of 50 µM ADO in 8 cells investigated. **P* < 0.05; paired Student's t-test. In order to evidence the ADO effect on A_{2A}AR and A_{2B}AR, all experiments were conducted in the presence of the selective A_1AR and A_3AR antagonists, DPCPX and MRS1523, respectively (both at 500 nM).

4.1 Effects of the new A_{2A}-A_{2B}AR antagonist, P626, on the inhibitory effect of adenosine on outward-K^+ currents in cultured OPCs

In order to characterize the effect of the new multi-target A_{2A}-A_{2B}ARs antagonist, P626, on outward currents in cultured OPCs, we applied 50 µM adenosine in the presence of P626. We tested the effects of adenosine on the voltage-ramp currents in the presence of A_1AR and A_3AR antagonists, in order to evaluate whether P626 was able to prevent its effects. As shown in figure 47, the multi-target compound P626 reduced the adenosine-inhibitory effect on outward-K^+ currents (Figure 47A). The 50 µM adenosine-sensitive currents were studied in the presence of increased P626 concentration. The multi-target compound significantly inhibited outward-K^+ reduction induced by 50 µM adenosine at all concentrations used. No statistical difference was found in all experimental conditions (Figure 47A-B). Of note, no

changes in evoked ramp current at +80 mV were recorded when the antagonist P626 was applied alone (data not shown).

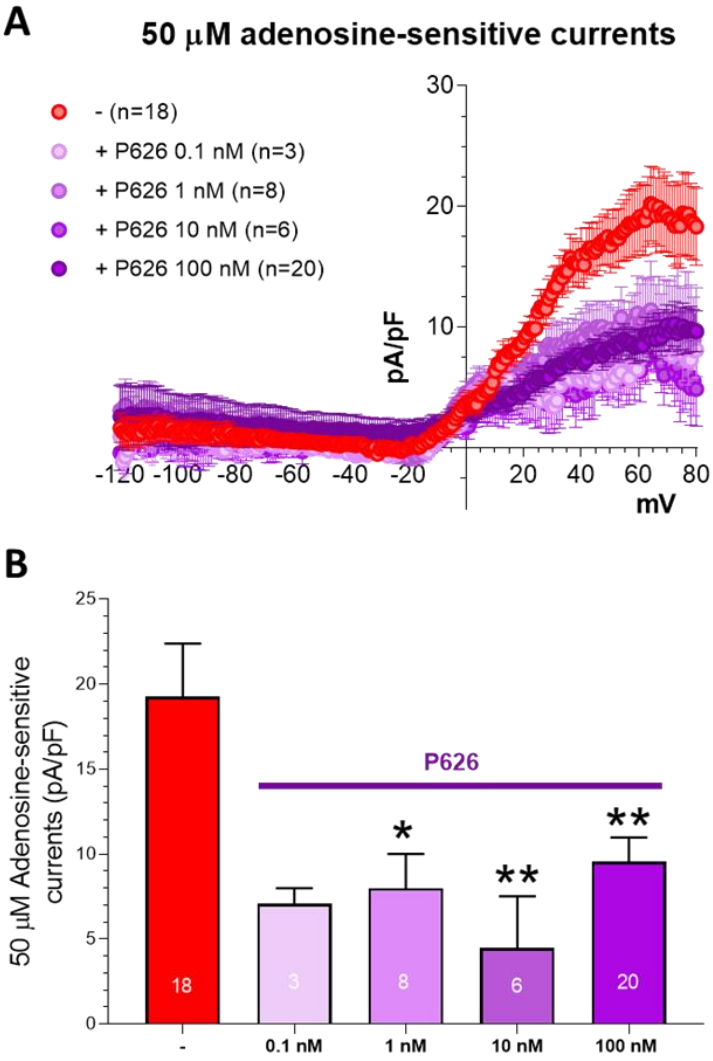


Figure 47. The inhibitory effect of adenosine (ADO), applied at 50 μM, on outward-K^+ currents is prevented by the newly synthesized A_{2A}-A_{2B}ARs antagonist P626. (A) ADO -sensitive currents evoked in OPCs by the voltage ramp protocol in the absence (-, n=18) or presence of different P626 concentrations: P626 0.1 nM (n=3), P626 1 nM (n=8), P626 10 nM (n=6) and P626 100 nM (n=20). (B) Pooled data (mean ± SEM) of ADO-sensitive currents (pA/pF) measured at +80 mV in the absence (-) or presence of increased P626 concentrations (0.1, 1, 10 and 100 nM) in OPCs cultures. Number of observations is written in the columns. In order to evidence the ADO effect on A_{2A}AR and A_{2B}AR, all experiments were conducted in the presence of the selective A_1AR and A_3AR antagonists, DPCPX and MRS1523, respectively (both at 500 nM).*$P < 0.05$, ** $P < 0.01$, paired Student's t-test.

