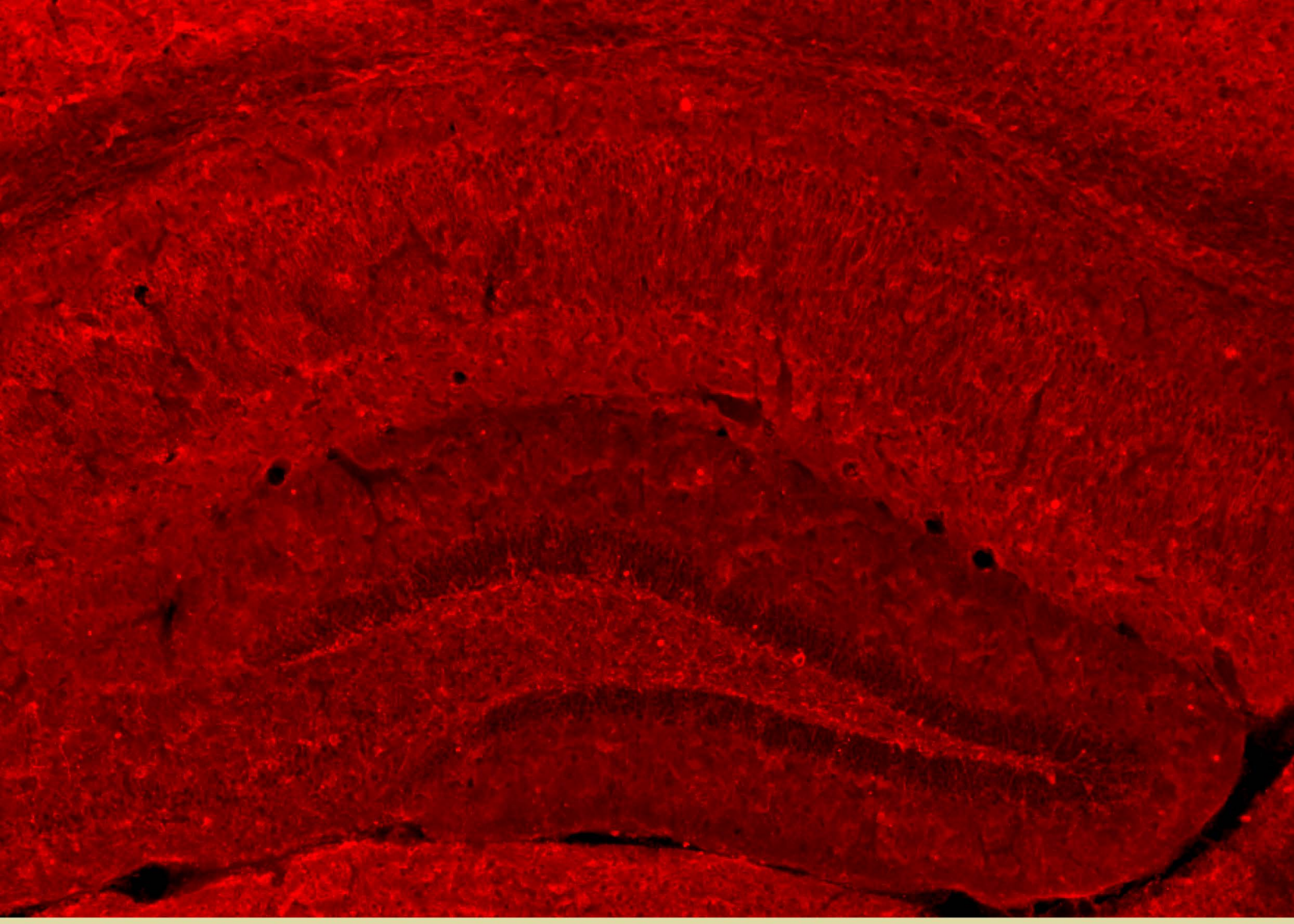

Francisco Manuel Queiroz Gonçalves

PHYSIO-PATHOLOGICAL ROLE OF ECTO-5'-NUCLEOTIDASE
A new target for neuroprotection

Tese de Doutoramento em Biociências, especialização em Neurociências,
orientada pelo Professor Doutor Ângelo José Ribeiro Tomé e Professor Doutor Rodrigo Pinto dos Santos Antunes da Cunha
e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Janeiro 2018

