2013

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Interaction between ecto-5'-nucleotidase and adenosine A_{2A} receptors in nerve terminals of mice prefrontal cortex

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Index

Abbreviations

5’-AMP – Adenosine-5’-monophosphate

5’-UMP – Uridine-5'-monophosphate

A_1R – Adenosine A_1 receptor

$A_{2A}R$ – Adenosine A_{2A} receptor

$A_{2B}R$ – Adenosine A_{2B} receptor

A_3R – Adenosine A_3 receptor

$A_{2A}R$-KO – Adenosine A_{2A} receptor knockout

ACh – Acetylcholine

aCSF – Artificial cerebrospinal fluid

ADK – Adenosine kinase

ADP – Adenosine diphosphate

AOPCP – α,β-methylene-adenosine diphosphate

AP – Alkaline phosphatase

APS – Ammonium persulphate

ATP – Adenosine triphosphate

BCA – Bicinchoninic acid

BSA – Bovine albumin serum

cAMP – Cyclic adenosine monophosphate

CAPS – (3-[cyclohexylamino]-1-propane-sulfonic acid)

CLAP – Cocktail of proteases inhibitors

CNS – Central nervous system

DTT – Dithiothreitol

E-5’N – Ecto-5’-nucleotidase

ECF – Enhanced chemifluorescence

EDTA – Ethylenediaminetetraacetic acid

ENT – Equilibrative nucleoside transporter

E-NPP – Ectonucleotide pyrophosphatase

E-NTPDases – Ectonucleoside triphosphate diphosphohydrolases

fEPSP - Field excitatory postsynaptic potentials

GABA – γ-aminobutyric acid

GFAP – Glial fibrillary acidic protein

GPI - Glycosyl-phosphatidyl-inositol

GTP – Guanosine triphosphate

HBM – HEPES buffered medium

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC – High Pressure Liquid Chromatography

IB – Isolation buffer

IgG – Immunoglobulin G

IP - Immunoprecipitation

LTP – Long-term potentiation

NHS – Normal horse serum

PBS – Phosphate buffered saline

PFA – Paraformaldehyde

PFC – Prefrontal cortex

Pi – Inorganic phosphate

PMSF – Phenylmethanesulfonylfluoride

PVDF – Polyvinilidene fluoride

SAH – S-adenosyl homocysteine

SCH 58261 – 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5 c)pyrimidine

SDS – Sodium dodecyl sulphate

SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM – Standard error of the mean

TBS – Trizma buffered saline

TBS-T – Trizma buffered saline with tween

TEMED – Tris(hydroxymethyl)aminomethane

UDP – Uridine diphosphate

UTP – Uridine triphosphate

VNUT - vesicular nucleotide transporter

Abstract

The activation of adenosine A_{2A} receptors ($A_{2A}R$) is mediated by adenosine that can be originated from the extracellular catabolism of released ATP. Ecto-5'-nucleotidase (e-5'N) plays a key role in the formation of ATP-derived adenosine and in the subsequent activation of $A_{2A}R$ to control synaptic plasticity. Upon brain injury, ATP is released as a stress signal and both e-5'N and $A_{2A}R$ are up-regulated in parallel. This prompts the hypothesis that e-5'N and $A_{2A}R$ could be co-localized and co-regulated.

The present study aims to define: i) the synaptic and sub-synaptic (pre-, post- and extra-synaptic regions) localization of e-5'N focusing in the prefrontal cortex (PFC) of adult C57Bl/6 mice, ii) the co-localization of e-5'N with $A_{2A}R$ in slices from the PFC and in cortical nerve terminals, iii) if the genetic deletion of $A_{2A}R$ affects the density of synaptic e-5'N in cortical regions, iv) the function of e-5'N in synaptic plasticity in the PFC, and finally v) if aging affects the density of synaptic e-5'N in PFC regions. The comparison by Western blot analysis of the density of e-5'N in prefrontal cortex total membranes and synaptosomes revealed that e-5'N, was present in nerve terminals (52.1 ± 2.3%, n=4), but was not as enriched as in the bulk of total membranes (72.5 ± 3.1%, n=4). The fractionation of prefrontal cortex synaptosomes unveiled the presence of two different isoforms of e-5'N, one being more present at the pre-synaptic and extra-synaptic fractions (~50 kDa) and the other (~70 kDa) at the post-synaptic fraction. By immunohistochemistry it was possible to observe an apparent co-localization of e-5'N with $A_{2A}R$, mainly associated with neurons and microglia but not with astrocytes. The pull-down of $A_{2A}R$ revealed a physical association of $A_{2A}R$ with e-5'-N in nerve terminals from the prefrontal cortex. The genetic deletion of $A_{2A}R$ did not affect significantly the levels of e-5'-N, although it was found a slight increase in the levels of

this enzyme. Electrophysiological studies in prefrontal cortex slices incubated with a selective antagonist of $A_{2A}R$ (SCH 58261) demonstrated that the activation of $A_{2A}R$ was necessary to obtain long term potentiation (LTP); however when e-5'N was blocked (with AOPCP), and consequently the adenosine formation was prevented, the basal synaptic transmission suffered an inhibitory effect but there were no significant changes in the LTP phenomenon, triggered by 5 trains of 300 Hz stimuli. We also evaluated by HPLC measurements the basal levels of AMP and adenosine in synaptosomes from the PFC and as expected it was observed that in the presence of AOPCP, the levels of AMP were increased, since this nucleoside was not converted into adenosine, nevertheless the levels of adenosine did not change significantly; suggesting that the contribution of e-5'N to adenosine formation in nerve terminals of PFC was not substantial. Finally we analysed whether the synaptic density of e-5'N was affected by the aging, thus we compared by immunoblot the density of this enzyme in young adult (8-12 weeks old) and in adult mice (36-40 weeks old); nevertheless, no significant differences were observed between these two groups of animals

Although this work have some question that need to be more deeply investigated, the results give new insights about the relation between e-5'N and $A_{2A}R$ in the PFC, and could be useful to tackle questions regarding frontal lobe brain dysfunctions.

Keywords: Ecto-5'-nucleotidase, prefrontal cortex, synaptic transmission, adenosine receptors, nerve terminals.

Sumário

A ativação dos recetores de adenosina A_{2A} ($A_{2A}R$) é feita através da adenosina que pode ser produzida através do catabolismo do ATP libertado no meio extracelular. A ecto-5'-nucleotidase (e-5'N) desempenha um papel importante na formação de adenosina proveniente do catabolismo do ATP, e subsequentemente na ativação dos $A_{2A}R$ controlando assim a plasticidade sináptica. Após uma lesão cerebral, o ATP é libertado como um sinal aversivo provocando o aumento em simultâneo da atividade da e-5'N e da densidade dos $A_{2A}R$. Isto levanta a hipótese de que a e-5'N e os $A_{2A}R$ podem encontrar-se co-localizados e poderá haver uma interação funcional entre eles.

Este estudo tem como objetivos definir: i) a localização sináptica e sub-sináptica (regiões pré-, pós- e extra-sinápticas) da e-5'N focando principalmente no córtex pré-frontal (PFC) de ratinhos C57Bl/6 adultos, ii) a co-localização da e-5'N com os $A_{2A}R$ em fatias do PFC e em terminais nervosos corticais, iii) se a deleção genética dos $A_{2A}R$ afeta a densidade sináptica da e-5'N em regiões corticais, iv) a função da e-5'N na plasticidade sináptica do PFC, e por fim, v) se o envelhecimento afeta a densidade sináptica da e-5'N em regiões do PFC. A comparação por análise de Western blot da densidade da e-5'N em membranas totais e sinaptossomas do córtex pré-frontal revelou que a e-5'N se encontrava nos terminais nervosos (52.1 ± 2.3%, n=4), mas não se encontrava tão enriquecida como nas membranas totais (72.5 ± 3.1%, n=4). O fracionamento dos sinaptossomas do córtex pré-frontal mostrou a presença de duas isoformas diferentes da e-5'N, estando uma mais presente nas frações pré-sináptica e extra-sináptica (~50 kDa) e outra presente (~70 kDa) na fração pós-sináptica. Através de imunohistoquímica foi possível verificar uma aparente co-localização entre a e-5'N e os $A_{2A}R$, estando mais presentes em neurónios e microglia, mas não com astrócitos. O

"pull-down" dos $A_{2A}R$ em terminais nervosos do córtex pré-frontal revelou uma associação física entre os $A_{2A}R$ e a e-5'-N. A delecção genética dos $A_{2A}R$ não afetou significativamente os níveis de e-5'-N, no entanto verificou-se um ligeiro aumento nos níveis desta enzima. Estudos de eletrofisiologia em fatias de córtex pré-frontal incubadas com um antagonista seletivo dos $A_{2A}R$ (SCH 58261) demonstraram que a ativação dos $A_{2A}R$ era necessária para obter potenciação de longa duração (LTP); porém quando a e-5'N era inibida (com AOPCP), prevenindo a formação de adenosina, a transmissão sináptica basal sofria um efeito inibitório mas não se observaram diferenças significativas no fenómeno da LTP, desencadeado por 5 "trains" de estímulos de 300 Hz. Também foram avaliados através de HPLC os níveis basais de AMP e adenosina em sinaptossomas de córtex pré-frontal, e como esperado observou-se níveis aumentados de AMP na presença de AOPCP, uma vez que este nucleósido não estava a ser convertido em adenosina, no entanto os níveis de adenosina não se encontravam alterados significativamente; o que sugere que a contribuição da e-5'N para a formação de adenosina nos terminais nervosos do PFC não é significativa. Finalmente, analisou-se se a densidade sináptica da e-5'N era afetada pelo envelhecimento, para tal comparámos por imunoblot a densidade desta enzima em ratinhos jovens adultos (8-12 semanas de idade) e em ratinhos adultos (36-40 semanas de idade); porém, não se observaram diferenças significativas entre estes dois grupos de animais.

Embora este trabalho tenha algumas questões que precisam de ser detalhadas com mais rigor, os resultados contribuem com novos dados sobre a relação entre a e-5'N e os $A_{2A}R$ no PFC, e poderão ser úteis para resolver questões relacionadas com disfunções do lobo frontal do cérebro.

Palavras-chave: Ecto-5'-nucleotidase, córtex pré-frontal, transmissão sináptica, receptores de adenosina, terminais nervosos.

1. Introduction

1.1. Purinergic system

Purines, like ATP, ADP and adenosine, and pyrimidines, such as UTP and UDP, are extracellular messengers widely distributed throughout the organism, promoting their effects through activation of membrane receptors (Ralevic and Burnstock, 1998; Burnstock, 2013). It has been known that the purinergic compounds play several different roles, such as cardiac function, platelet aggregation and vascular tone (Collins and Hourani, 1993), and also can act as trophic factors and endogenous regulators of growth and cell differentiation, both during development and in adulthood (Abbracchio *et al*., 1994). The purinergic compounds can activate two types of purinergic receptors known as P_1 and P_2 receptors. The P_1 receptors are all metabotropic while P_2 can be metabotropic (P_2Y receptors) or ionotropic receptors (P_2X receptors).The P_2 receptors are activated by ATP, UTP, ADP and UDP, whereas the P_1 receptors are activated by adenosine thus these are also known as adenosine receptors (Fig.1).

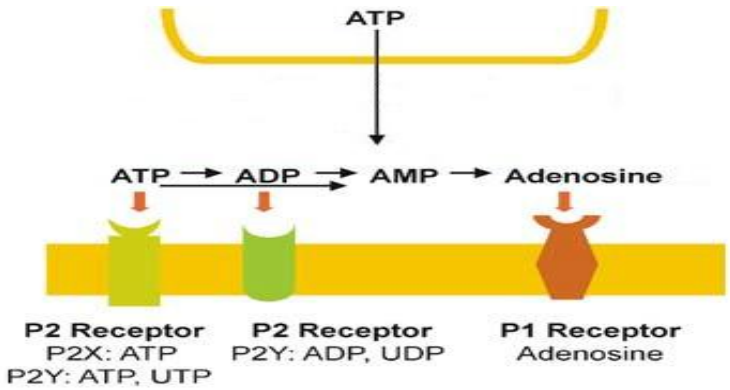

Fig.1 - Representative pathway of extracellular nucleotide metabolism and receptors for the different purinergic compounds. ATP released by cells can be metabolized into its different catabolites. ATP, ADP and AMP activate mainly P_2 receptors, whereas adenosine activates P_1 receptors. Adapted from Zimmermann, 2006.

1.2. ATP storage, release and breakdown

ATP is a ubiquitous molecule that is present in cytosol and in intracellular organelles, mainly in the secretory vesicles of neurons. The neurons contain ATP in their cytoplasm in a concentration around 2-5 mM, however in their synaptic vesicles a much higher concentration of ATP (100 mM) is stored (Burnstock, 2007). These vesicles also contain other nucleotides, such as ADP or GTP. It was reported the existence of a vesicular nucleotide transporter (VNUT) capable of transporting ATP into vesicles (Sawada *et al*., 2008). Apart from being co-stored with other nucleotides, ATP was found to be stored with acetylcholine (ACh) (Reigada *et al*., 2003) or with noradrenaline (von Kugelgen *et al*., 1994). In the central nervous system (CNS) the first evidence of calcium-dependent ATP release was obtained from synaptosomes (isolated nerve terminals), and this release was found to be dependent on membrane depolarization and Na^+ channels activation (North and Verkhratsky, 2006). Following these findings, it was then discovered that ATP could be released from specific brain regions, such as the cortex (North and Verkhratsky, 2006).

Synaptic vesicles contain a proton (H^+) pump which has the purpose of creating a high concentration of protons inside the secretory vesicle, making its interior positively charged in comparison to the cytosol. This electrochemical gradient is responsible for the uptake of several neurotransmitters by specific transporters (Pankratov *et al*., 2006). ATP, which is negatively charged, can be more easily stored into vesicles containing amines and ACh (Pankratov *et al*., 2006). This co-storage of ATP with different other neurotransmitters is highly dependent on the type of neuron in which the vesicles are stored (Volonté *et al*., 2003). The transportation of ATP into the vesicles is carried out through an ADP/ATP translocase, and this process occurs in

every stage of the vesicle formation and recycling. Also, ATP can be taken up by from pools of vesicles: i) vesicles still reserving ATP and/or vesicles that are ready to release ATP (Südhof, 2004); and more interestingly ATP is continuously taken up by vesicles even after the vesicular ACh transporter is blocked by inhibitors (Pankratov *et al*., 2006). There are evidences suggesting that the concentration of ATP within the secretory vesicles is directly related to vesicle lifetime (Pankratov *et al*., 2006). This could mean that only vesicles that spent more time in the reserve pool and/or vesicles that are formed in the cell body and transported along the axon contain large amounts of ATP.

Although controversial, there are already some evidences suggesting that ATP can be released separately from other neurotransmitters (reviewed in Pankratov *et al*., 2006). In experiments done with synaptosomes it was possible to observe that ATP and noradrenaline were stored in separate pools of vesicles and that their release depends on different pre-synaptic modulation (White and MacDonald, 1990). Other neurochemical studies performed in medial habernula showed that ATP is co-released with glutamate (Sperlágh *et al*., 1998). However, physiological studies clearly demonstrated that ATP and glutamate-mediated signals are from different axons (Robertson and Edwards, 1998). Thus, depending on the type of synaptic terminal we are studying, we may find different secretory vesicles with different contents; in some there may be co-storage of ATP with other neurotransmitters and in others only ATP.

ATP can be released from different types of cells, such as astrocytes, vascular cells and neurons; and this neurotransmitter can be release either by pre-synaptic or post-synaptic terminals (Burnstock, 2007). This release occurs not only by stimulation via neurotransmitters, but also from stressful stimulus such as hypoxia (Fields and Burnstock, 2006).The action of classical neurotransmitters are regulated by the release

and removal of the transmitter from the synaptic cleft, but ATP released in the synaptic cleft is hydrolyzed and each one of its catabolites can activate different types of receptors (see Fig.1). ATP and its final reaction product, adenosine, often display antagonistic actions, providing a well-balanced mechanism of homeostatic regulation (Cunha, 2001a; Fields and Burnstock, 2006).

There are several studies about the ectonucleotidases that breakdown ATP released from neurons and from non-neuronal cells. The enzymes capable of hydrolysing ATP are subdivided into four families: ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), of which E-NTPDase 1, 2, 3 and 8 are extracellular; ectonucleotide pyrophosphatase (E-NPP), which has 3 subtypes (E- NPP 1, 2, 3); alkaline phosphatases and ecto-5′-nucleotidase (see Table 1). NTPDase 1 hydrolyses ATP directly to AMP, and UTP to UDP, whereas NTPDase 2 converts ATP to ADP, and ecto-5′-nucleotidase catabolizes AMP into adenosine (Shirley *et al*., 2009). These enzymes have a wide distribution throughout the brain and can be present in the same cells (Zimmermann, 2001). In the nervous system, enzymes responsible for the hydrolysis of 5'-mononucleotides (like 5'-AMP or 5'-UMP) are mostly present in the glia cells, including astrocytes, microglia and oligodendrocytes (Zimmermann, 1992). Therefore, ectonucleotidases are involved in the modulation of synaptic transmission, microglial function and in glial ATP-derived calcium wave propagation (Fields and Burnstock, 2006).

Thus, nucleotides and nucleosides formed during the ATP breakdown mediate several trophic effects on neurons and glial cells, like cell proliferation, axonal growth and cell differentiation (Zimmermann, 2006). This suggests that cellular communication via nucleotides can implicate a variety of molecular interactions and cellular signaling pathways.

Table 1 - ATP hydrolysis derived from several ectonucleotidases (adapted de Shirley *et al*., 2009).

Enzyme	Hydrolysis pathways
E-NTPDases	
E-NTPDase 1	ATP → ADP + Pi → AMP + 2Pi
	ADP → AMP + Pi
E-NTPDase 2	ATP → ADP + Pi
	ADP → AMP + Pi
E-NTPDase 2 and E-NTPDase 8	ATP → ADP + Pi
	ADP → AMP + Pi
E-NPPs	
E-NPP 1 and E-NPP 3	ATP → AMP + PPi
	ADP → AMP + Pi
	3',5'-cAMP → AMP
E-NPP 2	ATP → AMP + PPi
	ADP → AMP + Pi
	3',5'-cAMP → AMP
Ecto-5'-Nucleotidase	AMP → adenosine + Pi
Alkaline phosphatases	ATP → ADP + Pi
	ADP → AMP + Pi
	AMP → adenosine + Pi

1.3. Adenosine as a neuromodulator and P_1 receptors

While ATP may act as a neurotransmitter in most brain regions (Burnstock, 2007), adenosine is neither stored in vesicles nor released like a classical neurotransmitter. Adenosine does not accumulate in synaptic vesicles and it is released into the extracellular medium through nucleoside transporters. Depending on the concentration of adenosine in intracellular and extracellular space, these transporters are also capable of doing adenosine re-uptake (Gu *et al*., 1995; Sebastião and Ribeiro, 2009). In fact, since adenosine is not released by exocytosis, it behaves as an

extracellular signaling molecule that exerts its influence in synaptic transmission; thus adenosine is not considered to be a neurotransmitter but rather a neuromodulator (Cunha, 2001a). Making use of G-protein-coupled mechanisms, adenosine not only leads to changes in secondary-messenger levels, like cyclic AMP (cAMP), but also modulates ion channels activity. Adenosine is also capable of modulating neuronal activity by facilitating or inhibiting neurotransmitters release in pre-synaptic and post-synaptic regions (Sebastião and Ribeiro, 2009). The contradictory pattern by which adenosine exerts its functions is due to the fact that it activates different G-protein-coupled receptors, known as P_1 or adenosine receptors. The P_1 receptors are subdivided into four subtypes which are commonly known as A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm *et al*., 1996). Receptors A_1 (A_1R) and A_3 (A_3R) possess an inhibitory function over adenylyl cyclase (enzyme responsible for the conversion of ATP into cAMP), and on the other hand, A_{2A} ($A_{2A}R$) and A_{2B} ($A_{2B}R$) have a facilitatory effect on adenylyl cyclase. There is much information regarding the activity and localization (Fig.2) of A_1R and $A_{2A}R$ since these receptors are the ones which have a higher affinity towards adenosine and are more abundant throughout the CNS (Dunwiddie and Masino, 2001; Fredholdm *et al*., 2001; Porkka-Heiskanen and Kalinchuk, 2011). A_1R and $A_{2A}R$ play opposite roles in neurotransmission because they are coupled to different G-proteins. A_1R is coupled to a G_i protein and mediates an inhibitory neuromodulation and this inhibitory effect inhibits adenylyl cyclase, activates inwardingly rectifying K^+ channels, blocks Ca^{2+} channels and activates phospholipase C, leading to the inhibition of excitatory neurotransmitters release, such as dopamine and serotonin (Benarroch, 2008). However, activation of A_1R lead them to rapid desensitization, thus leading to the internalization of the A_1R; therefore, activation of A_1R is not a useful tool to obtain neuroprotective effects (Cunha, 2001a), although it was proposed that activation of A_1R

is relevant against epilepsy (Boison, 2011). Contrasting the inhibitory action of A_1R, the $A_{2A}R$ are coupled to a G_s or a G_{olf} protein and stimulate adenylyl cyclase-cAMP-protein kinase signalling pathway facilitating the release of neurotransmitters (Wei *et al*., 2011). However, there is limited information about the mechanism by which $A_{2A}R$ exerts its function. It is known that $A_{2A}R$ receptor is highly abundant in striatal neurons; having a crucial function related with the control of dopaminergic neurotransmission (Cunha, 2005; Garção *et al*., 2013). Adenosine has different interactions with other neurotransmitter systems and because of its action with opposite effects through A_1R and $A_{2A}R$, it plays an essential role fine-tuning and modulating excitatory and inhibitory functions within the CNS.