4.2 The multi-target A_{2A}-A_{2B}ARs antagonist P626 reduces the inhibitory effect of BAY60-6583 on outward-K^+ currents in cultured OPCs

Coppi et al. (2020a) established that the A_{2B}ARs are involved in OPCs differentiation; indeed, it was demonstrated that the application of the selective A_{2B}AR, BAY60-6583 (BAY), inhibited OPCs *ex-vivo* differentiation by decreasing voltage-dependent K^+ currents. Therefore, we decided to evaluate the effect of the new multi-target antagonist, P626, in presence of BAY60-6583. We isolated BAY60-6583-sensitive current by applying the voltage ramp protocol described above in order to confirm our previously data. Of note, we demonstrated for the first time that the inhibitory effect of BAY60-6583 on outward-K^+ currents was prevented in the presence of 10 and 100 nM P626. As shown in Figure 48, the multi-target compound was able to significantly reduce the BAY60-6583-sensitive currents, which amplitude was 32.42 ± 4.06 pA/pF in absence of P626 (-, n=11), 15.67 ± 1.29 pA/pF after 5 min in the presence of 10 nM P626 (n=4) and, 10.13 ± 4.69 pA/pF in the presence of 100 nM P626 (n=6).

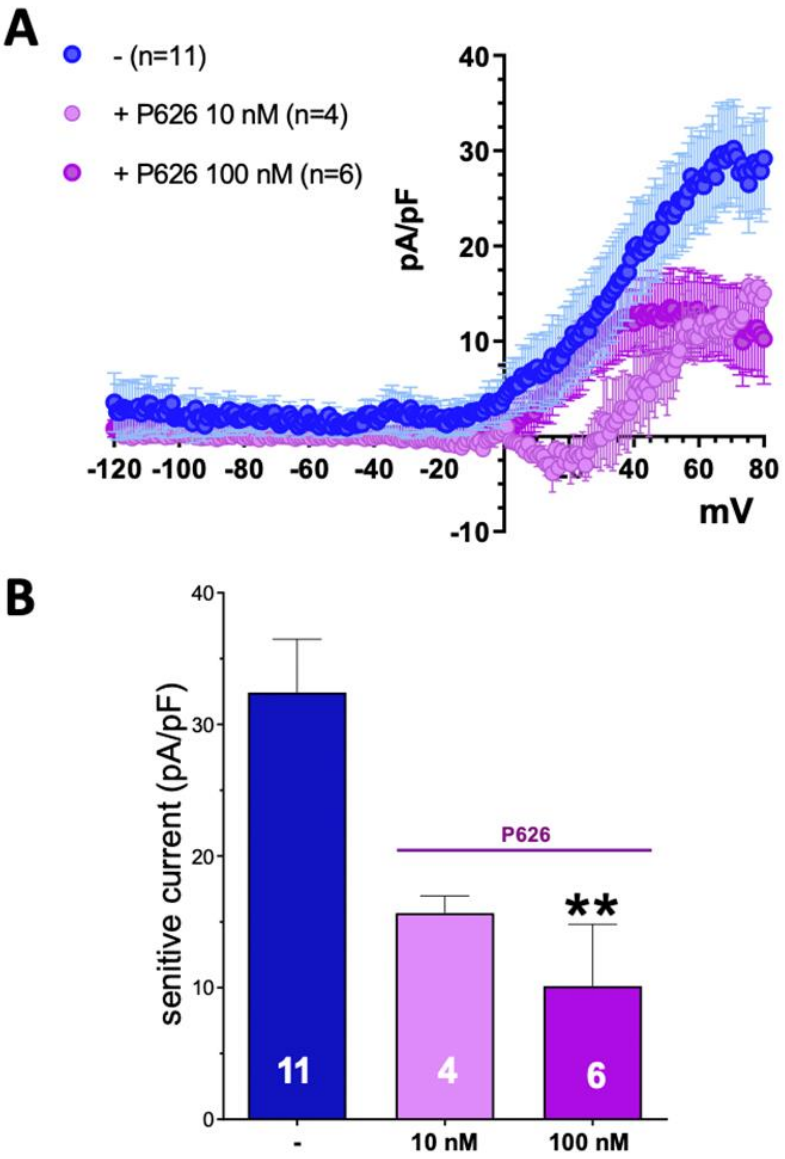
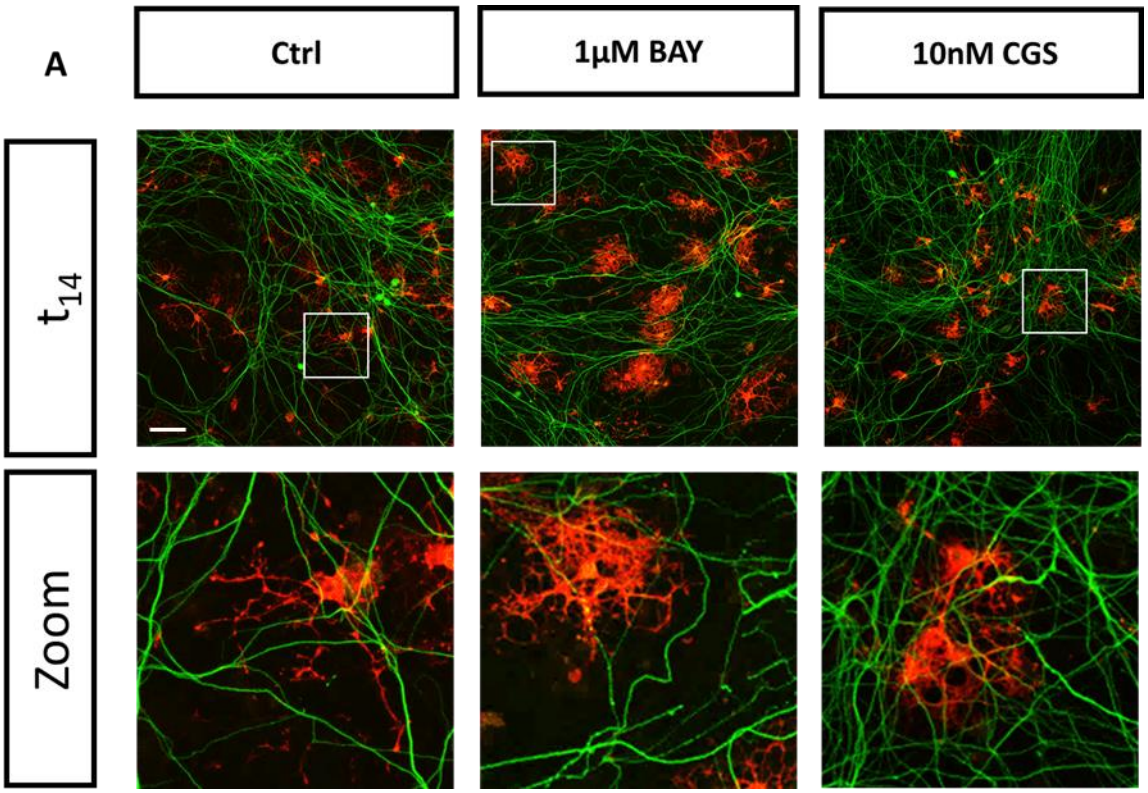


Figure 48. The inhibitory effect of BAY60-6583 (BAY), applied at 1 µM, on outward-K^+ currents is prevented by the newly synthesized A_{2A}-A_{2B}ARs antagonist P626. (A) BAY-sensitive currents evoked in OPCs by the voltage ramp protocol in the absence (-, n=11) or presence of different P626 concentrations: P626 10 nM (n=4) and P626 100 nM (n=6). (B) Pooled data (mean ± SEM) of BAY-sensitive currents (pA/pF) measured at +80 mV in the absence (-) or presence of 10 and 100 nM of P626 in OPCs cultures. Number of observations is written in the columns. ** $P < 0.01$, paired Student's t-test.

4.3 The selective stimulation of A_{2B}AR potentiates axons myelination in DRG-OPC co-culture

Our research group demonstrated the involvement of A_{2A}ARs and A_{2B}ARs in modulating OPCs differentiation (Coppi et al., 2013a; Coppi et al., 2020b). In order to investigate the role of the "A_2" adenosine receptors in the myelination processes, we set up an *ex-vivo* myelination system by co-culturing DRG neurons isolated from postnatal rats (PN5–PN6) with primary OPCs (PN1-PN2). The effect of BAY60-6583 and CGS21680 was evaluated after 14 days of co-culture treatment.

As shown in Figure 49, myelinated axons are positive to MBP, the mature OLs marker, and to β3-Tubulin, the axon marker. The selective stimulation of A_{2A}AR or A_{2B}AR by growing the co-culture in the continuous presence of one of the respective selective agonists, did not significantly change the total amount of MBP expressed in the co-culture, even if a tendency to reduction was observed (Figure 49B). Surprisingly, we observed that 1 µM BAY60-6583 significantly increased axonal myelination, quantified by the myelination coefficient or Mander's coefficient (M1), that represent the number of β3-Tubulin$^+$ pixel overlapping the MBP$^+$ pixel (Figure 49C). The effect of CGS21680 on myelination did not reach a significant level (Figure 49C).