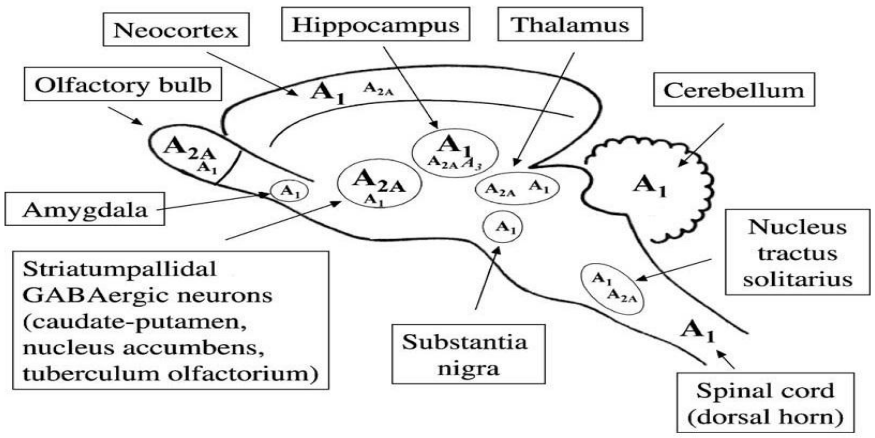

Fig.2 - Distribution of the higher affinity receptors for adenosine in the principal regions of the CNS. Adenosine has been proposed to play a major role in physiological and pathological functions in these regions. Adapted from Ribeiro *et al*., 2003.

There is evidence that points out that under certain conditions adenosine facilitates, rather than inhibits, the release of neurotransmitters such as ACh and dopamine (Cunha, 2005; Garção *et al*., 2013). This indicates that the release of neurotransmitters is not only regulated by the inhibitory A_1R, but also by the facilitatory $A_{2A}R$. This evidence also brought to light that both these receptors can be, and more

often are, localized in the same synapse (Fig.3) (Ribeiro *et al*., 2003; Rebola *et al*., 2005a). The presence of both receptors in the same nerve terminal suggests that each one operates under different physiological conditions (Cunha, 2001a).

It has been observed that at low-frequency stimulation of nerve terminals, the only receptor which had a role is the A_1R imposing its inhibitory action in the neurotransmission. This fact was further corroborated by studies in which A_1R were pharmacologically blocked or simply genetically deleted and the administration of adenosine was added to brain preparations did not affect the synaptic transmission, suggesting that low concentration levels of adenosine (basal levels) activate A_1R. These experiments proved that A_1R played a predominantly role in physiological brain processes (Cunha, 2008). Whereas $A_{2A}R$ obtain more "attention" when the nerve terminal receive high-frequency stimuli, favoring the release of ATP that is hydrolyzed into adenosine to activate the $A_{2A}R$. The $A_{2A}R$ activation served two purposes: the facilitation of neurotransmission and the down-regulation of A_1R which were active at the synapse (Cunha, 2008).

The inhibitory effects mediated by A_1R confer neuroprotection which has been shown in several experimental models (*in vivo* and *in vitro*), mainly in cases of hypoxia/ischemia and seizures (Von Lubitz, 1999; Latini and Pedata, 2001). The neuroprotective effects mediated by A_1R are due to the fact that the influx of Ca^{2+} in the pre-synaptic terminal is blocked and also because the release of neurotransmitters become inhibited. By acting on A_1R, adenosine hyperpolarizes neurons causing a reduced neuronal excitation and thus lowering the number of synapses being fired (Dunwiddie and Masino, 2001), provoking a reduction in cell metabolism and in energy consumption (Kawamura *et al*., 2010).

Little is known about the role of $A_{2A}R$ in neuroprotection, however several data

have shown that some compounds, such as caffeine (Cunha and Agostinho, 2010), had an antagonistic effect in these receptors conferring neuroprotection (Wardas, 2002; Gomes *et al*., 2011; Duarte *et al*., 2012). Also, studies performed using mice that had a genetic deletion of A_{2A} receptor (A_{2A}R knockout), showed that the absence of these receptors afford neuroprotective effects against Alzheimer's disease, Machado-Joseph disease, epilepsy and in conditions of ischemia/reperfusion (Wardas, 2002; Gui *et al*., 2009; Cunha and Agostinho, 2010; Gomes *et al*., 2011; Gonçalves *et al*., 2013), which strengthens the idea that the blockage or the genetic deletion of A_{2A}R confer neuroprotection in several models of disease (Stone *et al*,. 2001; Yaar *et al*., 2005; Cunha and Agostinho, 2010; Gomes *et al*., 2011). It has also been shown that the activation of A_{2A}R leads to an increase in the adenosine uptake (Pinto-Duarte *et al*., 2005). This regulation of extracellular adenosine levels by A_{2A}R is possibly very important in some brain areas, such as the hippocampus, that are under high-frequency neuronal firing, favoring the activation of A_{2A}R instead of A_1R (Cunha *et al*., 1996). It has also been shown that the activation of astrocytic A_{2A}R are responsible for the decrease of glutamate up-take into astrocytes by glutamate transporters (Matos *et al*., 2012). This finding provides evidence for the important role that astrocytic A_{2A}R play in adjusting the extracellular levels of glutamate, by modulating the glutamate transporter activity, consequently playing a key role in the control of neurotransmission (Matos *et al*., 2012). Nevertheless, the extracellular tonic adenosine levels are not the same in all brain regions, being maintained by both intracellular and extracellular mechanisms and also by astrocytes (Dunwiddie et al., 1997; Cunha *et al*., 1998a; Latini and Pedata, 2001; Martín *et al*., 2007).

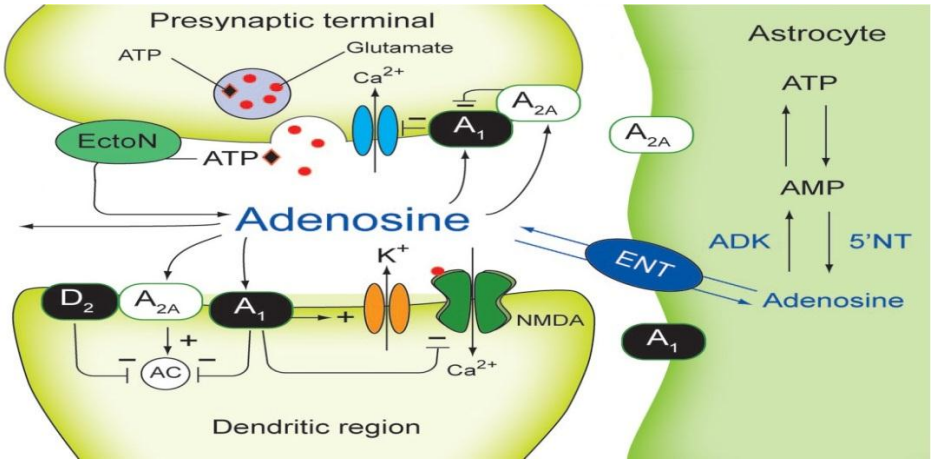

Fig.3 - Main pathways from which adenosine plays its role of signaling in the CNS represented in an excitatory synapse. Extracellular adenosine can be provided by two sources: i) transported through equilibrative nucleoside transporters (ENT) and ii) from ATP catabolism into adenosine through the action of ectonucleotidases (EctoN). A_1R and $A_{2A}R$ present at the same synapse mediate most of the physiological effects of adenosine. A_1R inhibit adenylyl cyclase (AC) whereas $A_{2A}R$ stimulate AC, thus mediating different physiological functions. Adapted from Benarroch, 2008.

1.4. Sources of adenosine

In the last few years there has been an increase in the studies supporting that astrocytes play an important role in neurotransmission, instead of just being relevant for metabolic and supportive functions (Pascual *et al*., 2005; Perea *et al*., 2009). The levels of extracellular adenosine are controlled by an astrocyte-based adenosine cycle (Boison *et al*., 2012). The major sources of extracellular adenosine present at the synaptic cleft are due to ATP released by astrocytes, which can occur by secretion via hemichannels (Kawamura *et al*., 2010) or by secretory vesicles (Pascual *et al*., 2005). The ATP released is subsequently degraded into adenosine by ectonucleotidases (Zimmermann, 2006). Apart from the release of ATP, astrocytes are also capable of releasing adenosine through nucleoside transporters; and the re-uptake of adenosine by these cells does not depend on energy-driven transporters, in contrast to classical neurotransmitters (Boison *et al*., 2010). Astrocytes have two types of equilibrative nucleoside transporters (ENT1

and ENT2), which allow the rapid exchange of adenosine between the extra- and intracellular spaces (Boison *et al*., 2012). The re-uptake of adenosine into astrocytes is done through the action of the equilibrative nucleoside transporters (ENT). Then adenosine is converted by adenosine kinase (ADK), an enzyme which converts adenosine into AMP, thus allowing it to became ATP once more and be ready to be released into the synaptic cleft (Fig.3) (Boison *et al*., 2012). There are data supporting that ADK present in astrocytes is the main regulator of extracellular adenosine levels, because it drives the astrocyte adenosine influx through bi-directional nucleoside transporters (Boison *et al*., 2010).

Neurons are also capable of releasing adenosine to the synaptic cleft, like astrocytes. In fact, neurons can release adenosine through bi-directional ENT and through uni-directional concentrative nucleoside transporters (Sweeney, 1996; Grey *et al*., 2004). The re-uptake of adenosine is carried out through ENT that are responsible for the intracellular *de novo* synthesis of nucleotides (Gu *et al*., 1995; Cunha *et al*., 1996; Latini and Pedata, 2001). Klyuch and colleagues (2012) showed that neurons can also release adenosine by exocytosis by blocking the refilling of synaptic vesicles with bafilomycin A1 (Klyuch *et al*., 2012). It was also shown that adenosine can be released through uni-directional concentrative nucleoside transporters driven by Na^+ (and proton) electrochemical gradients, which widely are distributed in the brain (Grey *et al*., 2004).

There are at least two different pathways from which adenosine can be formed intracellularly, one being ATP-derived adenosine by intracellular ectonucleotidases and the other via the hydrolysis of S-adenosyl homocysteine (SAH) by SAH hydrolase. The SAH hydrolase pathway only provides with one-third of the adenosine produced under physiological conditions. Nevertheless, SAH hydrolase is spread through the main brain

regions (hippocampus, neocortex and cerebellum) and may have some relevance in disease (Latini and Pedata, 2001). Adenosine can also be produced through another pathway that involves intracellular ectonucleotidases. The catabolism of intracellular ATP is carried out through a cytosolic 5'-nucleotidase (one isoform of 5'-nucleotidase family), however it is still difficult to know what is the contribution of this enzyme for adenosine production. Under hypoxic/ischemic conditions, it was described that extracellular adenosine origin is mainly dependent on adenosine release, which is derived from cytosolic ATP catabolism (Fig.4) (Latini and Pedata, 2001).

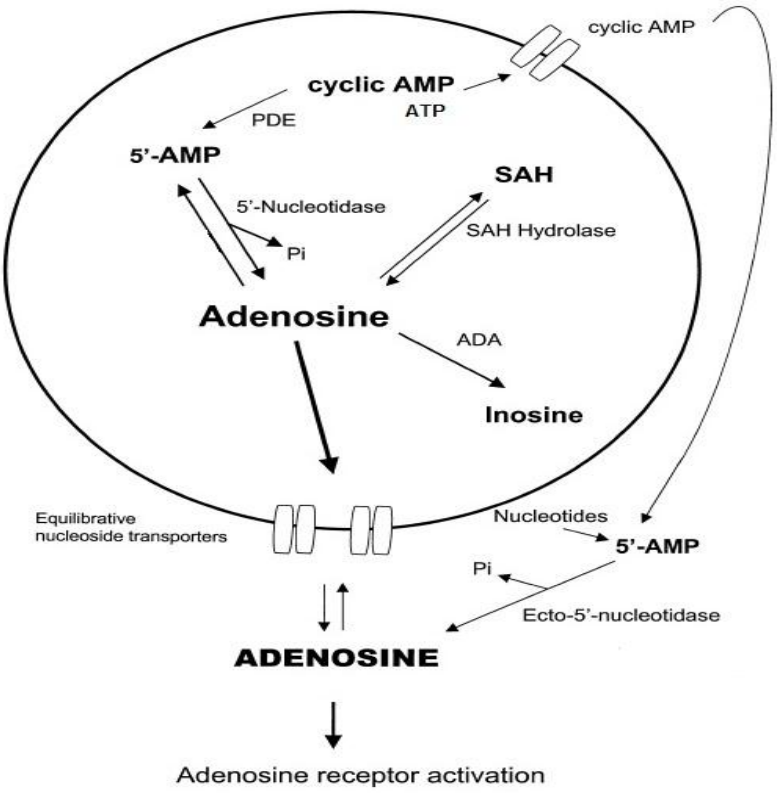

Fig.4 - Pathways of intracellular adenosine production, degradation and transport. Adapted from Latini and Pedata, 2001.

One other form to obtain extracellular adenosine is by the action of ectonucleotidases, a series of enzymes that among other functions are specialized in converting ATP into adenosine. This process is done by four major families of

ectonucleotidases (Fig.5). The first step of this cascade is the inactivation of ATP, which is mediated by the family of ectonucleoside triphosphate diphosphohydrolases (E-NTPDases, also known as ectoATPase or by apyrase) that are responsible by the hydrolysis of ATP into ADP and AMP (Shirley *et al*., 2009; Cognato and Bonan, 2010). These enzymes display a wide distribution throughout the whole brain (Langer *et al*., 2008). Apart from E-NTPDases, ATP can also be degraded by ecto-nucleotide pyrophosphatases (E-NPPs) and by alkaline phosphatases (AP), although both these families have broader substrate specificity, meaning that they can metabolize other purinergic compounds. However, like E-NTPDases, both these families are widely distributed in the brain (Langer *et al*., 2008). The extracellular conversion of AMP into adenosine is carried out through the action of ecto-5'-nucleotidase (e-5'N, also known as CD73) (Shirley *et al*., 2009; Sperlágh and Vizi, 2011). E-5'N is responsible for the limiting step of this cascade and is also distributed in most brain regions (Langer *et al*., 2008).

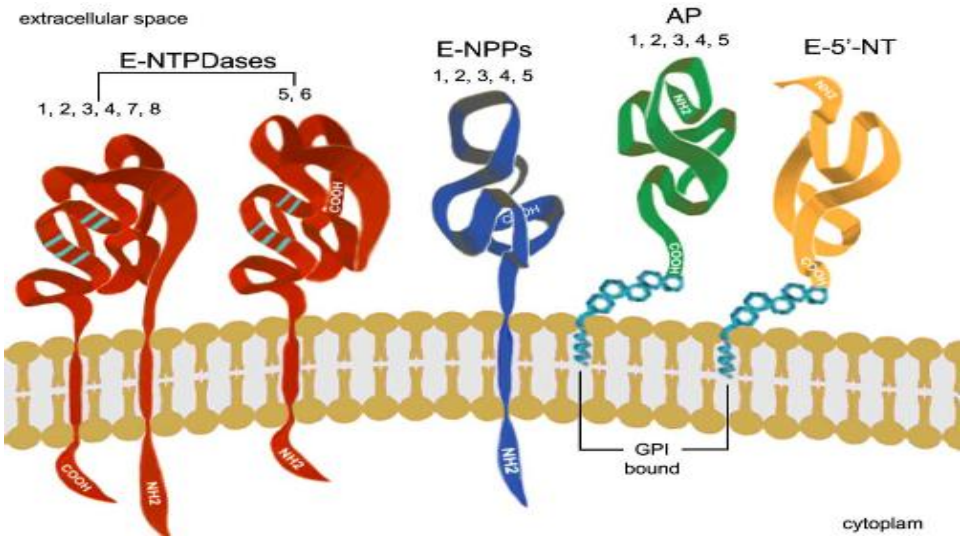

Fig.5 - Extracellular nucleotide-degrading enzymes. Ectonucleotidases present the catalytic site on the extracellular space. E-NTPDases and E-NPPs are integral membrane proteins, going through the whole cytoplasmic membrane, whereas alkaline phosphatases (AP) and ecto-5'-nucleotidase (e-5'N) are glycosyl-phosphatidyl-inositol-anchored proteins. Adapted from Cognato and Bonan, 2010.

1.5. Ecto-5’-nucleotidase – general properties and roles in disease

Until now at least 7 isoforms of the 5’-nucleotidase exists: i) ecto-5’-nucleotidase, ii) cytosolic 5'-nucleotidase IA, iii) cytosolic 5'-nucleotidase IB, iv) cytosolic 5'-nucleotidase II, v) cytosolic 5'(3') deoxyrribonucleotidase, vi) cytosolic 5'-nucleotidase III and vii) mitochondrial 5'(3') deoxyrribonucleotidase (Borowiec *et al*., 2006). The 5’-nucleotidase isoforms varies in their localization, five of them being cytosolic, one attached to the membrane, and the other being present at the mitochondrial matrix (Bianchi and Spychala, 2003; Hunsucker *et al*., 2005). However, the enzyme more involved in the extracellular formation of adenosine is considered to be the ecto-5’-nucleotidase.

E-5’N is codified by a single gene in mammalians although it has been reported the appearance of glycosylated forms (Cunha *et al*., 2000; Zimmermann, 2001). It is linked to the outer part of the plasmatic membrane through a glycosyl-phosphatidyl-inositol (GPI) anchor in its carboxylic terminal; however a soluble form can also exist if the GPI anchor is cleaved (Braun *et al*., 1997). Although it has a broad spectrum of substrates it seems to hydrolise preferentially AMP into adenosine (Zimmermann, 1992; Bianchi and Spychala, 2003). This ectoenzyme can be found in several cell types both in pathological and physiological conditions (Zimmermann, 1992; Resta *et al*., 1998; Bianchi and Spychala, 2003). The presence of e-5’N seems to be involved in events of synaptic plasticity in neurons (Rebola *et al*., 2008), which is in accordance with the fact that adenosine is a neuromodulator (Cunha, 2001a). One of the more obvious roles of the e-5’N is the purinergic recycling, in which by converting AMP into adenosine and inorganic phosphate (Pi) it allows the nucleosides produced extracellularly to be

transported into cells and then converted again by ADK into AMP, which can later be converted into ATP that can be again released into the synaptic cleft (Goding, 2000).

It was demonstrated that the bacterial e-5'N has a quite unique catalytic structure, and the same is thought to happen with the human e-5'N. The crystal structure of bacterial e-5'N has been given many insights about the catalytic mechanism of mammalian e-5'N (Knofel and Strater, 2001). It was seen that e-5'N was composed by two domains, the aminic terminal (N-terminal) and the carboxylic terminal (C-terminal), with its catalytic site holding a bi-metalic center (which binds Zn^{2+}) between the two terminals (Knofel and Strater, 1999). Although e-5'N has a very broad tissue distribution (Langer *et al*., 2008) its catalytic activity varies from tissue to tissue (Zimmermann *et al*., 2012). The e-5'N is mostly located in the hippocampus, namely in CA3 region, and in the hilus of the dentate gyrus, although it can also be present, in less amount in the pyramidal cells of CA1 and CA2 regions of rat hippocamus (Bjelobaba *et al*., 2007). It was also demonstrated that it e-5'N could be found in the olfactory bulb, caudoputamen and olfactory tubercle (Langer *et al*., 2008).

In studies regarding e-5'N molecular properties, it was difficult to detail an apparent molecular mass for the glycosylated form, varying from 60-80 kDa for the monomers and 160 kDa for the dimers, most likely because there are numerous isoforms which can be misleading in some analysis (Cunha *et al*., 2000). In fact, it was shown by two-dimensional electrophoresis that there are at least 13 different isoforms of e-5'N in the brain (Zimmermann, 1992). Moreover, there are evidences suggesting that e-5'N can interact with other components of the extracellular matrix, particularly to laminin and fibronectin. These matrix proteins are involved in several biological processes, such as, cell adhesion, growth, spreading and also migration, which might give us an insight about the other likely functions of e-5'N (Langer *et al*., 2008).

Although there are neurobiochemical studies reporting that e-5'N is active in neurons and in glial cells (Latini and Pedata, 2001), immunocytochemical analysis revealed that e-5'N is mostly associated with the plasmatic membrane of astrocytes, oligodendrocytes and microglial cells (Maienschein and Zimmermann, 1996; Zimmermann, 2006). However there is a lack of co-relation between the reported localization of this ectoenzyme in glial cells and the biochemical evidence regarding its activity in neurons. This is likely due the several e-5'N isoforms present at the CNS (Cunha *et al*., 2000).

There are some studies that show that e-5'N is involved in several pathological conditions, such as epilepsy and hypoxia. Indeed, it was observed in patients with temporal lobe epilepsy, an increase of e-5'N in the dentate gyrus and in the mossy fiber endings of CA4 and CA3 areas (Bonan *et al*., 2001). Similarly it was observed an increase in the activity of e-5'N in synaptosomes from hippocampus and cerebral cortex after the induction of seizure episodes with kainic acid (Bonan *et al*., 2001). In other study regarding the pathological condition of hypoxia, it was shown that the levels of e-5'N were incremented and the administration of α,β-methylene-adenosine diphosphate (AOPCP), an inhibitor of e-5'N, suppressed the augment of extracellular adenosine levels; the low extracellular levels of adenosine may favor the activation of A_1R, thus promoting a decrease of the stroke volume (Cui *et al*., 2013).

1.6. Ecto-5'-nucleotidase possible interaction with A_{2A} receptor in brain disorders - frontal lobe dysfunction

It has already been established the impact of adenosine on synaptic plasticity, mainly acting on A_1R and $A_{2A}R$ (Ribeiro *et al*., 2003), and also the involvement of both receptors in pathological and physiological conditions (see Burnstock *et al*., 2011). The e-5'N plays also a relevant role in synaptic plasticity, converting AMP into adenosine which in turn is directly used to activate $A_{2A}R$ (Rebola *et al*., 2008). However it is still under debate what is the relation of e-5'N with $A_{2A}R$ under pathological conditions.

It is known that ATP is stored in vesicles and released when nerve terminals are stimulated. This release is greater when the nerve terminal suffers high-frequency stimulation, or a noxious stimulus, thus leading to an increase in the concentration of ATP in the synaptic cleft that is further metabolized into adenosine (Burnstock, 2007). However there is another mechanism by which is possible to get adenosine in the synaptic cleft, the nucleoside transporters which are only predominantly active in cases of low-frequency stimulation of the nerve terminal (Boison *et al*., 2010). In the nerve terminal, depending on the levels of adenosine we may have two mechanisms that are responsible for the activation of adenosine receptors: the low-frequency stimulation of the nerve terminal leads to low levels of extracellular adenosine favoring the activation of A_1R, whereas the high-frequency stimulation, leads to high levels of adenosine in the synaptic cleft and activates predominantly $A_{2A}R$, as it can be seen in Figure 6 (Cunha, 2005). It was reported that both stressful (Cunha *et al*., 1996; Cunha *et al*., 2001) and pathological (Agostinho *et al*., 2000; Rebola *et al*., 2003) conditions provoked an increase in e-5'N activity and $A_{2A}R$ density (Napieralski *et al*., 2003), but did not affect A_1R.

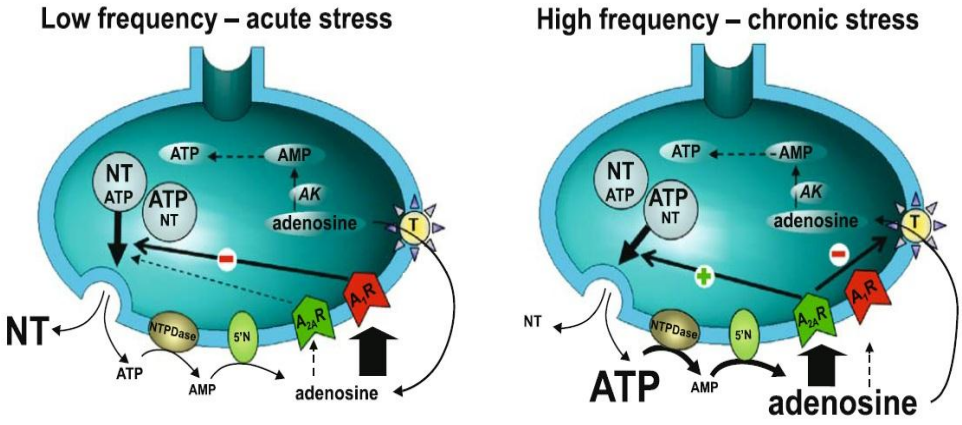

Fig.6 - Schematic representation of the ability of the extracellular metabolism of adenosine (and ATP) to determine which adenosine receptor will play a role in the mouse nerve terminal. At lower frequency stimulations less ATP is released, and thus lower amounts of adenosine is formed, which will allow nucleoside transporters to contribute for the accumulation of extracellular adenosine, favoring A_1R activation. At higher frequency stimulations there is abundant release of ATP, leading to more adenosine formed extracellularly that activate preferentially $A_{2A}R$. Adapted from Cunha, 2005.

The prefrontal cortex (PFC) is central to higher cognitive functions such as, working memory, decision-making and impulse suppression (Arnsten and Pliszka, 2011). Frontal lobe dysfunction is thought to be involved in schizophrenia and age-associated cognitive decline (Convit *et al*., 2001). In fact, because adenosine plays a neuromodulatory role and is important in learning and memory, it was suggested that antagonists of $A_{2A}R$ may prevent cognitive dysfunction (Cunha and Agostinho, 2010; Burnstock *et al*., 2011). This hypothesis is largely supported by the fact that adenosine, as a homeostatic bioenergetic network regulator, may act as neuromodulator of neurotransmitters release into the synaptic cleft; and a dysfunction in the purinergic system, or perhaps even in the purinergic metabolism, would result in a dysregulation of the neurotransmission leading to cognitive impairment (Boison *et al*., 2012).

In an experiment where Cunha and collaborators used aged rats, they observed a decrease of ACh release and impaired synaptic transmission in the hippocampus, probably due to the decrease of glutamate release with aging (Cunha *et al*., 1998b). The

decrease in neurotransmitters release such as glutamate could imply a decrease in ATP release, which subsequent cause a reduction in E-NTPDase activity. However, the formation of adenosine derived from ATP due to the action of e-5'N was incremented in aged rats (Cunha *et al*., 2001); probably because in aged rats there is an increased e-5'N activity (Fuchs, 1991). These evidences strengthen the idea that both e-5'N and $A_{2A}R$ share some association; and also suggests that there is an increase in the levels of $A_{2A}R$ with aging (Fig.7) (Cunha, 2001a).

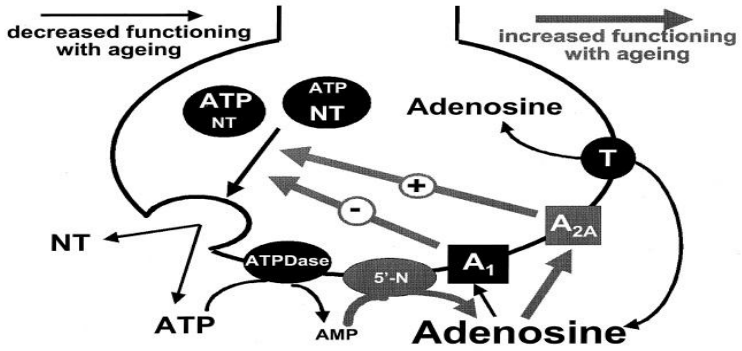

Fig.7 - Modification of the extracellular adenosine metabolism and neuromodulation in nerve terminals of the aged rat. The release of ATP and the activity of E-NTPDases are decreased; however, the $A_{2A}R$ in the nerve terminal are abundant and the activity of e-5'N is increased in aged rats. Adapted from Cunha, 2001a.

Considering the evidences that point out to the association between e-5'N and $A_{2A}R$ in physiological and pathological conditions, and knowing that $A_{2A}R$ play a neuromodulatory role and is also capable of conferring neuroprotection; it is of crucial importance to know if there is a co-regulation between $A_{2A}R$ and e-5'N and if the two proteins are co-localized in physiological conditions and in a context of cognitive impairment related to aging. If this scenario proves itself correct it seems obvious that e-5'N could be a novel target to for purinergic neuromodulation and to achieve neuroprotection.

2. Objectives

The activation of adenosine A_{2A} receptors ($A_{2A}R$) is mediated by adenosine mainly originated from the extracellular catabolism of released ATP. Ecto-5'-nucleotidase (e-5'N) plays a key role in the formation of ATP-derived adenosine and in the subsequent activation of $A_{2A}R$ to control synaptic plasticity (Rebola *et al*., 2008). Upon brain injury, ATP is released as a stressful signal and both e-5'N and $A_{2A}R$ are up-regulated in parallel (Cunha, 2005). This prompts the hypothesis that e-5'N and $A_{2A}R$ could be co-localized and co-regulated. Therefore this study aims to answer the following points:

- ✓ **To determine the synaptic and subsynaptic localization of e-5'N in cortical regions of adult C57Bl/6 mice**

- ✓ **To define the co-localization of e-5'N with $A_{2A}R$ in different cortical cell types and in nerve terminals of PFC**

- ✓ **To assess if the genetic deletion of $A_{2A}R$ affects the density of e-5'N in cortical regions**

- ✓ **To investigate if e-5'N plays an important role in the pre-frontal cortex synaptic plasticity**

- ✓ **To determine the impact of aging on e-5'N density in PFC nerve terminals**

3. Material & Methods

3.1. Material

3.1.1. Reagents

Table 2: Reagents used and respective suppliers.

Reagent	**Supplier**
30% Acrilamide/Bis Solutions	Bio Rad
Acetonitrile	Merck
Adenosine	Sigma-Aldrich
Adenosine 5'-monophosphate sodium salt	Sigma-Aldrich
Ammonuim persulfate (APS)	Sigma-Aldrich
BCA kit	Thermo scientific
Bicine	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
Calcium chloride ($CaCl_2$)	Sigma-Aldrich
CAPS (3-[cyclohexylamino]-1-propane-sulfonic acid)	Sigma-Aldrich
CLAP (cocktail of proteases inhibitors)	Sigma-Aldrich
DAKO Fluorescence mounting medium	DAKO
Dithiothreitol (DTT)	Sigma-Aldrich
ECF	GE Healthcare
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethyleneglycol	Sigma-Aldrich
Protein G PLUS-agarose gel beads	Santa Cruz Biotechnology
Glucose	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Halothane	Sigma-Aldrich
HEPES	Sigma-Aldrich
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich
Methanol	Sigma-Aldrich
Normal Horse serum (NHS)	Invitrogen
Hydrochloric acid (HCl)	Sigma-Aldrich
Paraformaldehyde (PFA)	Sigma-Aldrich
Percoll	GE Healthcare
Penylmethanesulfonylfluoride (PMSF)	Sigma-Aldrich
Potassium chloride (KCl)	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Bio Rad
Sodium azide	Sigma-Aldrich
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium hydroxide (NaOH)	Sigma-Aldrich
Sodium phosphate monobasic (NaH_2PO_4)	Sigma-Aldrich
Sodium thiopental	B.Braun Medical
Sucrose	Sigma-Aldrich
Tissue-tek	Sakura-Americas

Table 2 cont: Reagents used and respective suppliers.

Reagent	Supplier
TEMED	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Trizma base	Sigma-Aldrich
Tween	Sigma-Aldrich

3.1.2. Antibodies

Table 3: Primary and secondary antibodies for Western blotting.

Antibody	Supplier	Host	Dilution
$A_{2A}R$	Millipore	Mouse	1:500
E-5'N	Santa Cruz Biotechnology	Rabbit	1:500
β-actin	Sigma	Mouse	1:10000
PSD-95	Chemicon	Mouse	1:20000
Synaptophysin	Sigma	Mouse	1:20000
Snap-25	Sigma	Mouse	1:20000
Anti-Mouse alkaline phosphatase conjugated	GE Healthcare	Goat	1:20000
Anti-Rabbit alkaline phosphatase conjugated	GE Healthcare	Goat	1:20000

Table 4: Primary and secondary antibodies for immunohistochemistry.

Antibody	Supplier	Host	Dilution
$A_{2A}R$	Santa Cruz Biotechnology	Goat	1:200
E-5'N	Santa Cruz Biotechnology	Rabbit	1:50
GFAP	Cell Signalling	Mouse	1:200
β-tubulin III	Abcam	Mouse	1:2000
CD11b	ABD Serotec	Rat	1:100
Anti-Rabbit Alexa Fluor 594	Invitrogen	Donkey	1:1000
Anti-Goat Alexa Fluor 647	Invitrogen	Donkey	1:1000
Anti-Mouse Alexa Fluor 488	Invitrogen	Donkey	1:1000
Anti-Mouse Alexa Fluor 488	Invitrogen	Rat	1:1000

3.2. Animals

Male C57Bl/6 mice with 8-12 or 36-40 weeks old were obtained from Charles River (Barcelona, Spain). It was also used male C57Bl/6 mice 8-12 weeks old with a genetic deletion for the A_{2A} receptor (A_{2A}R-KO) from our group's animal colony. The animals were housed under controlled temperature (23 ± 2ºC), subject to a fixed 12 h light/dark cycle with free access to food and water. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort in accordance with the principles and procedures outlined as "Replacement, Refinement and Reduction of Animals in Research" (3Rs) in the guidelines of EU (86/609/EEC), FELASA.

For tissue preparation the animals were anesthetized with halothane before being killed by decapitation; pre-frontal cortices and the remaining cortex were quickly isolated from the mouse brain. In order to make synaptic preparations the brain structures were homogenized immediately after dissection.

3.3. Synaptic preparation

Synaptosomes are re-sealed nerve terminals (Fig.8), which enclose all the typical neuronal contents, including cytoplasm, synaptic vesicles and mitochondria, and present several advantages that make them one of the best models to study the molecular and functional properties of synapses. Their functions closely resemble nerve terminals *in vivo*: they can produce ATP and are capable of take up and release neurotransmitters, have enzymes and synaptic vesicles as well as functional ion channels, carriers and receptors on their plasma membranes, (Dunkley *et al*., 2008). The synaptosomal preparations we used are pure enough to study physiological and molecular aspects of synaptic function.

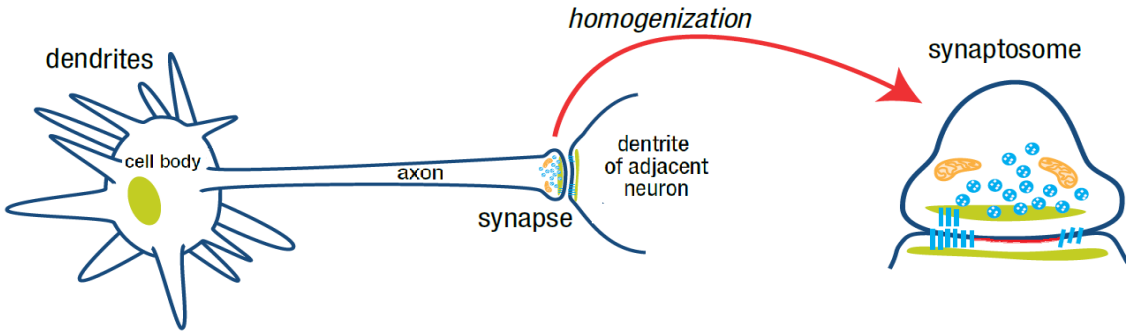

Fig.8 - Illustration of the formation process for synaptosomes (adapted from Wu *et al*., 2012).

Synapses represent about 1-2% of the total volume of the hippocampus and have high levels of proteins, which are mostly adhesion and cytoskeletal proteins responsible for sustaining the neuronal structure and its connections. In brain tissue preparations the neurochemical studies of synapses have a weak signal-to-noise ratio; and the antibodies have poor accessibility to their matching epitopes. The synaptosomes overcome these disadvantages and thus they are considered to be a good tool to study synaptic proteins or function (Cunha, 1998a).

3.3.1. Synaptosomes and total membranes

In order to compare the density of interest proteins in the synaptosomes and total membranes fractions of the same animal, half of the volume of the supernatant that resulted from the first centrifugation, which is common to both procedures (see detailed description below), was separated to prepare total membranes, and the other half was used to prepare synaptosomes.

3.3.1.1. Rapid isolation of synaptosomes

Synaptosomes from pre-frontal cortex were prepared using Percoll solution, as previously described (Canas *et al.*, 2009). The two pre-frontal cortices from one animal were homogenized at 4ºC in a sucrose solution (0.32 M) containing 1 mM EDTA, 10 mM HEPES, 1 mg/ml bovine albumin serum (BSA), and 0.25 mM dithiothreitol (DTT), pH 7.4 centrifuged at 3000 x*g* for 10 minutes at 4ºC (Sigma 3-18K centrifuge, rotor 12-158H). The supernatants were collected and centrifuged at 14000 x*g* for 12 minutes at 4°C (Sigma 3-18K centrifuge, rotor 12-158H) pellets were resuspended in 1 ml of a 45% (v/v) Percoll solution in Kreb's buffer (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM EDTA, 5 mM glucose, pH 7.4). After a centrifugation at 14000 x*g* for 2 minutes at 4°C (in an eppendorf centrifuge), the top layer was removed (synaptosomal fraction) and washed in 1 ml of Kreb's buffer. Another centrifugation was made at 11000 x*g* for 11 minutes at 4°C the supernatants were discarded and the remaining pellet was resuspended in RIPA buffer (radioimmunoprecipitation assay buffer) composed by 50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, supplemented with 2 μM PMSF (penylmethanesulfonylfluoride), 1% CLAP (cocktail of proteases inhibitors) and 0.25 mM DTT (dithiothreitol), and then stored at -20ºC.

3.3.1.2. Total membranes preparation

Isolated pre-frontal cortices were homogenized at 4ºC in a sucrose solution (0.32 M) containing 1 mM EDTA, 10 mM HEPES, 1 mg/ml bovine albumin serum (BSA), and 0.25 mM DTT, pH 7.4. The homogenized tissue was centrifuged at 3000 x*g* for 10 minutes, 4°C (Sigma 3-18K centrifuge, rotor 12-158H) then the supernatants were further centrifuged at 25000 x*g* for 60 minutes at 4°C (Avanti J-26X centrifuge, rotor JA-22-50). The supernatant were discarded and the pellet, mainly composed of the total

cytoplasmic membranes, was resuspended in RIPA buffer.