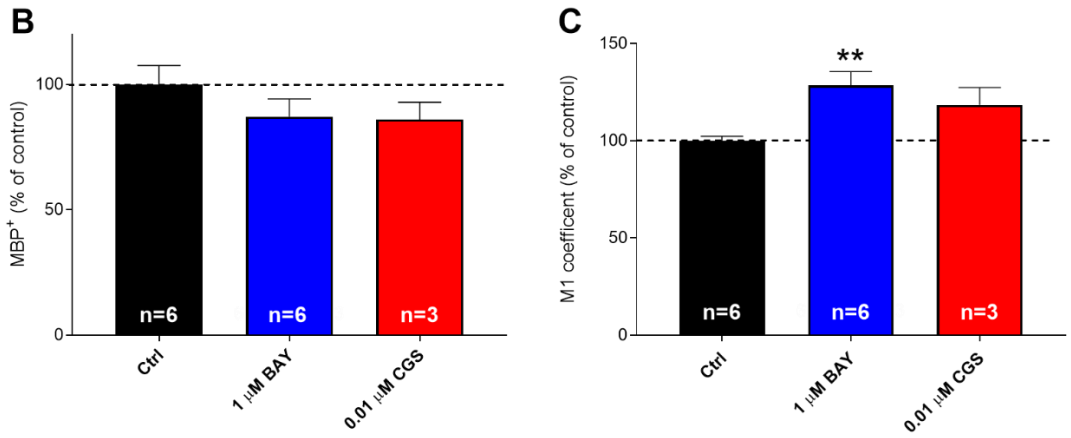


Figure 49. The selective stimulation of A_{2B}AR potentiates myelination of axons without modifying myelin basic protein levels in OPC-DRG co-culture. (A) Representative images of OPC-DRG cultures grown in the absence (ctrl) or presence of 1 µM BAY60-6583 (BAY) or 10 nM CGS21680 (CGS) for 14 days (t_{14}) and stained for anti-myelin basic protein (MBP, red) and anti-β3-Tubulin (green). Scale bar = 100 µm. Contacts between the axonal and oligodendrocyte membranes were evident and several wraps of oligodendrocyte membrane around the axon were visible. (B) Quantification of MBP^+ cells in different experimental conditions: in control (Ctrl) or following chronic exposure to "A_2" ligands. MBP is expressed as percentage respect to control. Values are expressed as mean ± SEM of at least three independent experiments. (C) Mander's coefficient (M1, expressed as percentage change respect to control) is indicative of overlapping between the number of MBP^+ pixels on β3-Tubulin $^+$ pixels.

4.4 DRG neurons express A_{2B}ARs which activation increases their action potential firing

As mentioned above, we recently demonstrated, and confirmed in the present work, that the selective activation of A_{2B}ARs inhibited outward-K^+ currents in purified cultures of OPCs, and by this mechanism, they inhibit their differentiation (Coppi et al., 2020a). Hence, results obtained in the co-culture assay showing increased axonal myelination in the presence of the A_{2B}AR agonist BAY60-6583 were at variance from our previous data observed in OPCs cultured alone. In order to explain this discrepancy, we evaluated whether the selective A_{2B}AR agonist modify DRG neurons responses, an effect never investigated. As shown in Figure 50, we confirmed that isolated rat DRG neurons express A_{2B}ARs by immunocytochemical analysis.