3.3.2. Fractionation of synaptic membranes (Pre-, Post-, Extra)

To isolate the synaptosomes into its subcellular components, like the pre-synaptic (active zone), post-synaptic and extra-synaptic fractions (non- active zone), mice cortical synaptosomes, were used using a methodology previously described by Rebola *et al*., 2005a. This sub-synaptic fractionation preparation allows a 90% effective separation of the pre-synaptic active zone, which is enriched in SNAP-25, post-synaptic density, enriched in PSD-95, and extra-synaptic fraction which has high levels of synaptophysin (Fig.9) (Pinheiro *et al*., 2003; Rebola *et al*., 2005a). We have also validated the sub-synaptic fractionation of our preparation by immunoblot using antibodies against SNAP-25, PSD-95 and synaptophysin.

Fig.9 - Schematic representation of the different synaptic fractions expected to be obtained in the sub-synaptic fractionation procedure. Adapted from Phillips *et al*., 2001.

For synaptosomes preparation, the cortical cortices were homogenized in 2.5 ml of isolation buffer (IB) (constituted by 0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% CLAP and 1 mM PMSF). The homogenate was transferred to 50 ml centrifuge tubes and resuspended in 2 M sucrose and 0.1 mM $CaCl_2$. The solution was carefully

agitated at 4ºC giving a 1.25 M sucrose solution. This solution was divided into 2 tubes UltraclearTM and 2.5 ml (per tube) of a 1 M sucrose solution (containing 0.1 mM $CaCl_2$) was carefully added to allow the formation of a gradient. The tubes were filled and equilibrated with IB and then centrifuged at 100,000 x*g*, 4ºC, for 3 hours (Beckman Coulter - Optima CL-100XP DU ultracentrifuge, rotor SW41Ti). The IB and the myelin layer present at the interface separating IB from 1 M sucrose was removed. The synaptosomes were collected at the interface between 1.25 and 1 M sucrose and then diluted 10 times in IB and centrifuged at 15,000 x*g* for 30 minutes (Avanti J-26X centrifuge, rotor JA-22-50). The resulting pellet was resuspended in 1.1 ml of IB (100 µl of the supernatant, corresponding to synaptosomes fraction, was kept at -20ºC for control analysis), and diluted 10 times in cooled 0.1 mM $CaCl_2$. A similar volume of 2x solubilization buffer pH 6.0 (40 mM Tris, 2% (v/v) Triton X-100, pH 6.0 adjusted at 4ºC) was added. The mixture was softly stirred for 30 minutes on ice and divided into 2 UltraclearTM tubes for a centrifugation at 40,000 x*g* for 30 minutes, 4ºC (Avanti J-26X centrifuge, rotor JA-22-50). The pellet corresponds to synaptic junctions and the supernatant to extra-synaptic fraction. The supernatants were kept on ice while the pellet was washed in 1x solubilization buffer pH 6.0 (20 mM Tris, 1% (v/v) Triton X-100, pH 6.0 adjusted at 4ºC) and resuspended in 5 ml of solubilization buffer pH 8.0 (20 mM Tris, 1% (v/v) Triton X-100, pH 8.0 adjusted at 4ºC). This mixture was stirred softly for 30 minutes on ice and centrifuged at 40,000 x*g* for 30 minutes at 4ºC (Avanti J-26X centrifuge, rotor JA-22-50). The pellet corresponds to the post-synaptic density and the supernatant to pre-synaptic density. The supernatant was transferred to centrifuge tubes and the pellet resuspended in a minimal volume of RIPA buffer and kept at -20ºC. To concentrate even further the extra-synaptic and pre-synaptic fractions, a maximum volume of cold acetone (-20ºC) was added to the supernatants and kept

overnight at -20ºC. Both fractions were centrifuged at 18,000 x*g* for 30 minutes at -15ºC (Sorvall RC6, rotor SS34). Pellets were resuspended in a minimal volume of RIPA buffer.

3.3.3. Co-immunoprecipitation

Synaptosomal fraction of prefrontal cortex of mice was used to do co-immunoprecipitation assays. The samples (150 µg protein/150 µl RIPA) were divided into three eppendorfs: 1- for incubation with e-5'N, 2- for $A_{2A}R$ incubation and 3- for incubation with irrelevant IgG (Immunoglobulin G) (negative control) and then an equal volume of 50 µl of protein G PLUS-agarose beads were added to each one and incubated for 30 minutes at 4 ºC. After incubation, the solution was centrifuged at 1000 x*g* for 5 minutes at 4 ºC. From the supernatant we took 20 µl that would later correspond to our input sample (positive control). Still from the supernatant 20 µl were taken and incubated with the primary antibody or IgG for 1 hour at 4 ºC. Afterwards, 20 µl of protein G PLUS-agarose beads was added to the mixture and left overnight for incubation at 4 ºC in a rotational shaker.

After a series of centrifugations (1000 x*g* for 5 minutes) and washes with isotonic solution to remove the major portion of the G PLUS-agarose beads, the pellet was re-suspended in RIPA buffer and the samples were prepared for Western blot analysis.

3.4. Protein quantification and Western Blot analysis

3.4.1. Protein quantification by the BCA method and sample preparation

Protein quantification was done using the Bicinchoninic acid (BCA) protein assay reagent kit. This assay uses a colorimetric method which is compatible with high concentrations of most components of the lysis solution used. A standard curve was made in miliQ water, using the following concentrations of BSA: 2; 1; 0.5; 0.25; 0.125; 0.0625 and 0 µg/µl. The samples and the lysis solution were diluted 10 times. In a 96 multiwell, the standard curve was prepared by pipetting 15 µl of each different concentration of BSA, in triplicate. In each of those wells containing the BSA, 15 µl of the lysis solution was also added, as well as 200 µl of BCA reagent. The diluted samples were prepared in the same manner, but instead of adding the lysis solution, 15 µl of miliQ water was added. The multiwell was wrapped in tinfoil to protect from the light and it was placed in an incubator at 35 ºC for 30 minutes. Protein was read at 570 nm in a spectrophotometer (SpectraMax plus 384).

For Western blot assays the samples were normalized to 2 µg of protein/µl by adding sample buffer (500 mM Tris, 600 mM DTT, 10.3% SDS, 30% glycerol and 0.012% (w/v) of bromophenol blue, usually it is used 1/6 of a 6x concentrated sample buffer) and rectifying with milliQ water. The samples were then denaturated by boiling at 95 ºC for 5 minutes.

3.4.2. Western Blot

Western blot assays were done using the Bio-Rad system. Protein were separated by SDS-PAGE electrophoresis, using a 10 % polyacrylamide resolving gel with 4% polyacrylamide stacking gel (see Table 5), after loading different protein amounts of each sample. Then, the proteins were electrotransferred to PVDF (polyvinylidene difluoride) membranes (GE Healthcare), which were further blocked for one hour at room temperature with 5% low-fat milk (w/v) in Tris-buffered saline (20 mM Tris, 140 mM NaCl, pH 7.6) or 3% bovine serum albumin (depending on the antibodies specifications), pH 7.6, containing 0.1% (v/v) Tween 20 (TBS-T). Afterwards membranes were incubated overnight at 4°C with primary antibodies (see table 3). After this incubation membranes were rinsed three times with TBS-T, and incubated with phosphatase-linked secondary antibodies for 2 hours at room temperature. Finally, membranes were rinsed in TBS-T and then incubated with enhanced chemi-fluorescence substrate (ECF) and the proteins were visualized in a VersaDoc 3000 system (Bio Rad, USA) and quantified using the and Quantity One software (Bio Rad, USA).

Table 5 - Gel formula for the Western Blot analysis.

Gel formulation (1 Gel)	10% (Resolving gel)	4% (Stacking gel)
Water	4.1 ml	6.1 ml
Tris-HCl 1.5M; pH 8.8 (Resolving gel)	2.5 ml	--------
Tris-HCl 0.5M; pH 6.8 (Stacking gel)	--------	2.5 ml
Acrilamide 30%	3.3 ml	1.3 ml
APS 10%	50 µl	50 µl
TEMED	5 µl	10 µl

3.5. Immunohistochemistry of brain slices

3.5.1. Preparation of fixed brain slices

Perfusion of mice with PFA was done as previously described by our group (Canas *et al*., 2009). Male C57Bl/6 mice with 8-12 weeks of age were anesthetized with thiopental (50 mg/kg) via intraperitoneal, the heart was exposed, and then a catheter was inserted into the left ventricle. The right ventricle was slightly cut open to allow the outflow of the perfusate and prevent the swelling of the tissue. The animal was perfused with 72 ml of PBS (8.01% NaCl, 0.20% KCl, 1.78% Na_2HPO_4, 0.27% KH_2PO_4 [w/v], pH 7.4) followed by 90 ml of 4% PFA solution (prepared in saline solution). After perfusion the mouse brain was extracted and maintained in 4% PFA solution overnight at 4°C. The brains were transferred to PBS containing 30% of sucrose and were kept in this solution until the slicing. After this procedure the brains were embedded in Tissue-Tek, frozen at -20°C and cut into 50 µm coronal sections using a cryostat (Leica CM3050 S). Slices were store at 4ºC in Walter´s antifreeze solution (30% glycerol (v/v), 30% ethyleneglycol (v/v) in 0.5 M phosphate buffer).

3.5.2. Immunohistochemistry

The perfused brain sectioned coronally (50 µm) were rinsed three times for 10 minutes period with PBS at room temperature. Afterwards, slices were blocked with PBS containing 5% normal horse serum for 45 minutes and then incubated with PBS containing 0.25% Triton X-100 and 5% normal horse serum in the presence of the primary antibodies for 48 hours (see table 4 for antibodies and respective dilutions).

The slices were rinsed three times with PBS containing 0.25% Triton X-100 for

periods of 10 minutes and then were incubated with the respective AlexaFluor secondary antibodies diluted in PBS with 0.25% Triton X-100 and 5% normal horse serum for 2 hours at room temperature (see table 4). After washing three times with PBS, slices had their nuclei stained with DAPI (diluted in PBS 1:5000) for a period of 10 minutes. After rinsing slices with PBS, they were mounted in a glass slide with DAKO fluorescent mounting medium. Images were acquired in a Zeiss Imager Z2 fluorescence microscope equipped with 63x (Plan Neofluar 33 objective, 0.75 numerical aperture) objectives and Axiovision SE64 4.8.2 software. It was confirmed that none of the secondary antibodies produced any signal in slices by using slices that were not incubated with primary antibodies.

3.6. Electrophysiological recordings in prefrontal cortex slices

3.6.1. Diverse components present in evoked extracellular potentials

Neuronal activity in the brain gives rise to trans-membrane currents that can be measured in the extracellular medium (Buszáki *et al*., 2012). Electric current contributions from all active cellular processes within a volume of brain tissue superimpose at a given location in the extracellular medium and generate a potential (measured in Volts), with respect to a reference potential, which can be measured by an extracellular electrode (Buszáki *et al*., 2012). This recorded potential (evoked by external stimuli in brain slices) is also known as field excitatory post-synaptic potential (fEPSP).

In this work, we recorded the population spike which is the shift in electrical potential as a consequence of the movement of ions involved in the generation and propagation of action potentials. It represents the sum of action potentials generated in a

synchronous manner by the population of cell bodies in the neighborhood of the recording electrode. As the population spike often reflects synaptically induced firing, they can be classified as a type of field excitatory post-synaptic potentials.

The role of A_{2A}Rs and e-5'N was evaluated on basal synaptic transmission as well as on synaptic plasticity, particularly on long-term potentiation (LTP). LTP is a long-lasting enhancement in signal transmission between neurons that results from stimulating them in synchrony. It is one of several long-term plasticity phenomena allowing chemical synapses to change the strength of their connection. LTP is widely considered one of the major cellular mechanisms underlying learning and memory (Mendonça and Ribeiro, 2001). LTP of the PFC can be obtained by stimulating a population of presynaptic fibers with a short-duration train of high-frequency stimulation (Huang *et al*., 2004). In this work the trains consisted of 300 Hz stimuli repeated 5 times every 3 minutes for a total period of 15 minutes.

3.6.2. Preparation of prefrontal cortex brain slices

Male C57BL/6 mice were anesthetized under halothane atmosphere, decapitated and, the brain rapidly removed from the skull and submerged in ice-cold artificial cerebrospinal fluid aCSF solution (NaCl 125 mM, KCl 3 mM, $MgSO_4$ 1 mM, $CaCl_2$ 2 mM, Na_2HPO_4 1.25 mM $NaHCO_3$ 25 mM and glucose 11 mM, pH 7.4) and bubbled with a 95% O_2 + 5% CO_2 mixture. Coronal slices (250 µm - thick) containing the prelimbic medial prefrontal cortex were cut with a Vibratome 1500 sectioning system (Vibratome, Germany). The slices were then transferred to a pre-chamber containing aCSF under continuous oxygenation to recover at 32ºC for at least 1 h.

3.6.3. Recordings in the prefrontal cortex

Slices were then transferred to a submerged recording chamber where they were continuously perfused at a rate of 2-3 mL/min with oxygenated aCSF.

A bipolar concentric stimulation electrode SNE-100 (Kopf, Germany) was placed on the layer II/III of the prelimbic PFC (Fig.10 A) delivering rectangular pulses (60 – 150 µA) of 0.1 ms duration applied with a Digitimer DS3 stimulator (Digitimer LTD, United Kingdom) once every 20 s. Population spike was recorded through an extracellular microelectrode (4 M NaCl, 1–2 MΩ resistance) placed in the layer V of the prelimbic PFC (Fig.10 A). Recordings were obtained with an amplifier ISO-80 (World precision instruments, U.S.A.) coupled to an ADC-42 analogue/digital acquisition board (Pico Technology's, United Kingdom). Responses were digitalized at 10 KHz and continuously monitored on a personal computer with the WinLTP 1.1 program (Anderson and Collingridge, 1997). Responses were quantified as the amplitude of the population spike recordings.

After stabilizing the signal, the input/output curve was obtained (Fig.10 D). Then the intensity of the stimulus was regulated to obtain 40 – 50% of the maximum response. When drugs were applied, the input/output curves were obtained for both before drug treatment and 30 minutes after drug treatment (Fig.10 C).

LTP was induced after recording a baseline for 10 min. The protocol consisted of a 5 trains of 300 Hz tetanus every 3 minutes as described in Huang *et al*., 2004 (Fig.10 B and C).

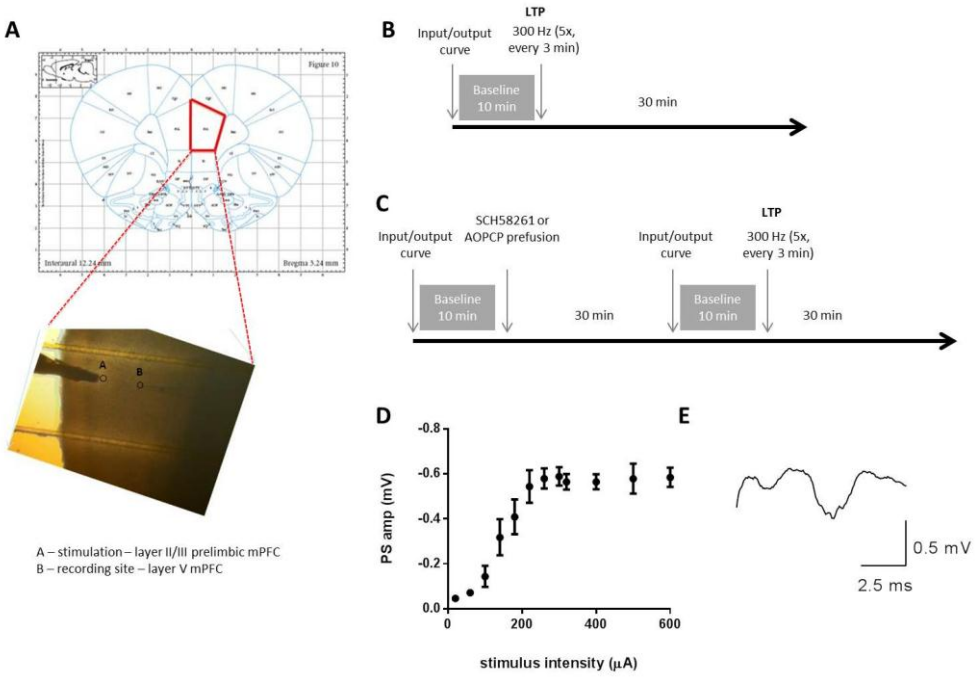

Fig.10 - Experimental design for the recording of population spike at the prelimbic medial prefrontal cortex (mPFC) **(A)** Coronal slices containing the prelimbic mPFC. In the schematic representation in the upper panel the prelimbic mPFC is delimited by the red lines. The lower panel shows a coronal slice containing the prelimbic mPFC in the recording chamber. The stimulation electrode was placed on layers II/III, and the recording electrode was placed on layer V. **(B)** Experimental design for control slices. **(C)** Experimental design for the slices subjected to 30 minutes pretreatment with the e-5'N inhibitor AOPCP (100 µM) or the $A_{2A}R$ antagonist SCH 58261 (50 nM). LTP was induced by five trains of tetanus (300 Hz; one train every 3 minutes). **(D)** Input/output (I/O) synaptic relation from prelimbic (mPFC). The I/O curve was traced before and after drug treatment and no changes were observed. The basal synaptic transmission was set to 40% of the maximum response before inducing LTP. (E) A sample population spike from the prelimbic mPFC of a mouse slice. Figure kindly supplied by Joana Real and Samira Ferreira.

3.7. HPLC measurement of adenosine release from mouse prefrontal cortex synaptosomes

Adenosine release was assayed in batch-like conditions. Half of the prefrontal cortex synaptosomes (~2.3 mg protein × mL^{-1}) were incubated at 25 ºC for 5 minutes in the presence of the e-5'N inhibitor (AOPCP) while the other half was incubated at 25 ºC for 5 minutes in the absence of AOPCP (basal control).

The separation and quantification of adenosine and its metabolites was carried out by High Pressure Liquid Chromatography (HPLC), as previously described (Cunha

and Sebastião, 1993) with slight modifications, employing a LiChroCart-RT 125-4 C-18 reverse-phase column (particle size, 5 μm), combined with an UV detector set to 254 nm. The mobile phase consisted of KH_2PO_4 (100 mM) and acetonitrile (92/8 v/v%) at pH 6.50, with the flow rate of 1 mL × min^{-1}, and a loop volume of 50 μL. The identification and quantification of adenosine and its metabolites was achieved by calculating the peak areas then converted to concentration values (expressed as μmol × mg $protein^{-1}$) by calibration with known standards ranging from 3 to 20 μM.

3.8. Data presentation

Whenever possible, the data is presented as mean ± standard error of the mean (SEM) of the number (n) of experiments indicated in figure legends. In experiments with one variable it was used one-sample *t*-test comparing with the control (100%). To test the significance of the difference between two groups, a Student's *t* test was used considering a statistical difference for a $p<0.05$. In experiments with more than two groups it was used one-way analysis of variance (ANOVA), followed by Mann-Whitney's/Tukey's multiple comparison test. A value of $p<0.05$ was considered to represent a significant difference.

4. Results and Discussion

4.1. Synaptic and subsynaptic distribution of e-5'N in mice prefrontal cortex

4.1.1. Synaptic distribution of e-5'N

To analyse whether the e-5´N is enriched in nerve terminals in relation to the bulk of total membranes, two different types of brain tissue preparation were used: re-sealed nerve terminals (synaptosomes) and total membranes. The advantages of synaptosomes were already discussed in the material and methods section; however, total membranes were not. Total membranes from the PFC comprehend all the membranes that exist in a particular tissue, meaning, that there are present cell membranes and organelle membranes, whereas synaptosomes are re-sealed nerve terminals. Before assessing the localization of e-5'N both preparations in synaptosomes and total membranes, we validated the purity of our preparations by Western blot, by using antibodies that label the pre- (SNAP-25) and post-synaptic (PSD-95) components that make part of the synaptosomes. As observable in Figure 11 the synaptosomal preparations had higher levels of PSD-95 and SNAP-25 than the total membranes, which ensure that these preparations were enriched in components of nerve terminals.

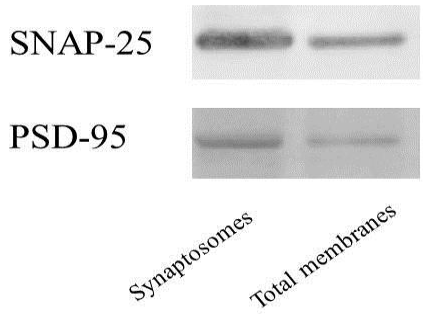

Fig.11 – Validation of the purity for synaptosomes and total membranes obtained from mice prefrontal cortex. 20 µg of protein were loaded and incubated with specific antibodies which are known to be present at the synapse, therefore being more enriched in the synaptosomes preparation. The synaptosomes presented enrichment of SNAP-25 (upper blot) and of PSD-95 (bottom blot) when compared to the total membranes.

The anti-e-5'N antibody used recognize a portion of the C-terminal of this enzyme and display an immunoreactive band with an apparent molecular mass of approximately 71 kDa. In the same immunoblot three amounts of protein of synaptosomes and total membranes were loaded (10, 20 and 40 µg), which were obtained from the same PFC samples (see material and methods section). This first approach was done in order to detect the e-5'N immunoreactivity for non-saturating amounts of protein (Fig.12 A). The relative amount of e-5'N immunoreactivity was achieved for 20 µg of loaded protein and it was observed that the density of e-5'N in synaptosomes was lower (52.1% ± 2.3%, n=4) than in total membranes (72.5% ± 3.1%, n=4) (Fig.12 B). The ratio between e-5'N immunoreactivity in synaptosomes and in total membranes was 0.72 ± 0.03 (n=4), indicating that e-5'N albeit present in prefrontal cortex nerve terminals was not as enriched when compared with the bulk of total membranes.

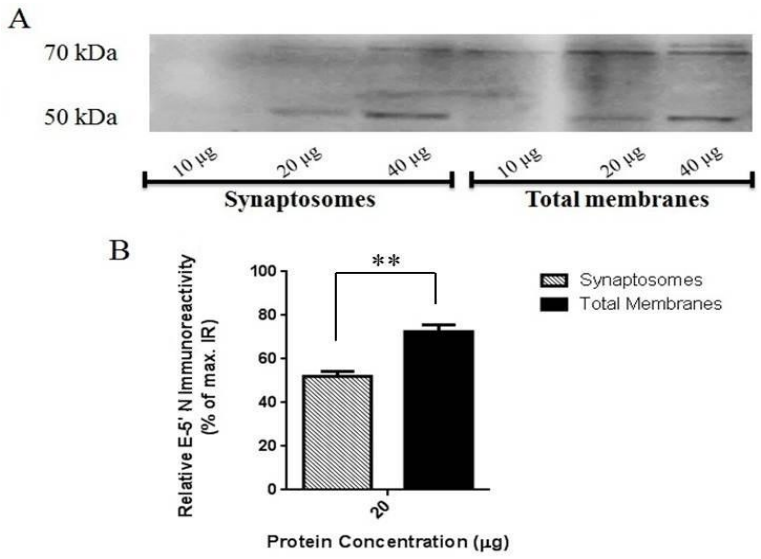

Fig.12 – Levels of e-5'N in nerve terminals (synaptosomes) and in total membranes of mice prefrontal cortex. **(A)** Representative Western blot of the e-5'N levels in synaptosomes and total membranes for different amounts of protein. Two immunoreactive bands were achieved, one at 70 kDa and other at 50 kDa, which might represent two different isoforms of e-5'N **(B)** Graphic represents the percentage of immunoreactivity for the band detect at 70 kDa for 20 µg of loaded protein, which was calculated considering the maximal immunoreactivity value obtained as 100%. The results are presented as mean ± SEM of 4 independent experiments. **P<0.05; paired *t*-test.

From the immunoblot it is possible to observe two different bands, one with an apparent molecular mass of approximately 50 kDa and another around 70 kDa (Fig.12 A). The band with 70 kDa corresponds to the e-5'N that was described earlier in the section 1.5. The band with an apparent molecular mass of 50 kDa is most likely a form of e-5'N that suffered post-translational modifications. It is know that e-5'N has several isoforms present in the CNS (Zimmermann, 1992; Cunha *et al*., 2000); also e-5'N has at least 4 different sites capable of being glycosylated (Zimmermann *et al*., 2012) so it is possible that the anti-e-5'N antibody used in this study recognized one form of e-5'N that went through post-translational modifications.

The presence of e-5'N at the pre-synaptic region of the synapse reinforces the hypothesis that e-5'N is essential for the activation of $A_{2A}R$ present at the pre-synaptic nerve terminal in cortical areas (Rebola *et al*., 2005b). However, the higher relative abundance of e-5'N in total membranes in comparison with synaptosomes indicates that e-5'N might have other roles in the CNS. In fact, e-5'N interacts with proteins, such as laminin and fibronectin, which are present in the extracellular matrix and are also present in microglia (Chamak and Mallat, 1991) and astrocytes (Xu *et al*., 2008). Fibronectin and laminin are involved in several biological processes, such as cell growth and adhesion and also in cell spreading and migration (Langer *et al*., 2008); thus e-5'N may also play important roles in microglia and astrocytes and that could possibly be why it is not enriched in the synaptosomal fraction.

To further analyse these hypothesis and to be more thorough about the synaptic localization of e-5'N, another type of tissue preparation should have been used in these experiments, the gliossomes, which are a preparation enriched in glial plasmalemmal vesicles (Matos *et al*., 2012).

4.1.2. Subsynaptic distribution of e-5'N

Since it was observable the presence of e-5'N in synaptosomes, the next objective was to detail the exact location of e-5'N in the synapse. For that purpose, we used a fractionation procedure that allows an effective separation of the synaptosome in its pre-synaptic, post-synaptic and extra-synaptic zones, which was previously validated by our group (Rebola *et al*., 2005a). This procedure has the advantage of giving better accessibility of antibodies to the epitopes located in the synapses though the solubilization of different sub-synaptic components (Phillips *et al*., 2001; Rebola *et al*., 2005a). Before assessing the localization of e-5'N in the sub-synaptic preparation, we validated the purity of the sub-synaptic fractions by Western blot, by using antibodies that label the pre-synaptic active zone (anti-SNAP-25), of post-synaptic (anti-PSD-95) zone and of extra-synaptic regions (anti-synaptophysin) (see Fig.13). In these controls, it is expected an enrichment of the sub-synaptic proteins in the corresponding sub-synaptic fractions. Hence, SNAP-25 (synaptosomal-associated protein 25) should be located in the pre-synaptic fraction, PSD-95 (post-synaptic density 95) should have a high density in the post-synaptic fraction and synaptophysin (synaptic vesicle marker) should be enriched in the extra-synaptic fraction.

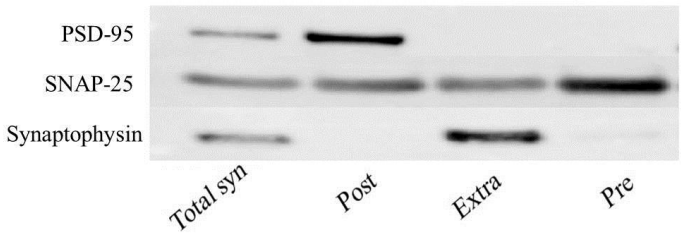

Fig.13 – Purity validation of sub-synaptic samples. Representative Western blot of the control purity of sub-synaptic preparations, where it is expected an enrichment of subsynaptic proteins in the respective membrane fractions: in pre-synaptic (SNAP-25, with 25 kDa), in post-synaptic (PSD-95, with 95 kDa) and extra-synaptic (synaptophysin with 38kDa).We observed that SNAP-25 has its highest expression in the pre-synaptic fraction, PSD-95 is only present in the post-synaptic fraction and synaptophysin is enriched in the extra-synaptic fraction. The first lane, corresponding to the total synaptosome, is an internal (positive) control, which comprehends all fractions.

Regarding the determination of e-5'N density in the different sub-synaptic fractions, the data obtained showed the presence of two bands, one with an apparent molecular mass of approximately 50 kDa in the pre-synaptic and extra-synaptic fractions and the other with 70 kDa in the post-synaptic fraction (Fig.14 A). Though e-5'N was present in all subfractions, it presented an enrichment in the extra-synaptic and post-synaptic fractions.

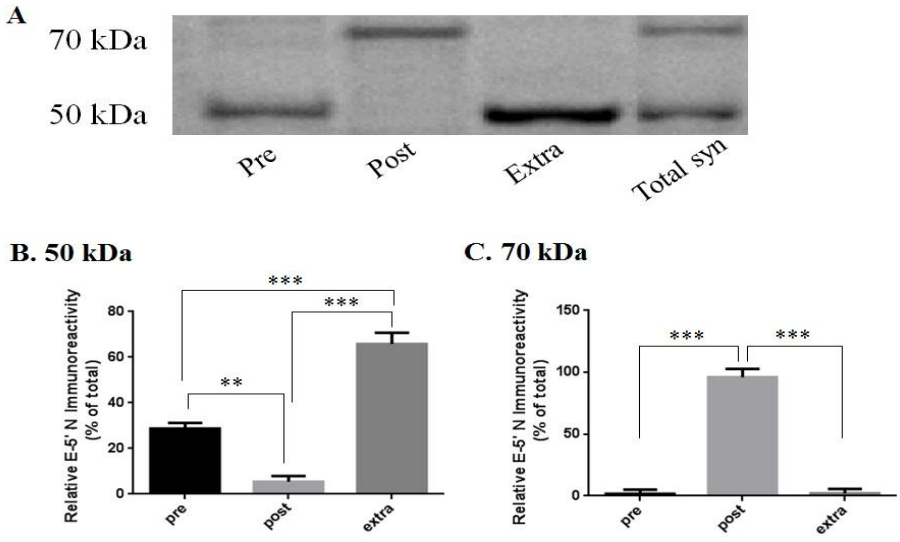

Fig.14 – Sub-synaptic levels of e-5'N in mice brain cortex. **(A)** Representative immunoblot of the e-5'N levels in sub-synaptic fractions and total synaptosomes. 20 µg of protein were loaded in each lane of the gel. The percentage of e-5'N (50 kDa in panel **B**; 70 kDa in panel **C**) immunoreactivity for each sub-synaptic fraction was calculated considering the sum of the pre- (Pre), post- (Post) and extra-synaptic (Extra) immunoreactivities. The total synaptosomes (Total syn) were analysed as an internal positive control of the experiment. The results are presented as mean ± SEM of 3 independent experiments. **p<0.01; ***p<0.001 One-way ANOVA, followed by Tukey post hoc test.

The analysis of this result was carried out comparing the immunoreactivity for the two isoforms with different apparent molecular mass in separate; thus, when we compared the enrichment of the isoform with 70 kDa we got a higher density of e-5'N in the post-synaptic region (96.1% ± 3.9%, n=3), than in the pre-synaptic (1.8% ± 1.8%, n=3) and extra-synaptic fractions (2% ± 2%, n=3) (Fig.14 C). However, when we

compared the 50 kDa isoform of e-5'N the results showed an increase in the protein density at the extra-synaptic fraction (65.8% ± 4.9%, n=3) and in the pre-synaptic fraction (28.7% ± 2.5%, n=3), while the post-synaptic region had almost no (5.4% ± 2.5%, n=3) immunoreactivity (Fig.14 B).

The result suggests that e-5'N had a wide distribution within the nerve terminal. E-5'N, as other GPI-anchored proteins, is synthesized at the endoplasmic reticulum and passes through the Golgi complex to reach the cell surface (Heilbronn *et al*., 1995); thus the presence of e-5'N in the extra-synaptic sub-fraction possibly indicates that e-5'N can move from the non-synaptic region to the synapse. Our results showed that e-5'N is also present at the pre- and post-synaptic fractions, which supports the hypothesis that e-5'N is the enzyme responsible for the hydrolysis of extracellular AMP into adenosine in the synaptic cleft, which is then directed to the activation of $A_{2A}R$ (Cunha *et al*., 1996); that were already described as being present both at the pre-synaptic and post-synaptic regions (Rebola *et al*., 2005b). Furthermore, the presence of e-5'N in the synapse (pre- and post-synaptic fractions) is consistent with studies that indicate that e-5'N may be involved with the formation of new synapses (synaptogenesis) (Heilbronn *et al*., 1995; Zimmermann, 1996; Bailly *et al*., 1998). Additionally, there is enrichment of e-5'N in the extra-synaptic fraction. This implicates that e-5'N is required for other functions other than providing adenosine for the activation of $A_{2A}R$ present at the active site of the synapse. Indeed, some of the other functions of e-5'N are the interactions with other proteins present at extracellular matrix (discussed in section 4.1.1.).

However, the appearance of two bands with different molecular mass (similar to the immunoblot demonstrating the synaptic levels of e-5'N, (Fig.12 A) further suggested that the two bands (50 and 70 kDa) corresponded to different isoforms of e-5'N. In accordance with our results the presence of two isoforms with different

molecular mass was already described, by Zimmermanm (1992), using 2-dimensional SDS-PAGE analysis. The isoform with 50 kDa presents a molecular mass different from the theoretical molecular mass attributed to e-5'N, but it is possible that the isoform with 50 kDa had suffered post-translational modifications. Vogel and colleagues (1992) had already demonstrated that the GPI anchor present in e-5'N can be cleaved (Braun *et al*., 1997). Also it has been reported that e-5'N can exist as truncated forms (Fini *et al*., 2003). The truncated form is most likely the soluble form of e-5'N, ecto-5'-nuclelotidase without the GPI anchor (Fini *et al*., 2003).

4.2. Co-localization of e-5'N and A_{2A} receptor in prefrontal cortex slices and nerve terminals

4.2.1. Co-localization of e-5'N and A_{2A} receptors in different cell populations from PFC slices

Since the data so far have shown that e-5'N was localized in synapses, but also elsewhere, we decided to perform triple immunohistochemistry in the prelimbic region of the prefrontal cortex. This technique allowed us to further analyse the localization of e-5'N within different brain cell populations, like astrocytes (labeled with GFAP), neurons (labeled with β-tubulin III) and microglia (labeled with CD11b) and if there is a co-localization of e-5´N with the $A_{2A}R$, which were labeled with anti-e-5'N and anti-$A_{2A}R$ antibodies. To label microglia it was used an antibody against CD11b, which is a subunit of integrin beta 2 protein involved in the recruitment of microglial cells. Astrocytes can be distinguished from other glial cells because they possess glial fibrillary acidic protein (GFAP), an astrocytic cytoskeleton protein. Beta-III Tubulin, also known as tubulin beta-4, is regarded as a neuron-specific marker. Antibodies

against all these proteins (CD11b, GFAP and β-tubulin III) used to label microglia, astrocytes and neurons, respectively, were validated by our group (see, Sweeney *et al*., 1990; Canas *et al*., 2009; Matos *et al*., 2012)

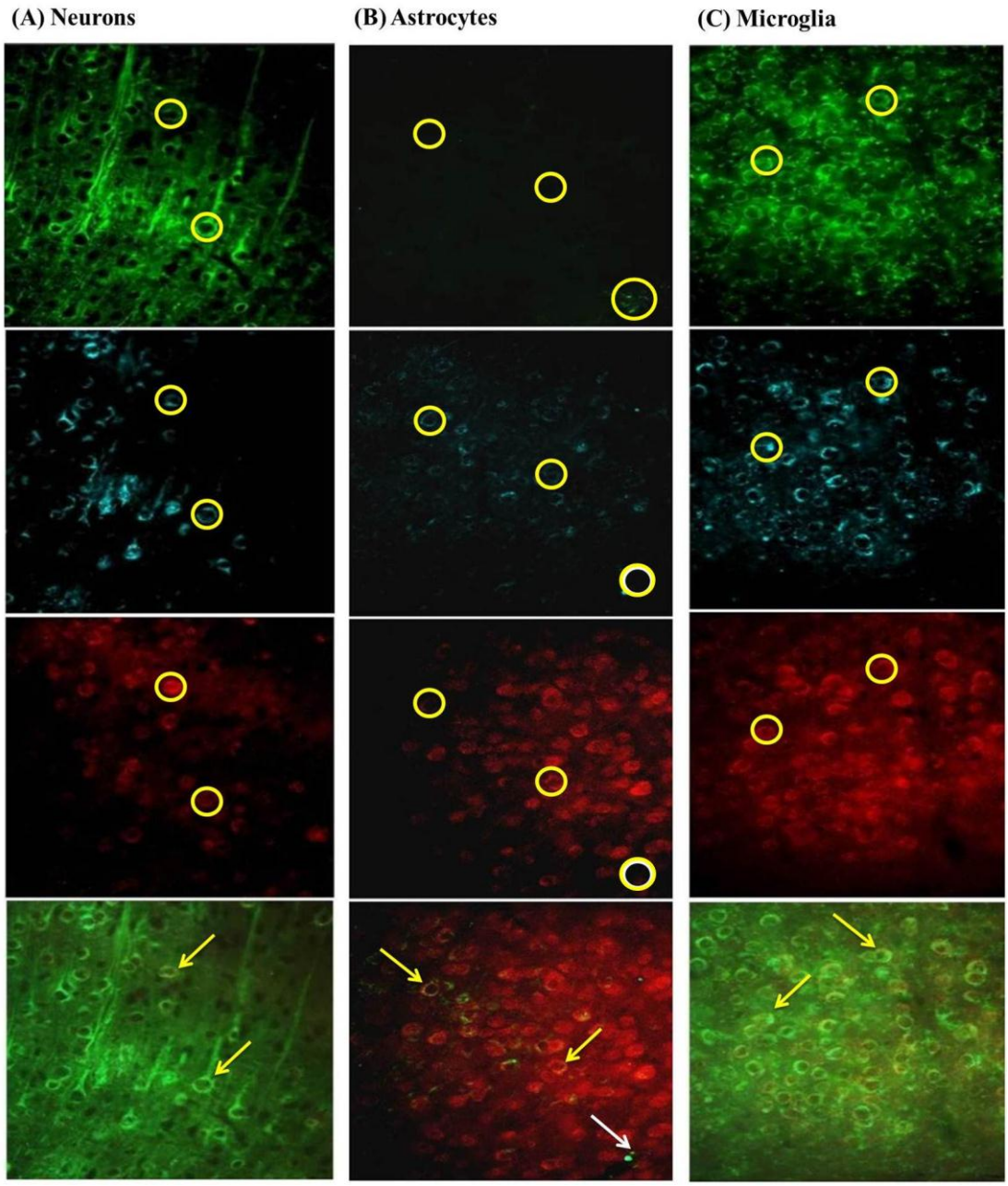

Fig.15 - Triple immunohistochemistry analysis of e-5'N (labeled in red) and $A_{2A}R$ (labeled in blue – far-red) with different markers for different cell populations (labeled in green), β-tubulin III for neurons, GFAP for astrocytes and CD11b for microglia, in mouse prefrontal cortex brain slices. Yellow circles highlight protein/cell of interest. White arrow indicate a negative co-localization of e-5'N and $A_{2A}R$ with a type of cell (astrocytes). The yellow arrows indicate a positive co-localization between e-5'N, $A_{2A}R$ and the cell type marked (neurons and macroglia). **(A)** Cells labeled with β-tubulin III (neurons marker) with yellow arrows indicate positive immunoreactivity for e-5'N and $A_{2A}R$. **(B)** Cells labeled with GFAP (astrocytes marker) with white arrow indicate negative immunoreactivity for e-5'N and $A_{2A}R$. However, there was still co-localization between e-5'N and $A_{2A}R$. **(C)** Cells labeled with CD11b (microglial marker) with yellow arrows indicate positive immunoreactivity for e-5'N and $A_{2A}R$. Magnification: 630x (epifluorescence microscope). Images are representative of 4 independent experiments.