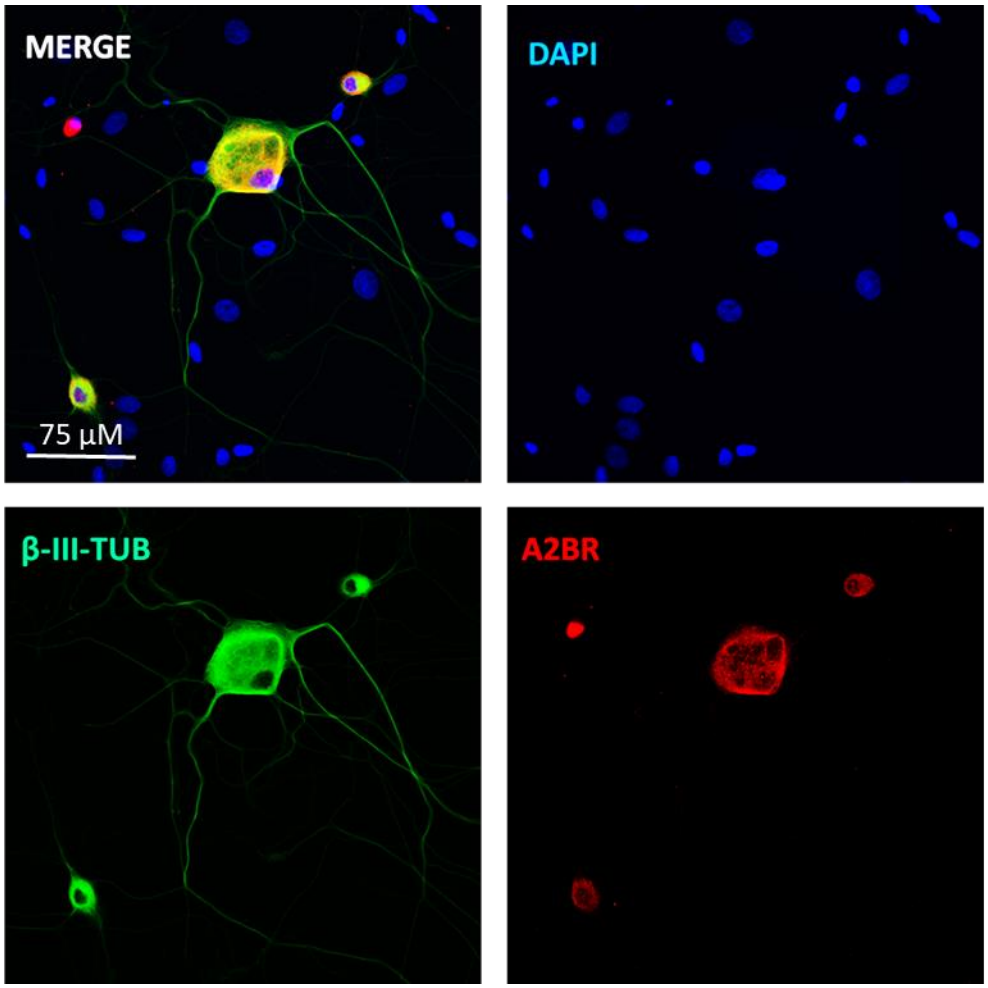


Figure 50. A_{2B}AR expression on cultured rat DRG neurons. Confocal images (63X) of immunofluorescence staining for anti-A_{2B}AR (red) and anti- β3-Tubulin (green) in primary DRG neurons cultures. Cell nuclei are marked with DAPI (blue). Scale bar: 75 µm.

Moreover, to further investigate the role of A_{2B}ARs on DRG neurons, we performed *current clamp* recordings on isolated rat DRG cultures since the release of glutamate by a firing neuron represents one of the most powerful stimuli to OPC migration and differentiation in myelinating OLs (Cherchi et al, 2021a). Patch clamp recordings were performed on 18 DRG neurons showing, on average, a Cm of 17.7 ± 1.7 pF and a Rm of 352.7 ± 39.9 MΩ. We recorded the number of action potentials (APs) generated by DRG neurons stimulated by depolarizing current steps from -10 pA to +40 pA (1200 s step duration, 10 mV increment) (Figure 51A). This protocol was applied in the absence or in the presence of BAY60-6583 and we found a significant increase of APs number at +10 pA and +20 pA in the presence of A_{2B}AR agonist (Figure 51).

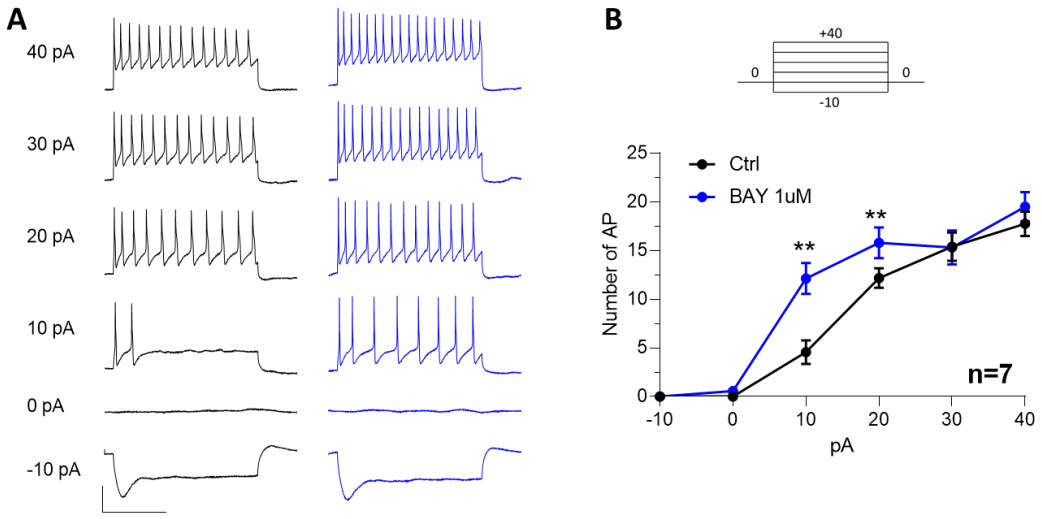


Figure 51. The selective activation of A_{2B}AR increases the number of action potentials (APs) evoked by a current-step protocol in cultured rat DRG neurons. (A) Original voltage traces evoked by a current-step protocol (from −10 to +40 pA, 1200 ms: inset in B) in a representative DRG before (black traces, left panels) or after the application of BAY60-6583 (BAY, 1 µM, 5 min, blue traces, right panels). (B) Averaged number of APs-to-current injected relationship recorded in the absence (Ctrl: black circles) or in the presence (blue circles) of 1 µM BAY in 7 cells investigated. ***P* < 0.01 paired Student's t-test.