The results obtained suggest that e-5'N and $A_{2A}R$ present an apparent co-localization as it can be seen in Figure 15 pointed out by the yellow arrows. Furthermore our results showed that apart from the apparent co-localization of e-5'N and $A_{2A}R$, they were both associated with neurons and microglia (Fig.15 A, C) However, the results suggest that e-5'N and $A_{2A}R$ albeit apparently co-localized were not observed in astrocytes as it is pointed out by the white arrow (Fig.15 B). However this lack of association of e-5'N and $A_{2A}R$ with astrocytes may be due to the fact that astrocytes and e-5'N seemed to be located in different planes (results not shown).

Our results are somewhat contradictory to what is present in the literature. In some studies with cultured cortical neurons it was reported the presence of e-5'N in astrocytes (Zamzow *et al*., 2008) and it was also demonstrated by immunohistochemical and Western blot analyses the presence of e-5'N in astrocytes (Zimmermann, 1996; Langer *et al*., 2008; Augusto *et al*., 2013). However, the work of Bjelobaba and colleagues showed almost no presence of e-5'N in astrocytes (Bjelobaba *et al*., 2011); while in other studies it was demonstrated that e-5'N was more present in neurons and microglia (Braun and Zimmermann, 2001; Bjelobaba *et al*., 2011; Stanojevi´c *et al*., 2011). Nonetheless it was reported that after a noxious stimulus in the cortex, e-5'N protein levels were decreased in neurons and increased in astrocytes near the injured area (Bjelobaba *et al*., 2011). However, to be thorough and to obtain detailed results from these preparations there is the need to further analyse these samples using confocal microscopy.

Nonetheless, our results demonstrated an apparent co-localization of e-5'N with $A_{2A}R$, and observing that they are mainly present in neurons and microglia, the next task was to investigate if e-5'N and $A_{2A}R$ presented a close association in nerve terminals of mice prefrontal cortex.

4.2.2. Co-localization of e-5'N and A_{2A} receptors in synaptosomes from PFC

The data obtained with the fractionation procedure have shown the presence of e-5'N in different regions of nerve terminals, more precisely in the pre-synaptic and post-synaptic regions. It is known that $A_{2A}R$ is also present in the active zone of the synapse (Rebola *et al*., 2005b). To further prove the apparent co-localization of e-5'N with $A_{2A}R$, obtained with the immunohistochemistry, we performed a co-immunoprecipitation assay. From the same sample of synaptosomes obtained from mice prefrontal cortex, we did a "pull-down" of e-5'N, and a "pull-down" of $A_{2A}R$ in separate experiments (for a detailed explanation see material and methods). The results we got are displayed in the Figure 16.

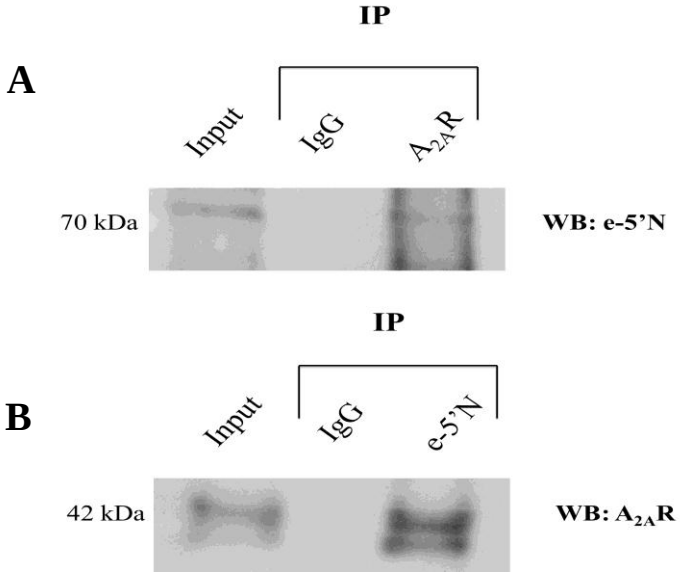

Fig.16 – Immunoprecipitation (IP) of e-5'N and A_{2A} receptor in synaptosomes samples of PFC. **(A)** Proteins were immunoprecipitated with anti-A_{2AR} antibody and labeled with antibody against e-5'N. **(B)** Proteins were immunoprecipitated with anti-e-5'N antibody and labeled with antibody against $A_{2A}R$. The input corresponds to the internal positive control, and the IgG corresponds to an irrelevant protein (negative control) Immunoblots are representative of 2 independent experiments.

From the results obtained with the immunoblot it is possible to say that there is a physical interaction between e-5'N and $A_{2A}R$ in nerve terminals from the prefrontal cortex. These data corroborate the idea that e-5'N might indeed provide the adenosine necessary for the activation of $A_{2A}R$ (Cunha *et al*., 1996; Cunha, 2005); and are in accordance with a recent work, that showed a co-localization of e-5'N and $A_{2A}R$ in the striatum thus supporting the hypothesis that e-5'N and $A_{2A}R$ have a physical interaction (Augusto *et al*., 2013). In this work they further proved this physical association using an e-5'N knockout mice model in which they obtained no interaction between e-5'N and $A_{2A}R$ (Augusto *et al*., 2013).

There is also evidence obtained using co-immunoprecipitation assays for $A_{2A}R$ and tyrosine kinase B, indicating that $A_{2A}R$ may be located in lipid rafts (Mojsilovic-Petrovic *et al*., 2006). Lipid rafts are membrane microdomains highly enriched with GPI-anchored proteins (Lasley, 2011), such as e-5'N, therefore, this further supports a close association that we observed between $A_{2A}R$ and e-5'N and furthermore this result may imply some co-regulation between e-5'N and $A_{2A}R$.

4.3. Synaptic distribution of e-5'N in PFC from $A_{2A}R$-KO mice

From the results obtained with the co-immunoprecipitation assay we showed that there was a physical interaction between e-5'N and $A_{2A}R$. This result led us to investigate if mice with a global genetic deletion for $A_{2A}R$, $A_{2A}R$ knockout mice ($A_{2A}R$-KO) displayed different levels of e-5'N in nerve terminals. The $A_{2A}R$-KO mice model used in this assay was well-characterized and validated by our group prior to its utilization (Lopes *et al*., 2004).

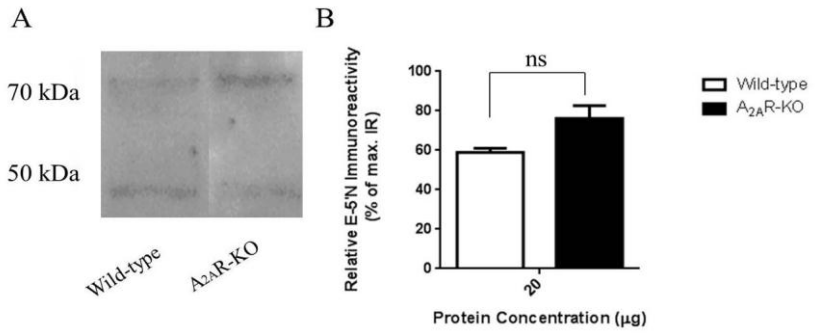

Fig.17 – Levels of e-5'N in synaptosomes of prefrontal cortex from wild-type and A_{2A}R-KO mice. **(A)** Representative immunoblot of the e-5'N levels in synaptosomes from wild-type and A_{2A}R-KO mice. **(B)** Graphic represents the percentage of immunoreactivity for the band detect at 70 kDa for 20 µg of loaded protein, which was calculated considering the maximal immunoreactivity value obtained as 100%. The results are presented as mean ± SEM of 3 independent experiments. ns: non-significant; unpaired *t*-test.

For this analysis we only used PFC synaptosomes from wild-type and from A_{2A}R-KO mice (Fig.17 A). The relative amount of e-5'N immunoreactivity was achieved for 20 µg of loaded protein and it was observed that the density of e-5'N in synaptosomes from wild-type mice was lower (58.9% ± 2.0%, n=3) than in synaptosomes from A_{2A}R-KO mice (76.1% ± 6.5%, n=3), however the differences were statistically non-significant (Fig.17 B). The ratio between e-5'N immunoreactivity in synaptosomes from wild-type and from A_{2A}R-KO mice was 0.79 ± 0.1 (n=3), indicating that e-5'N density levels did not alter in the absence of the A_{2A}R.

Although the results obtained with A_{2A}R-KO and wild-type mice showed no statistical differences in the synaptic density levels of e-5'N in these two animal groups, it seems to exist a slight increase of e-5'N levels in nerve terminals of A_{2A}R-KO, which might be due to a compensatory mechanism. In a recent study where it was used e-5'N knockout mice, the authors observed no changes in the binding density of A_{2A}R as compared with the wild-type mice (Augusto *et al*., 2013). Although they used e-5'N knockout mice, instead of A_{2A}R-KO mice, these data corroborate our findings.

Taken together these evidences may suggest that A_{2A}R and e-5'N do not regulate one another.

4.4. Role of e-5'N in mice prefrontal cortex synaptic transmission

4.4.1. Impact of A_{2A} receptors in PFC synaptic transmission

The data so far has shown a clear physical association between e-5'N and $A_{2A}R$ in PFC synaptosomes; however, in $A_{2A}R$-KO mice the density levels of e-5'N was similar to that found in wild-type mice. The data also showed that e-5'N was present throughout the active region of the synapse (pre-synaptic fraction) and in the non-active region of the synapse (post- and extra-synaptic fractions), thus suggesting that e-5'N might have some role in synaptic plasticity (Rebola *et al*., 2008).

It is already known that $A_{2A}R$ play an important role in synaptic transmission and in plasticity phenomenona in several brain regions (for a review see Dias *et al*., 2013). It is also known that the activation of $A_{2A}R$ in the cortico-striatal region leads to the release of neurotransmitters, like dopamine that may regulate the excitatory neurotransmission (Cunha, 2005; Wang *et al*., 2012). However, in the prefrontal cortex the particular role played by $A_{2A}R$ in synaptic transmission is still unclear.

First we tried to define the role of $A_{2A}R$ in the synaptic transmission in the PFC, and for that we used an electrophysiological approach (see material and methods section 3.6). To define whether the synaptic transmission was dependent from the activation of $A_{2A}R$ we used a selective antagonist of this receptor, SCH 58621 (50 nM) and we explored its action in LTP phenomenon (Fig.18 A).

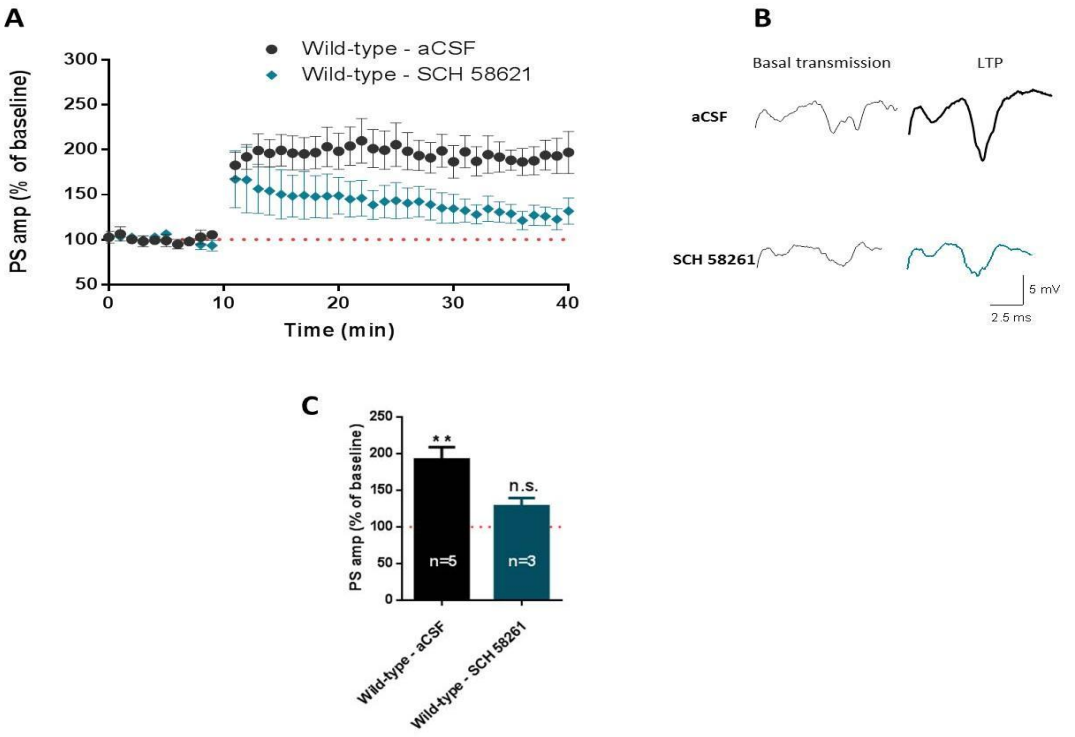

Fig.18 – Blockade of $A_{2A}R$ impairs long-term potentiation (LTP) at the prelimbic mPFC from C57Bl/6 mice. **(A** and **C)** In the control slices (only artificial cerebrospinal fluid – aCSF), 5 trains of 300 Hz stimuli induced a long-term increase in the population spike amplitude (PSamp) that was 191.90 ± 16.87 % of the amplitude of the basal synaptic transmission (black dots / black bar graph; $p<0.01$ – one-sample t-test, n = 5). Under 30 minutes pre-treatment with the $A_{2A}R$ antagonist SCH 58261 (50 nM), the LTP induced by 5 trains of 300 Hz stimuli was no longer significant (blue diamonds / blue bar graph; 128.30 ± 11.47 % of the basal synaptic transmission, $p>0.05$ – one-sample t-test, n = 3). **(B)** Representative population spike recordings obtained before and 30 minutes after LTP induction in control slices (aCSF) and in slices pretreated with the $A_{2A}R$ antagonist SCH 58261. Data are presented as mean ± SEM of n ≥3 independent experiments. Bar graphs are mean ± SEM of the last 10 minutes of the recordings. **p<0.01; ns: non-significant. Graph kindly supplied by Joana Real and Samira Ferreira.

The results comparing the effect of the selective antagonist of the $A_{2A}R$, SCH 58261 (50 nM) in PFC slices clearly show that the $A_{2A}R$ need to be activated to obtain LTP. The presence of this $A_{2A}R$ antagonist abolished almost completely the LTP effect (128.30 ± 11.47 %) when compared to the LTP (191.90 ± 16.87 %) in control slices (Fig.18 A, C). This is in accordance with a previous study using cortico-striatal slices and pharmacological tools where it was shown that when the slices were subjected to an agonist of the $A_{2A}R$ there was an increase in the synaptic transmission, whereas when the slices were incubated with an antagonist of $A_{2A}R$ occurs a decrease in the synaptic

transmission (Quiroz *et al*., 2009). There are also other studies showing that the activation of $A_{2A}R$ is required to achieve a long-term potentiation effect in striatal slices (Higley and Sabatini, 2010; Wang *et al*., 2012). It was also reported that $A_{2A}R$ are required to achieve LTP phenomenon in hippocampus by using the selective antagonist (SCH 58621) of these receptors (Rebola *et al*., 2008). However, to prove that the adenosine that activated $A_{2A}R$ was originated from the extracellular catabolism of ATP, the authors used an inhibitor of e-5'N (AOPCP) and observed reduced amplitude of hippocampal LTP (Rebola *et al*., 2008). This led us to hypothesize that the LTP phenomenon in PFC slices might also depend on the activation of $A_{2A}R$ through the activity of e-5'N. Thus, we further investigated if we could mimic the effect of SCH 58621 in synaptic plasticity of PFC with the inhibitor of e-5'N (AOPCP).

4.4.2. Impact of the e-5'N inhibitor (AOPCP) in PFC synaptic transmission

It is known that e-5'N is the enzyme responsible for the hydrolysis of extracellular AMP into adenosine, which is further directed to the activation of $A_{2A}R$ (Cunha *et al*., 1996; Rebola *et al*., 2008). Thus, we hypothesize that if the adenosine formation was blocked indirectly, the activation of $A_{2A}R$ would not occur and thus the LTP would be blunted. To explore this hypothesis we performed electrophysiological recordings in two groups of PFC slices, one that was subjected to aCSF and the other was perfused with the e-5'N inhibitor, AOPCP (100 µM) (Fig.20).

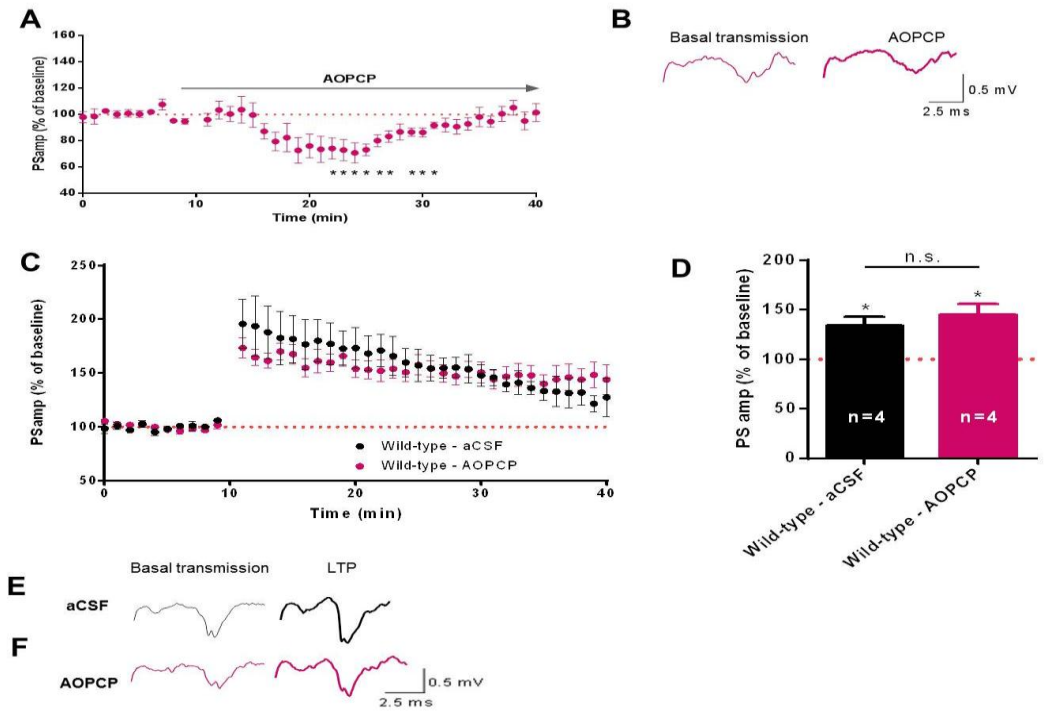

Fig.19 – Inhibition of e-5'N induces a transient decrease of the basal synaptic transmission but fails to affect long-term potentiation at the prelimbic mPFC from C57Bl/6 mice. **(A)** 30 minutes pretreatment with the e-5'N inhibitor AOPCP (100 µM) induced a transient decrease in the population spike amplitude (PSamp; *p<0.05 – one-sample t-test). **(B)** Representative population spike recordings obtained before and after 15 minutes of AOPCP perfusion. **(C – E)** 30 minutes pretreatment with the e-5'N inhibitor AOPCP (100 µM) did not affect the LTP induced by 5 trains of 300 Hz stimuli. In the control slices (only artificial cerebrospinal fluid – aCSF), the population spike amplitude was 134.10 ± 9.24 % of the basal synaptic transmission (black dots / black bar graph; p<0.05 – one-sample t-test, n = 4). Under 30 minutes pretreatment with AOPCP, the population spike amplitude was 145.30 ± 10.53 % of the baseline (pink dots / pink bar graph; p<0.05, n = 4), which was not significantly different from the control (aCSF) slices (p>0.05 - Mann-Whitney test). **(D)** Representative population spike recordings obtained before and 30 minutes after LTP induction in control slices (aCSF) and in slices pretreated with the e-5'N inhibitor AOPCP (100 µM). Data are presented as mean ± SEM of n = 4 independent experiments. Bar graphs are mean ± SEM of the last 10 minutes of the recordings. *p<0.05; ns: non-significant. Graph kindly supplied by Joana Real and Samira Ferreira.