In order to better clarify A_{2B}AR effect we applied a depolarizing step at +10 pA (1200 ms), each 30s, and we confirmed that 1 µM BAY60-6583 increased the number of APs, compared to control condition, in 9 cells tested (Figure 52A, B). However, BAY60-6583 did not modify the membrane potentials (Vm) that is, on average, -60.0 ± 3.7 mV in control conditions and -55.2 ± 4.7 in the presence of the selective A_{2B}AR agonist (data not shown). Similar results were found in the presence of a lower concentration of BAY60-6583 (300 nM, Figure 52B). In this conditions the increase in the number of APs induced by the A_{2B}AR was prevented by the application of the selective A_{2B}AR antagonist, PSB603 (100 nM, Figure 52B).

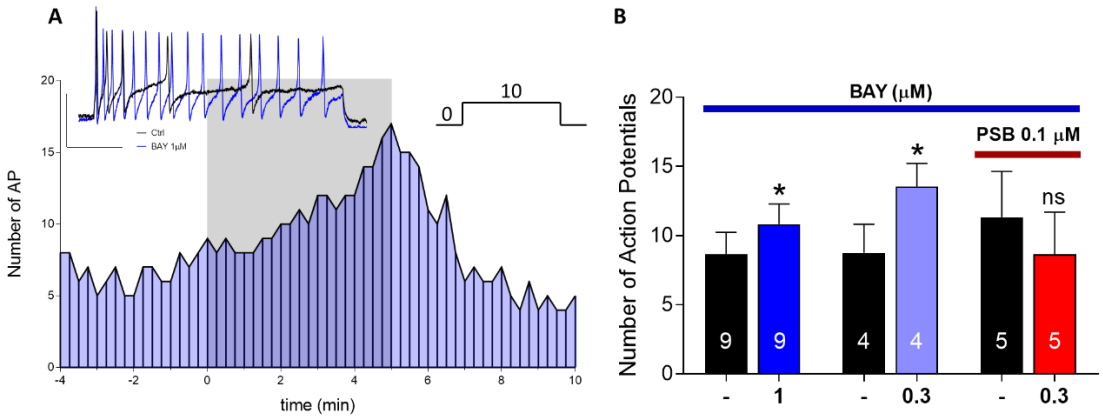


Figure 52. The A_{2B}AR-mediated increase in the number of action potentials (APs) is prevented by the selective A_{2B}AR antagonist, PSB603. (A) Time course of APs number changes evoked in a typical DRG neuron by a 10 pA depolarizing step current injection (every 30 s) recorded before (black trace) or after (blue trace) 5 min 1 μM BAY606583 (BAY) application (inset). (B) Pooled data of APs number measured before or after 5 min BAY606583 application (1 μM: blue column; 0.3 μM: purple column), respectively, alone or in presence of the selective A_{2B}AR antagonist (0.1 μM PSB603). **P* < 0.05 paired Student's t-test.

4.5 DISCUSSION

The stimulation of adenosine "A_2" receptor subtypes by the endogenous ligand adenosine reduced outward-K^+ currents in cultured OPC. The experiments were conducted in the presence of the A_1AR and A_3AR antagonists, DPCPX and MRS1523, respectively, in order to highlight adenosine effects on A_{2A}ARs and A_{2B}ARs. The effect of adenosine in these experimental conditions was similar to what observed by applying the selective A_{2B}AR agonist in cultured OPCs and was prevented by the new multi-target A_{2A}-A_{2B}AR antagonist, P626. Furthermore, we demonstrated, for the first time, that the selective stimulation of A_{2B}AR increased axonal myelination in OPC-DRG co-cultures.