The data obtained with the administration of AOPCP in PFC slices in basal transmission showed an inhibitory effect after the administration of AOPCP that is later recovered (Fig.19 A). This recovery suggests that the adenosine derived from the extracellular catabolism of ATP did not affect the basal transmission at a long term, thus this result might led us to speculate that in the presence of AOPCP the adenosine receptor that is activated seems to be A_1R. This idea was strengthened by a study where the authors used rat hippocampal slices incubated with AOPCP, and it was observed

that the inhibition of e-5'N reduces the adenosine levels in the synaptic cleft; therefore the concentration of adenosine was not enough to activate $A_{2A}R$ thus favoring the activation of A_1R (Cunha *et al*., 1996; Cunha, 2001b).

The administration of AOPCP did not affect significantly the LTP when compared to control slices (administrated with aCSF, Fig.19 C, D). This suggests that even upon high-frequency stimulation, adenosine derived from the extracellular catabolism of ATP, through ectonucleotidases, did not present an impact on plasticity phenomena in PFC regions; thus the effect of $A_{2A}R$ may not be observable here. This led us to prompt the hypothesis that in PFC regions subjected to the administration of AOPCP, the adenosine receptor that is mainly activated seems to be A_1R, because AOPCP inhibits the formation of ATP-derived adenosine, thus maintaining the adenosine concentration low enough to activate only A_1R (Cunha *et al*., 1996; Cunha, 2001b; Rebola *et al*., 2008). This was further corroborated by several studies performed in hippocampal slices where the authors observed that the endogenous adenosine stimulates the A_1R, modifying the excitatory synaptic transmission and attenuating LTP plasticity (Mendonça and Ribeiro, 2000; Mendonça and Ribeiro, 2001; Wei *et al*., 2011). An effect of ATP receptors (P_2) on synaptic transmission did not seem to be present in our experimental conditions, since it was shown that P_2 receptors only exerted their action in synaptic plasticity if the A_1R were blocked (Almeida *et al*., 2003). However, in a study done in hippocampal slices showed that even if the A_1R was not blocked the P_2 would still have an impact on synaptic plasticity (Fujii, 2004). The administration of ATP (10 µM) slowly induced LTP in hippocampal slices (Fujii, 2004); although, the observed LTP could be due to the activation of $A_{2A}R$, because the ATP catabolism was still taking place in the synaptic cleft. In fact, they explored this possibility by removing all ADP present in the synaptic cleft, by washout with aCSF

solution, and observed no potentiation; thus indicating that LTP was not induced by P_1 receptors but by P_2 receptors (Fujii, 2004).

4.5. Adenosine release from mouse PFC synaptosomes

Upon observing that AOPCP did not affect the synaptic plasticity significantly we wanted to investigate if the inhibitor of e-5'N, AOPCP (50 µM), changes the amount of adenosine formed in nerve terminals. For that we measured by HPLC the levels of AMP and adenosine in synaptosomal preparations exposed or not (control) to AOPCP. Through this measurement we were able to assess the contribution of e-5'N in adenosine formation in PFC nerve terminals.

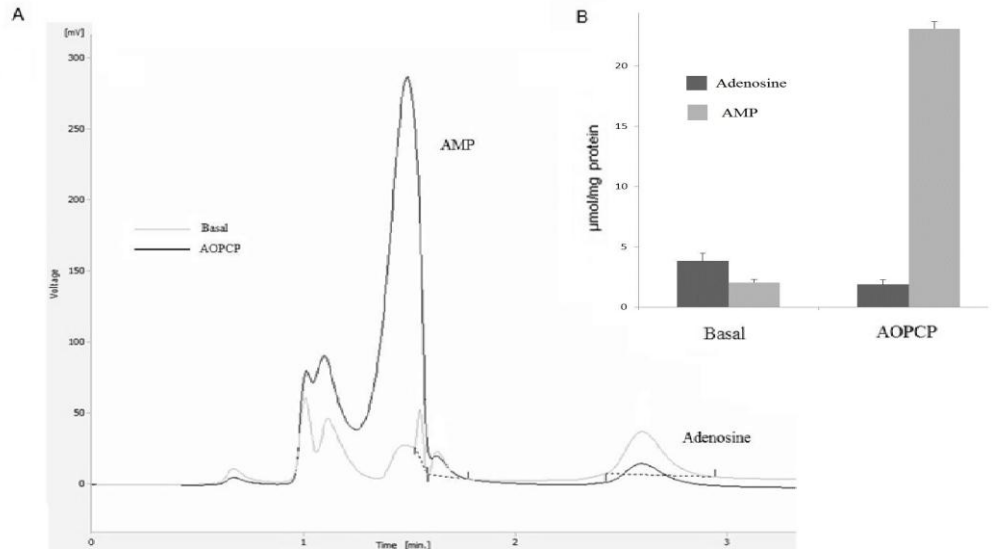

Fig.20 – HPLC measurements of AMP and adenosine in synaptosomes from prefrontal cortex. **(A)** Chromatogram showing the measurement of AMP and adenosine in basal conditions (grey line) and in the presence of AOPCP (50 µM) (black like). In the presence of AOPCP the adenosine formed is less and AMP concentration increases. **(B)** Quantification of AMP (2.03 ± 0.25 µmol × mg protein^{-1}) and adenosine (3.83 ± 0.65 µmol × mg protein^{-1}) in basal conditions and in the presence of AOPCP (50 µM) AMP (23.08 ± 0.56 µmol × mg protein^{-1}) and adenosine (1.87 ± 0.38 µmol × mg protein^{-1}). The results are representative of 2 independent experiments.

From the data obtained with the HPLC measurements we observed low levels of AMP and adenosine in basal conditions, which is in accordance with the literature

(Cunha, 2001b; Latini and Pedata, 2001; Volonté *et al*., 2003). However, when the last enzyme of the ectonucleotidase cascade (e-5'N) is inhibited by AOPCP, we observed increased levels of AMP (23.08 ± 0.56 μmol × mg protein^{-1}) when compared to the control (2.03 ± 0.25 μmol × mg protein^{-1}) and a slight decrease of the adenosine levels (1.87 ± 0.38 μmol × mg protein^{-1}) when compared to the control (3.83 ± 0.65 μmol × mg protein^{-1}) in the synaptic cleft (Fig.20). Indeed it was previously shown that the blockade of e-5'N causes a slight increase in AMP levels (Sakowicz-Burkiewicz *et al*., 2010). These data showing that in presence of AOPCP the levels of adenosine in nerve terminals are reduced, are in accordance with our electrophysiological data (Fig.19), and supports our hypothesis that in the presence of AOPCP the low levels of adenosine preferentially favors A_1R activation. Moreover, this result may lead us to prompt the hypothesis that in basal conditions the primary source of adenosine may not be through ectonucleotidases pathway but rather through the action of ENT or through exocytosis (Cunha *et al*., 1996; Boison *et al*., 2012; Klyuch *et al*., 2012).

Since no statistical analysis was performed due to the number of experiments (n=2), no definite conclusions can be obtained.

4.6. Synaptic density of e-5'N in PFC from aged mice

Frontal lobe dysfunction is thought to be involved in schizophrenia and age-associated cognitive decline (Convit *et al*., 2001). It is known that the formation of adenosine from the catabolism of ATP by e-5'N is incremented in aged rats (Cunha *et al*., 2001). Also, there are evidences that e-5'N has increased activity in aged rats (Fuchs, 1991; Mackiewicz *et al*., 2006). Thus, the last goal of this work was to investigate what was the impact of aging on synaptic density of e-5'N.

To explore if the synaptic density of e-5'N increased with aged, we used mice 36-40 weeks old and assessed the levels of e-5'N in synaptosomes from the PFC by Western blot analysis (Fig.21 A).

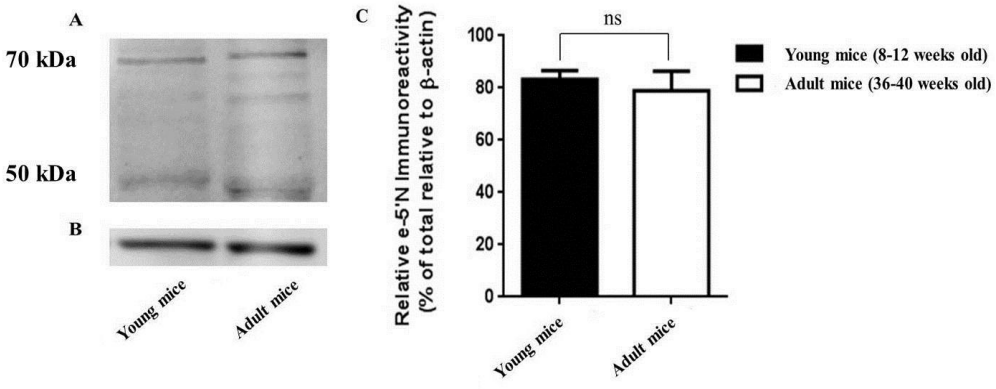

Fig.21 – Levels of e-5'N in synaptosomes of prefrontal cortex from young (8-12 weeks old) and old mice (36-40 weeks old). **(A)** Representative immunoblot of the e-5'N levels in synaptosomes from young and adult mice. **(B)** Representative immunoblot of β-actin density (control for protein loading). **(C)** Graphic represents the ratio, in percentage, of e-5'N immunoreactivity (70 kDa), and β-actin immunoreactivity for 20 µg of loaded protein. The results are presented as mean ± SEM of 3 independent experiments. ns: non-significant comparing young and adult mice; unpaired *t*-test.

The relative amount of e-5'N immunoreactivity was achieved for 20 µg of loaded protein and it was observed that the density of e-5'N in synaptosomes from adult mice was lower (78.9% ± 8.1%, n=3) than in young mice (83.2% ± 3.3%, n=3) (Fig.21 C). No significant differences between the two groups of mice were observed, probably because the difference in the age of the two animal groups is not enough to detect alterations in e-5'N. Indeed, we are aware that we should have used animals with bigger age differences. In a study regarding the activity of e-5'N it was shown that only from the 50 weeks of age mice would start to display changes (Mackiewicz *et al*., 2006). Furthermore, Cunha and colleagues showed that the catabolism of ATP into adenosine, through the action of e-5'N, increases with aging (Cunha *et al*., 2001); however, this study did not explored alterations in density of e-5'N, they only probed for its activity,

so it is not certain whether e-5'N density is affected during aging. It should be referred that in these old animals (36-40 weeks old) it was also observed two immunoreactive bands, one with 70 kDa and the other with 50 kDa, which we think to correspond to two e-5'N isoforms (as previously discussed), and from the sub-synaptic fraction data (see section 4.1.2), we can conclude that the 70 kDa and 50 kDa immunoreactive bands might correspond to e-5'N in the post-synaptic fraction and in the extra- and pre-synaptic fractions, respectively.

5. Conclusions and Final remarks

The present work has brought to light several conclusions about the role of e-5'N in the prefrontal cortex of mice and has showed that e-5'N shared an association with the $A_{2A}R$. It was observed that e-5'N is present in synaptosomes (purified nerve terminals) from the prefrontal cortex, albeit it was not enriched in nerve terminals when compared with total membranes. By performing sub-synaptic fractionation we detailed in which fractions (pre- post- and extra-synaptic regions) the e-5'N was more enriched. We discovered the clear presence of at least two different isoforms of e-5'N in synaptosomes from the PFC; one isoform with 70 kDa that was present mainly in the post-synaptic fraction, whereas the isoform with 50 kDa was enriched in pre- and extra-synaptic components. Through immunohistochemistry analysis in PFC of mice, we observed that e-5'N and $A_{2A}R$ exhibit an apparent co-localization. Apart from the apparent co-localization between e-5'N and $A_{2A}R$, they seemed to be located preferentially in neurons and microglia. To further analyse the apparent co-localization of $A_{2A}R$ and e-5'N we did a co-immunoprecipitation assay where we found a clear physical association between $A_{2A}R$ and e-5'N in nerve terminals. However, when we used $A_{2A}R$-KO mice (mice model where the $A_{2A}R$ is genetically deleted) to investigate if the density levels of e-5'N were altered, no difference was observed. Moreover, it was shown through electrophysiological recordings that $A_{2A}R$ played an important role in the LTP phenomenon in the PFC. In fact the antagonist of $A_{2A}R$ decrease the LTP in PFC slices, in contrast to what was observed with the inhibitor of e-5'N. Therefore, these results suggest that the formation of adenosine through e-5'N action may not be important to $A_{2A}R$ activation and subsequent modulation of the synaptic plasticity in the PFC. Although it is known that aging affects the activity of e-5'N, when we compared the density levels of e-5'N in synaptosomes of young (8-12 weeks old) and adult (36-40 weeks old) mice no significant changes were observed.

The work so far has brought several evidences about the interplay between e-5'N and $A_{2A}R$ in the PFC, however there are still questions that remain unanswered. It is necessary to observe if there are changes in the density levels of e-5'N in the sub-synaptic fractions in $A_{2A}R$-KO mice model. It would also be of great advantage to use forebrain-$A_{2A}R$-knockout mice to tackle the same questions. In forebrain-$A_{2A}R$-knockout mice model, only the forebrain neurons do not present $A_{2A}R$, so it would be a useful tool to observe changes in synaptic density of e-5'N and also in the synaptic transmission of PFC region. The same should be done in e-5'N-knockout mice models to observe if there are changes in the density levels of adenosine receptors and if the synaptic transmission presents alterations. Both mice models (forebrain-$A_{2A}R$-knockout and e-5'N-knockout) should be used to investigate the interplay between e-5'N and $A_{2A}R$ with more detail. The use of these mice models will allow to detail with more precision what is the role of e-5'N in pathological conditions that are related to the PFC degeneration, namely epilepsy; thus providing a more clear interaction between e-5'N and $A_{2A}R$ in conditions of pathology.

Nevertheless, this thesis paved the way to a better understanding of the relation between e-5'N and $A_{2A}R$ in the PFC, and therefore, may lead to some novel tactics to tackle questions regarding frontal lobe dysfunctions.

6. References

Abbracchio, MP., Ceruti, S., Langfelder, R., Cattabeni, F., Saffrey, MJ., Burnstock, G. (1994). Trophic actions of purines. *J. Neurochem.* **63**:S40.

Agostinho, P., Caseiro, P., Rego, AC., Duarte, EP., Cunha, RA., Oliveira, CR. (2000). Adenosine modulation of D-[^{3}H]aspartate release in cultured retina cells exposed to oxidative stress. *Neurochem Int.* **36**:255-265.

Almeida, T., Rodrigues, RJ., Mendonça, A., Ribeiro, JA., Cunha, RA. (2003). Purinergic P2 Receptors Trigger Adenosine Release Leading to Adenosine A_{2A} Receptor Activation and Facilitation of Long-term Potentiation in Rat Hippocampal Slices. *Neuroscience*. **122**:111-121.

Anderson, WW., Collingride, GL. (1997). A data acquisition program for on-line analysis of long-term potentiation and long-term depression. *Neurosci Abst.* **23**:665.

Arnstern, AFT., Pliszka, SR. (2011). Catecholamine influences on prefrontal cortical function: Relevance to treatment of attention deficit hyperactivity disorder and related disorders. *Pharmacol Biochem Behav.* **99**:211–216.

Augusto, E., Matos, M., Sévigny, J., El-Tayeb, A., Müller, CE., Cunha, RA., Chen, JF. (2013). Ecto-5'-Nucleotidase (CD73)-Mediated Formation of Adenosine Is Critical for the Striatal Adenosine A_{2A} Receptor Functions. *J Neurosci.* **33**:11390-11399.

Bailly, YJ., Siegfried, WS., Mariani, J., Kreutzberg, GW., Delhaye-Bouchaud, N. (1998). Immature Chemodifferentiation of Purkinje Cell Synapses Revealed by 5'-Nucleotidase Ecto-Enzyme Activity in the Cerebellum of the *Reeler* Mouse. *Synapse*. **29**:279-292.

Benarroch, EE. (2008). Adenosine and its receptors: multiple modulatory functions and potential therapeutic targets for neurologic disease. *Neurology* **70**:231-236.

Bianchi, V., Spychala, J. (2003). Mammalian 5'-nucleotidases. *J Biol Chem.* **278**:195–198.

Bjelobaba, I., Stojiljkovic, M., Pekovic, S., Dacic, S., Lavrnja, I., Stojkov, D., Rakic, L., Nedeljkovic, N. (2007). Immunohistological determination of ecto-nucleoside triphosphate diphosphohydrolase1 (NTPDase1) and 5'-nucleotidase in rat hippocampus reveals overlapping distribution. *Cell Mol Neurobiol.* **27**:731–743.

Bjelobaba, I., Parabucki, A., Lavrnja, I., Dtojkov, D., Dacic, S., Pkovic, S., Rakic, L., Stojiljkovic, M., Nedeljkovic, N. (2011). Dynamic Changes in the Expression Pattern of Ecto-5'-Nucleotidase in the Rat Model of Cortical Stab Injury. *J Neurosci Res.* **89**:862-873.

Boison, D., Chen, JF., Fredholm, BB. (2010). Adenosine signalling and function in glial cells. *Cell Death Diff.* **17**:1071-1082.

Boison, D. (2011). Modulators of Nucleoside Metabolism in the Therapy of Brain Diseases. *Curr Top Med Chem*. **11**:1068-1086.

Boison, D., Singer, P., Shen, HY., Feldon, J., Yee, BK. (2012). Adenosine hypothesis of schizophrenia - Opportunities for pharmacotherapy. *Neuropharmocology*. **62**:1527-1543

Bonan, CD., Schtinger, MRC., Battastini, AMO., Sarkis, JJF. (2001). Ectonucleotidases and Synaptic Plasticity: Implications in Physiological and Pathological Conditions. *Drug Develop Res.* **52**:57-65.

Borowiec, A., Lechward, K., Tkacz-Stachowska, K., Skladanowski, AC. (2006). Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5'-nucleotidases. *Acta Biochim Pol*. **53**:269-278.

Braun, N., Lenz, C., Gillardon, F., Zimmermann, M., Zimmermann, H. (1997). Focal cerebral ischemia enhances glial expression of ecto-5'-nucleotidase. *Brain Res.* **766**:213-226.

Braun, N., Zimmermann, H. (2001). Microglial Ectonucleotidases: Identification and Functional Roles. *Drug Develop Res.* **53**:208-217.

Burnstock, G. (2007). Physiology and Pathophysiology of Purinergic Neurotransmission. *Physiol Rev.* **87**:659–797.

Burnstock, G., Fredholm, BB., Verkhratsky, A. (2011). Adenosine and ATP receptors in the nrain. *Curr Top Med Chem*. **11**:973-1011.

Burnstock, G. (2013). Introduction to purinergic signaling in the brain. *Adv Exp Med Biol*. **986**:1-12.

Buzsáki, G., Anastassiou, CA., Koch, C. (2012). The origin of extracellular fields and currents — EEG, ECoG, LFP and spikes. *Nat Rev Neurosci*. **13**:407-420.

Canas, PM., Porciuncula, LO., Cunha, GMA., Silva, CG., Machado, NJ., Oliveira, JMA., Oliveira, CR., Cunha, RA. (2009). Adenosine A_{2A} receptor blockade prevents synaptotoxicity and memory dysfunction caused by β-amyloid peptides via p38 mitogen-activated protein kinase pathway. *J Neurosci*. **29**:14741-14751.

Chamak, B., Mallat, M. (1991). Fibronectin and laminin regulate the in vitro differentiation of microglial cells. *Neuroscience.* 45:513-527.

Collins, MG., Hourani, SMO. (1993). Adenosine receptor subtypes. *Trends Pharmacol. Sci.* **14**:360-366.

Cognato, GP., Bonan, CD, (2010). Ectonucleotidases and epilepsy. *Open Neurosci J*. **4**:44-52.

Cui, M., Bai, X., Li, T., Chen, F., Dong, Q., Zhao, Y., Liu, X. (2013). Decreased Extracellular Adenosine Levels Lead to Loss of Hypoxia-Induced Neuroprotection after Repeated Episodes of Exposure to Hypoxia. *PLoS One*. **2**: e57065.