As mentioned above, adenosine is an important neuromodulator that exerts its function by the activation of four selective receptor subtypes. All the four adenosine receptors are expressed at each stage of OL maturation and exert a crucial role in oligodendrogliogenesis (Stevens et al., 2002; Fields, 2004). It is known that the Gi-coupled A_1AR stimulation reduces OPC proliferation and promotes their differentiation in pre-myelinating OLs (Stevens et al., 2002). Furthermore, the activation of these receptors is known to stimulate OPCs migration (Othman et al., 2003). Concerning the A_3AR subtypes, no data are present in literature about their role in OPCs differentiation. However, it is known that the selective A_3AR agonist, 2-Cl-IB-MECA, induces apoptosis of cultured rat OLs (González-Fernández et al., 2014). Concerning the Gs-coupled "A_2" receptors, Coppi et al. (2013a) demonstrated that the selective A_{2A}AR stimulation by CGS21680 inhibited TEA-sensitive delayed rectifying K^+ currents (I_K) in cultured OPCs. Moreover, treatment of cultured OPCs with 3 mM TEA significantly decreased their differentiation (Coppi et al., 2013a). Furthermore, it was demonstrated that the selective A_{2B}AR agonist, BAY60-6583, not only reversibly inhibited outward I_K but also 4-AP-sensitive transient K^+ (I_A)

conductance, differently from the A_{2A}AR stimulation, an effect prevented by the selective A_{2B}AR antagonist, MRS1706 (Coppi et al., 2020a). It was also observed that the AC activator forskolin inhibited K^+ currents and occluded the effects of a further application of BAY60-6583, suggesting that A_{2B}ARs affect K^+ currents by increasing intracellular [cAMP]. Nevertheless, it was demonstrated in the same work that A_{2B}AR downregulation by small interference RNA (siRNA) increased OPCs differentiation, indicating that the selective activation of these receptors reduced the maturation of these cells.

In this Thesis, we evaluated the effect of the endogenous ligand adenosine on ramp-evoked K^+ currents (from -80 mV to + 120 mV) in cultured OPCs. Similar to what observed in the presence of CGS21680 or BAY60-6583, adenosine (0.1-50 µM) inhibited outward-K^+ currents in a concentration-independent manner. In particular, a low concentration of adenosine (0.1 µM) reduced only the sustained K^+ current $I_{K,}$ consistent with the A_{2A}AR activation, while the application of high concentration of adenosine (50 µM) inhibited also the transient K^+ current I_A, in line with the relative differences in adenosine sensitivity (high sensitivity for A_{2A}ARs, low sensitivity for A_{2B}ARs), of the "A_2" receptor subtypes.

While the I_K role in OPC differentiation is clear (Coppi et al., 2013a; Coppi et al., 2020a), the modulation of I_A currents has not been extensively studied in glial cells (Olsen and Sontheimer, 2004). However, it has been postulated that I_A modulation could have substantial impact on the ability of Schwann cell processes to contact axons (Peretz et al., 1999), since downregulation of this current is permissive for axonal contact (Despeyroux et al., 1994).

As I_A is widely diffused along demyelinated axons and contributes to multiple sclerosis (MS) symptoms (Waxman et al., 1994), in 2010 dalfampridine, an extended-release form of 4-AP, has been approved by the FDA to improve walking in MS patients (Zhang et al., 2021). For all these reasons, it is important to deepen the role of I_A modulation by A_{2B}ARs in OPCs. The A_{2B}ARs, the more enigmatic subtypes that present a low affinity for the endogenous ligand, differently from A_{2A}Rs, may also activate Gq proteins (Antonioli et al., 2019), and this could explain the difference between "A_2" receptors on K^+ currents.

In addition, we demonstrated for the first time that, in the presence of the selective A_1AR and A_3AR antagonists, the effects of adenosine in reducing outward-K^+ currents was significantly antagonized in the presence of the multi-target A_{2A}-A_{2B}ARs antagonist P626. However, P626 did not totally prevented the adenosine effect and this was probably related with the high concentration of the endogenous ligand (in the order of µM). Nevertheless, a lower concentration of adenosine (0.1 µM) is able to activate only A_{2A}ARs, hence the effect of the multi-target antagonist, P626, on A_{2B}AR subtype would not be investigated under these experimental conditions. Another hypothesis could be the presence of an A_{2A}AR-A_{2B}AR heterodimer in OPCs where the activation of one of the two AR subtypes might occlude the effect of the other or that both effects converge on the same cellular pathway. However, further experiments will have to be performed to support this hypothesis.

In addition, we demonstrated that also the effect of the selective A_{2B}AR agonist, BAY60-6583, in inhibiting K^+ currents were blocked by the application of P626 in cultured OPCs.

On the basis of results from Coppi et al., (2013a, 2020a), we can suppose that these effects of adenosine may be also related to a reduction of OPC *ex-vivo* differentiation. Hence, the multi-target antagonist, P626, could represent a possible new compound to test in demyelinating pathologies, such as MS, as it could be able to prevent the effects of endogenous adenosine, released in pathological condition, on "A_2" AR subtypes (Safarzadeh et al., 2016).

Moreover, in I part of this Thesis, we demonstrated that P626 delayed the AD onset during OGD in the CA1 hippocampus, an event that is correlated to neuronal injury. Therefore, in relation of these results, P626 could be also efficacious in preventing the demyelinating effects related to a hypoxic-ischemic insult.

Consistently with these data, in literature it was demonstrated that the A_{2B}AR blockade ameliorates the detrimental effects in a rat model of sensorineural HL (SNHL) and in a mouse model of MS (Manalo et al., 2020). Also the A_{2A}AR antagonism seems to be advantageous in preventing the symptoms in a mouse model of EAE. However, other studies suggested that "A_2" agonists promote myelination (Ma et al., 2022), indicating a controversial role of Gs-coupled ARs in oligodendrogliogenesis and myelination.

On these basis, we evaluated the effects of the "A_2" subtypes on myelinating processes. To this purpose, we chronically treated (14 days) OPC-DRG co-cultures with selective A_{2A}AR or A_{2B}AR agonists, in order to evaluate their role in myelination. Surprisingly, the chronic treatment with BAY60-6583 (1 μM) increased the myelination index without affecting the total expression of MBP, a marker of mature OLs. This myelination index was calculated by the Mander's coefficient (M1), which represents the fraction of MBP^+ pixels overlapping the β3-$tubulin^+$ pixels in each region of interest (ROI) (Igado et al., 2020). Similar, but non-significant, results were obtained in the presence of the selective A_{2A}AR agonist, CGS21680, possibly due to the lower number of experiments (n=3).

As mentioned above, in the literature it was demonstrated that A_{2B}AR activation prevented OPCs differentiation (Coppi et al., 2020a). In order to explain the discrepancy between results obtained in in primary OPCs cultured alone or in the presence of DRG neurons, we investigated the functional role of this receptor subtype on DRG neurons.

First, we found, by immunocytochemical analysis, that DRG neurons expressed A_{2B}ARs, accordingly to what observed by Li et al. (2022) on ST36 DRG neurons. In addition, we demonstrated that the selective stimulation of A_{2B}AR with BAY60-6583, at the same concentrations used in OPC-DRG co-cultures, increased the action potentials (APs) *firing* in DRG neurons. However, BAY60-6583 did not modify the membrane potentials (Vm) and this could indicate that it modulated cell excitability. This effect was reversible and blocked by the application of the selective A_{2B}AR antagonist, PSB603. Hence, we can affirm that the selective stimulation of A_{2B}ARs on DRG neurons was able to modulate their *firing*, and, as reported in literature, this effect could be associated with the release of chemoattractant compounds (i.e. glutamate, adenosine, ATP) that are involved in OPCs differentiation and myelin deposition (Cherchi et al., 2021a). However, as mentioned above, we did not find a significantly difference in total MBP expression in co-culture and it could be due to the inhibitory effect of A_{2B}AR in OPCs differentiation. The insights provided by these results might uncover that the different cellular localization of the A_{2B}ARs (i.e. on OPCs or on DRG neurons) could be key to justify distinct effects of BAY60-6583 on

oligodendrogliogenesis and myelination process depending on the experimental model implicated.

In conclusion, we can postulate that the A_{2A}AR and A_{2B}AR are necessary to oligodendrogliogenesis regulation and could represent a promising target for the treatment of demyelinating disorders.

PUBLICATIONS

Dr. Martina Venturini

Publications in international Journals

Cherchi F., **Venturini M**., Magni G., Scortichini M., Jacobson K.A., Pugliese A.M., Coppi E. (2023). Covalently Binding Adenosine A3 Receptor Agonist ICBM Irreversibly Reduces Voltage-Gated Ca2+ Currents in Dorsal Root Ganglion Neurons. Purinergic Signal. doi: 10.1007/s11302-023-09929-y. Epub ahead of print. IF=3.95

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Cherchi F., **Venturini M.**, Santalmasi C., Frulloni L., Dettori I., Coppi E. and Pugliese A.M. Pharmacological and functional characterization of adenosine "A2" receptors by using the endogenus neuromodulator adenosine and the novel dual A2A/A2B receptor antagonist, P626, in OPC culture.

Cherchi F., **Venturini M.**, Santalmasi C., Frulloni L., Dettori I., Coppi E. and Pugliese A.M. Pharmacological and functional characterization of adenosine "A2" receptors in myelination processes in vitro and in vivo cuprizone demyelination model.

Article in submission

Venturini M., Cherchi F., Santalmasi C., Frulloni L., Dettori I., Catarzi D., Pedata F, Colotta V., Varano F., Coppi E. and Pugliese A.M. Pharmacological characterization of P626, a novel dual adeno-sine A2A/A2B receptor antagonist, on synaptic plasticity and during an ischemic-like insult in CA1 rat hippocampus. Under submission.

Abstracts at national and international meetings

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