Convit, A., Wolf, OT., de Leon, MJ., Patalinjug, M., Kandil, E., Caraos, C., Scherer, A., Saint Louis, LA., Cancro, R. (2001). Volumetric analysis of the pre-frontal regions: findings in aging and schizophrenia. *Psych Res: Neuroimag*. **107**:61-73.

Cunha, RA., Sebastião, AM. (1993). Adenosine and adenine nucleotides are independently released from both the nerve terminals and the muscle fibers upon electrical stimulation of the innervated skeletal muscle of the frog. *Eur J Physiol.* **424**:503-510.

Cunha, RA., Correia-de-Sá, P., Sebastião, AM., Ribeiro, JA. (1996). Preferential activation of excitatory adenosine receptors at rat hippocampal and neuromuscular synapses by adenosine formed from released adenine nucleotides. *BrJ Pharmacol.* **119**:253–260.

Cunha, RA. (1998). On slices, synaptosomes and dissociated neurones to study in vitro ageing physiology. *Trends Neurosci.* **21**:286., author reply 287.

Cunha, RA., Sebastião, AM., Ribeiro, JA. (1998a). Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabolism by ectonucleotidases into adenosine and channeling to adenosine A_1 receptors. *J Neurosci*. **18**(6):1987-1995.

Cunha, RA., Almeida, T., Constantino, MD., Ribeiro, JA. (1998b). Aging modifies adenosine modulation of [^{3}H]acetylcholine release from the rat hippocampus. *Drug Dev Res*. **43**:52.

Cunha, RA., Brendel, P., Zimmermann, H., Ribeiro, JA. (2000). Immunologically distinct isoforms of ecto-5'-nucleotidase in nerve terminals of different areas of the rat hippocampus. *J Neurochem.* **74**:334–338.

Cunha, RA., Almeida, T., Ribeiro, JA. (2001). Parallel modification of adenosine extracellular metabolism and modulatory action in the hippocampus of aged rats. *J Neurochem.* **76**:372–382.

Cunha, RA. (2001a). Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int.* **38**:107-125.

Cunha, RA. (2001b). Regulation of the Ecto-Nucleotidase Pathway in Rat Hippocampal Nerve Terminals. *Neurochem Res.* **26**:979-991.

Cunha, RA. (2005). Neuroprotection by adenosine in the brain: From A_1 receptor activation to A_{2A} receptor blockade. *Purin Sign.* **1**:111-134.

Cunha, RA. (2008). Different cellular sources and different roles of adenosine: A_1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A_{2A} receptor-mediated facilitation of plasticity. *Neurochem Int.* **52**:65-72.

Cunha, RA., Agostinho PM. (2010). Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline. *J AlzDis.* **20**:95-116.

Dias, RB., Rombo, DM, Ribeiro, JA., Henley, JM., Sebastião, AM. (2013). Adenosine: setting the stage for plasticity. *Trends Neurosci.* **36**:248-257.

Duarte, JMN., Agostinho, PM., Carvalho, RA., Cunha, RA. (2012). Caffeine Consumption Prevents Diabetes-Induced Memory Impairment and Synaptotoxicity in the Hippocampus of NONcZNO10/LTJ Mice. *PLoS ONE*. **7**:e21899.

Dunkley, PR., Jarvie, PE., Robinson, PJ. (2008). A rapid Percoll gradient procedure for preparation of synaptosomes. *Nature Prot.* **3**:1718-1728.

Dunwiddie, TV., Masino, SA. (2001). The Role and Regulation of Adenosine in the Central Nervous System. *Annu Rev Neurosci.* **24**:31-55.

Dunwiddie, TV., Diao, L., Proctor, WR. (1997). Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. *J Neurosci.* **17**:7673–7682.

Fields, RD., Burnstock, G. (2006). Purinergic signalling in neuron–glia interactions. *Nat Rev Neurosci*. **7**:423-436.

Fini, C., Talamo, F., Cherri, S., Coli, M., Floridi, A., Ferrara, L., Scaloni, A. (2003). Biochemical and mass spectrometric characterization of soluble ecto-5_-nucleotidase from bull seminal plasma. *Biochem J.* **372**:443-451.

Fredholm, BB., Burnstock, G., Harden, TK., Spedding, M. (1996). Receptor nomenclature. *Drug Develop Res.* **39**:461-466

Fredholm, BB., IJzerman, AP., Jacobson, KA., Klotz, KN., Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev.* **53**:527-552.

Fuchs, JL. (1991). 5'-Nucleotidase Activity Increases in Aging Rat Brain *Neurobiol of Aging.* **12**:523-530

Fujii, S. (2004). ATP- and Adenosine-Mediated Signaling in the Central Nervous System: The Role of Extracellular ATP in Hippocampal Long-Term Potentiation. *J Pharmacol Sci.* **96**:103-106.

Garção, P., Szabó, EC., Wopereis, S., Castro, AA., Tomé, ÂR., Prediger, RD., Cunha, RA., Agostinho, P., Köfalvi, A. (2013). Functional interaction between pre-synaptic α6β2-containing nicotinic and adenosine A_{2A} receptors in the control of dopamine release in the rat striatum. *Brit J Pharmacol.* **169**:1600-1611.

Goding, JW. (2000). Ecto-enzymes: physiology meets pathology. *J Leukoc Biol.* **67**:285–311.

Gomes, CV., Kaster, MP., Tomé, AR., Agostinho, PM., Cunha, RA. (2011). Adenosine receptors and brain diseases: Neuroprotection and neurodegeneration. *Biochim et Biophys Acta*. **1808**:1380–1399.

Gonçalves, N., Simões, AT., Cunha, RA., de Almeida, LP. (2013). Caffeine and Adenosine A_{2A} Receptor Inactivation Decrease Striatal Neuropathology in a Lentiviral-Based Model of Machado–Joseph Disease. *Ann Neurol.* **73**:655–666.

Gray, JH., Owen, RP., Giacomini, KM. (2004). The concentrative nucleoside transporter family, SLC28. *Eur J Physiol.* **447**:728-734.

Gu, JG., Foga, IO., Parkinson, FE., Geiger, JD. (1995). Involvement of bidirectional adenosine transporters in the release of L-[^{3}H]adenosine from rat brain synaptosomal preparations. *J Neurochem.* **64**:2105–2110

Gui, L., Duan, W., Tian, H., Li, C., Zhu, J., Chen, JF., Zheng, J. (2009). Adenosine A_{2A} receptor deficiency reduces striatal glutamate outflow and attenuates brain injury induced by transient focal cerebral ischemia in mice. *Brain Res.* **1297**:185–193.

Heilbronn, A., Krapohl, A., Zimmermann, H. (1995). 5'-Nucleotidase in PC12 cells as revealed by immunocytochemistry. *Cell Tissue Res.* **280**:123-131.

Higley, MJ., Sabatini, BL. (2010). Competitive regulation of synaptic Ca^{2+} influx by D2 dopamine and A_{2A} adenosine receptors. *Nat Neurosci.***13**:958-966.

Huang, YY., Simpson, E., Kellendonk, C., Kandel, ER. (2004). Genetic evidence for the bidirectional modulation of synaptic plasticity in the prefrontal cortex by D1 receptors. *PNAS*. **101**:3236-3241.

Hunsucker, SA., Mitchell, BS., Spychala, J. (2005). The 5'-nucleotidases as regulators of nucleotide and drug metabolism *Pharmacol Therap.* **107**:1–30.

Klyuch, BP., Dale, N., Wall, MJ. (2012). Deletion of Ecto-5'-Nucleotidase (*CD73*) Reveals Direct Action Potential-Dependent Adenosine Release. *J Neurosci.* **32**:3842-3847.

Kawamura, M.Jr.., Ruskin, DN., Masino, SA. (2010). Metabolic autocrine regulation of neurons involves cooperation among pannexin hemichannels, adenosine receptors, and K_{ATP} channels. *J Neurosci.* **30**:3886–3895.

Knöfel, T., Sträter, N. (1999). X-ray structure of the *Escherichia coli* periplasmic 5'-nucleotidase containing a dimetal catalytic site. *Nature Struct. Biol.* **6**:448–453.

Knöfel, T., Sträter, N. (2001). Mechanism of hydrolysis of phosphate esters by the dimetal center of 5'-nucleotidase based on crystal structures. *J Mol Biol.* **309**:239-254.

Langer, D., Hammer, K., Koszalka, P., Schrader, J., Robson, S., Zimmermann, H. (2008). Distribution of ectonucleotidases in the rodent brain revisited. *Cell Tiss Res.* **334**:199-217.

Lasley, RD. (2011). Adenosine receptors and membrane microdomains. *Biochim et Biophys.* **1808**:1284-1289.

Latini, S., Pedata, F. (2001). Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem.* **79**:463-484.

Maienschein, V., Zimmermann, H. (1996). Immunocytochemical localization of ecto-5'-nucleotidase in cultures of cerebellar granule cells. *Neuroscience.* **70**:429-38.

Mackiewicz, M., Nikonova, EV., Zimmermann, JE., Romer, MA., Cater, J., Galante, RJ., Pack, AI. (2006). Age-related changes in adenosine metabolic enzymes in sleep/wake regulatory areas of the brain. *Neurobiol Aging.* **27**:351-360.

Marco, M., Augusto, E., dos Santos-Rodriges, A., Schwarzschild, MA., Chen, JF., Cunha, RA., Agostinho, P. (2012). Adenosine A_{2A} Receptors Modulate Glutamate Uptake in Cultured Astrocytes and Gliosomes. *Glia.* **60**:702-716.

Martín, ED., Fernández, M., Perea, G., Pascual, O., Haydon, PG., Araque, A., Ceña, V. (2007). Adenosine released by astrocytes contributes to hypoxia-induced modulation of synaptic transmission. *Glia*. **55**:36–45.

Mendonça, A., Ribeiro, JA. (2000). Long-term potentiation observed upon blockade of adenosine A_1 receptors in rat hippocampus is N-methyl-d-aspartate receptor-dependent. *Neurosci Lett.* **291**:81-84.

Mendonça, A., Ribeiro, JA. (2001). Adenosine and Synaptic Plasticity. *Drug Develop Res.* **52**:283-290.

Napieralski, R., Kempkes, B., Gutensohn, W. (2003). Evidence for coordinated induction and repression of ecto-5´-nucleotidase (CD73) and the A_{2A} adenosine receptor in a human B cell line. *Biol. Chem.* **384**:483–487.

North, RA., Verkhratsky, A. (2006). Purinergic transmission in the central nervous system. *Eur J Physiol.* **452**:479-485.

Pankratov, Y., Lalo, U., Verkhratsky, A., North, RA. (2006). Vesicular release of ATP at central synapses. *Eur J Physiol.* **452**:589-597.

Pascual, O., Casper, KB., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, JY., Takano, H., Moss, SJ., McCarthy, K., Haydon, PG. (2005). Astrocytic Purinergic Signaling Coordinates Synaptic Networks. *Science.* **310**:113-116.

Pedata, F., Corsi, C., Melani, A., Bordoni, F., Latini, S. (2001). Adenosine extracellular brain concentrations and role of A_{2A} receptors in ischemia. *Ann N Y Acad Sci.* **939**:74-84.

Perea, G., Navarrete, M., Araque, A. (2009). Tripartite synapses: astrocytes process and control synaptic information. *Trends in Neurosci.* **32**:421-431.

Phillips, GR., Huang, KJ., Wang, Y., Tanaka, H., Shapiro, L., Zhang, W., Shan, WS., Ardnt, K., Frank, M., Gordon, RE., Gawinowicz, MA., Zhao, Y., Colman, DR. (2001). The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. *Neuron.* **32**:63-77.

Pinheiro, PS., Rodrigues, RJ.,Silva, AP., Cunha, RA., Oliveira, CR., Malva, JO. (2003). Solubilization and immunological identification of presynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in the rat hippocampus. *Neurosci Lett.* **336**: 97-100.

Pinto-Duarte, A., Coelho, JE., Cunha, RA., Ribeiro, JA., Sebastião, AM. (2005). Adenosine A_{2A} receptors control the extracellular levels of adenosine through modulation of nucleoside transporters activity in the rat hippocampus. *J Neurochem.* **93**:595-604.

Porkka-Heiskanen, T., Kalinchuk, AV. (2011). Adenosine, energy metabolism and sleep homeostasis. *Sleep Medic Rev.* **15**:123-135.

Quiroz, C., Luján, R., Uchigashima, M., Simões, AP., Lerner, TN., Borycz, J., Kachroo, A., Canas, PM., Orru, M., Schwarzschild, MA., Rosin, DL., Kreitzer, AC., Cunha, RA., Masahiko, W., Ferré, S. (2009). Key Modulatory Role of Presynaptic Adenosine A_{2A} Receptors in Cortical Neurotransmission to the Striatal Direct Pathway. *ScientificWorldJournal.* **9**:1321-1344.

Ralevic, V., Burnstock, G. (1998). Receptors for Purines and Pyrimidines. *Pharmacol Rev.* **50**:413-492.

Rebola, N., Coelho, JE., Costenla, AR., Lopes, LV., Parada, A., Oliveira, CR., Soares-da-Silva, P., de Mendonça, A., Cunha, RA. (2003). Decrease of A_1 receptor density and of adenosine neuromodulation in the hippocampus of kindled rats. *Eur J Neurosci.* **18**:820-828.

Rebola, N., Rodrigues, RJ., Lopes, LV., Richardson, PJ., Oliveira, CR., Cunha, RA. (2005a). Adenosine A_1 and A_{2A} receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. *Neuroscience*. **133**:79-83.

Rebola, N., Canas, PM., Oliveira, CR., Cunha, RA. (2005b). Different Synaptic and Subsynaptic Localization of Adenosine A_{2A} Receptors in the Hippocampus and Striatum of the Rat. *Neuroscience.* **132**:893-903.

Rebola, N., Lujan, R., Cunha, RA., Mulle, C. (2008). Adenosine A_{2A} receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. *Neuron.* **57**:121-134.

Reigada, D., Díez-Pérez, I., Gorostiza, P., Verdaguer, A., de Aranda, IG; Pineda, O; Vilarrasa, J; Marsal, J., Blasi, J., Aleu, J., Solsona, C. (2003). Control of neurotransmitter release by an internal gel matrix in synaptic vesicles. *PNAS.* **100**:3485-3490.

Resta, R., Yamashita, Y., Thompson, LF. (1998). Ecto-enzyme and signaling functions of lymphocyte CD73. *Immuno Rev.***161**:95-109.

Ribeiro, JA., Sebastião, AM., Mendonça, A. (2003). Adenosine receptors in the nervous system: pathophysiological implications. *Prog Neurobiol.* **68**:377-392.

Robertson, SJ., Edwards, FA. (1998). ATP and glutamate are released from separate neurones in the rat medial habenula nucleus: frequency dependence and adenosine-mediated inhibition of release. *J Physiol.* **508**:691-701.

Sakowicz-Burkiewicz, M., Kocbuch, K., Grden, M., Szutowicz, A., Pawelczyk, T. (2010). Adenosine 5'-Triphosphate it the Predominant Source of Peripheral Adenosine in Human B Lymphoblasts. *J Physiol and Pharmacol.* **61**:491-499.

Sawada, K., Echigo, N., Juge, N., Miyaji, T., Otsuka, M., Omote, H., Yamamoto, A., Moriyama, Y. (2008). Identification of a vesicular nucleotide transporter. *PNAS*. **115**:5683-5686.

Sebastião, AM., Ribeiro, JA. (2009). Adenosine receptors and the central nervous system. *Hand Exper Pharmacol.* **193**:471-534.

Shirley, DG., Vekaria, RM., Sévigny, J. (2009). Ectonucleotidases in the kidney. *Purin Sign.* **5**:501–511.

Sperlágh, B., Sershen, H., Lajtha, A., Vizi, ES. (1998). Co-release of endogenous ATP and [^{3}H]noradrenaline from rat hypothalamic slices: origin and modulation by α_2-adrenoreceptors. *Neuroscience.* **82**:511–520.

Sperlágh, B., Vizi, ES. (2011). The role of extracellular adenosine in chemical neurotransmission in the hippocampus and basal ganglia: Pharmacological and clinical aspects. *Curr Top in Med Chem.* **11**:1034-1046.

Stone, TW., Jones, PA., Smith, RA. (2001). Neuroprotection by A_{2A} receptor antagonists. *Drug Dev. Res.* **52**:323-330.

Südhof, TC. (2004). The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27**:509-47.

Sweeney, TM., Frankfurter, A., Little, CD. (1990). In vitro differentiation of neuron-like cells. *Mol Cell Neurosci.* **1**:193-201.

Sweeney, MI. (1996). Adenosine Release and Uptake in Cerebellar Granule Neurons Both Occur via an Equilibrative Nucleoside Carrier that Is Modulated by G Proteins. *J Neurochem.* **67**:81-88.

Vogel, M., Kowalewski, H., Zimmermann, H., Hooper, NM., Turner, AJ. (1992). Soluble low-K_m 5'-nucleotidase from electric-ray (*Torpedo marmorata*) electric organ and bovine cerebral cortex is derived from the glycosyl-phosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. *Biochem J.* **284**:621-624.

Volonté, C., Amadio, S., Cavaliere, F., D'Ambrosi, N., Vacca, F., Bermardi, G. (2003). Extracellular ATP and neurodegeneration. *Curr Drug Targets.* **2**:403-12.

von Kugelgen, I., Allgaier, C., Schobert, A., Starke, K. (1994). Co-release of noradrenaline and ATP from cultured sympathetic neurons. *Neuroscience.* **61**:199-202.

Von Lubitz, D.K.J.E. (1999). Adenosine and cerebral ischemia: Therapeutic future or death of a brave concept?. *Eur J Pharmacol.* **365**:9-25.

Wang, W., Dever, D., Lowe, J., Storey, GP., Bhansali, A., Eck, EK., Nitulescu, I., Weimer, J., Bamford, NS. (2012). Regulation of prefrontal excitatory neurotransmission by dopamine in the nucleus accumbens core. *J Physiol.* **590**:3743-3769.

Wardas, J. (2002). Neuroprotective role of adenosine in the CNS. *Pol J Pharmacol.* **54**:313-326.

Wei, CJ., Li, W., Chen, JF. (2011). Normal and abnormal functions of adenosine receptors in the central nervous system revealed by genetic knockout studies. *Biochim et Biophys Acta.* **1808**:1358-1379.

White, TD., MacDonald, WF. (1990). Neural release of ATP and adenosine. *Ann N Y Acad Sci.* **603**:287-300.

Wu, HY., Opperman, K., Kaboord, B. (2012). Method to isolate functional synaptosomes - Enrich neuronal synaptic proteins while maintaining phosphoprotein integrity. *Piercenet. E-pub.*

Xu, H., Hu, F., Sado, Y., Ninomyia, Y., Borza, DB., Ungvari, Z., LaGamma, EF., Csiszar, A., Nedergaard, M., Ballabh, P. (2008). Maturational Changes in Laminin, Fibronectin, Collagen IV, and Perlecan in Germinal Matrix, Cortex, and White Matter and Effect of Betamethasone. *J Neurosci Res.* **86**:1482-1500.

Yaar, R., Jones, MR., Chen, JF., Ravid, K. (2005). Animal models for the study of adenosine receptor function. *J Cell Physiol.* **202**:9–20.

Zamzow, CR., Xiong, W., Parkinson, FE. (2008). Astrocytes Affect the Profile of Purines Released From Cultured Cortical Neurons. *J Neurosci Res.* **86**:2641.2649.

Zimmermann, H. (1992). 5'-nucleotidase: molecular structure and functional aspects. *Biochem J.* **285**:345-365.

Zimmermann, H. (1996). Biochemistry, Localization and Functional Roles of Ecto-nucleotidases in the Nervous System. *Prog Neurobiol.* **49**:589-618.

Zimmermann, H. (2001). Ectonucleotidases: some recent developments and a note on nomenclature. *Drug Develop Res.* **52**:44–56.

Zimmermann, H. (2006). Nucleotide signaling in nervous system development. *Eur J Physiol.* **452**:573-588.

Zimmermann, H., Zebbisch M., Sträter N. (2012). Cellular function and molecular structure of ecto-nucleotidases. *Purin Sign.* **8**:437-502.