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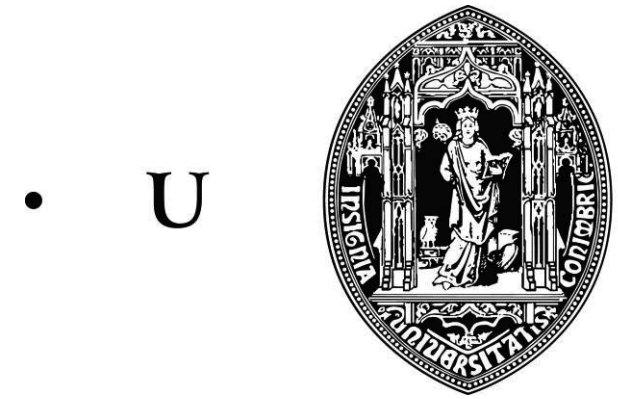
Universidade de Coimbra

Faculdade de Ciências e Tecnologia da Universidade de Coimbra

(FCTUC)



Physio-pathological role of ecto-5'-nucleotidase – a new target for neuroprotection

Francisco Manuel Queiroz Gonçalves

Janeiro de 2018

Tese de Doutoramento apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para prestação de provas necessárias à obtenção do grau de Doutor em Biociências (especialidade em Neurociências), realizada sob orientação científica do Professor Doutor Ângelo José Ribeiro Tomé (Faculdade de Ciências e Tecnologia da Universidade de Coimbra) e do Professor Doutor Rodrigo Pinto dos Santos Antunes da Cunha (Faculdade de Medicina da Universidade de Coimbra).

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Doubt is the principle of wisdom

Aristotle

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Table of Contents

Abbreviations List

A_1R- Adenosine A_1 receptors

$A_{2A}R$- Adenosine A_{2A} receptors

$A_{2B}R$- Adenosine A_{2B} receptors

A_3R- Adenosine A_3 receptors

ACh- Acetylcholine

ADA- Adenosine deaminase

AD- Alzheimer's disease

ADP- Adenosine-5´-diphosphate

AK- Adenosine kinase

AMP- Adenosine-5´-monophosphate

AMPA- α -Amino-3-hydroxi-5-methyl-4-isoxazolepropionic acid

AOPCP- α,β-Methylene-ADP- adenosine-5′-O-[(phosphonomethyl)phosphonic acid]

AP- Alkaline phosphatase; antero-posterior

APP- Amyloid precursor protein

ARL67156- 6-N,N-Diethyl-D-β,γ-dibromomethylene ATP

ATP- Adenosine-5'-triphosphate

$A\beta_{1-42}$- Beta-amyloid

cAMP- Cyclic adenosine-5´-monophosphate

CAPS- 3-(Cyclohexylamino)-1-propanesulfonic acid

CD73 or e5'NT- Ecto-5'-nucleotidase

CNS- Central nervous system

CS- Conditioned stimulus

DG- Dentate gyrus

DMSO- Dimethylsulfoxide

DNA- Deoxyribonucleic acid

DRG- Dorsal root ganglia

DTT- Dithiothreitol

DV- Dorso-ventral

E-NPP- Ecto-nucleotide pyrophosphatase

E-NTPDases- Ecto-nucleoside triphosphate diphosphohydrolases

EC- Entorhinal cortex

EC_{50}- Concentration of a ligand eliciting 50% of the maximal response

EDTA- Ethylenediaminetetraacetic acid

eNT- Ecto-nucleotidase

ENT- Equilibrative nucleoside transporters

FAD- Familial AD mutations

fEPSP– Field excitatory postsynaptic potentials

FDA- Food and Drug Administration

GABA- γ-Aminobutyric acid

GFAP- Glial fibrillary acidic protein

GLT-I- Astrocyte glutamate transporter-I

HEPES- N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)

HPLC- High performance liquid chromatography

IC_{50}- Concentration of a ligand that causes 50% of the maximal inhibition

ICV- Intracerebroventricular

IP3- Inositol-(1,4,5)- triphosphate

KO- Knock out

LDCV- Large dense core vesicles

LPP- lateral perforant pathway

LTP- Long-term potentiation

LV- Lentivirus

MAP-2- Microtubule-associated protein 2

MAPK- Mitogen-activated protein kinases

mGluR- Metabotropic glutamate receptors

ML- Meso-lateral

MPP- medial perforant pathway

MTLE- Mesial temporal lobe epilepsy

NADH- Reduced form of nicotinamide adenine dinucleotide

NFT- Neurofibrillary tangles

NMDA- N-methyl-D-aspartate

NMDAR- NMDA receptor

NPP- Ecto-pyrophosphatase/phosphodiesterases

NTPDase- diphosphohydrolases

P1R- Purinergic receptors type 1

P2R- Purinergic receptors type 2

P2XR- Ionotropic P2X receptors

P2YR- Metabotropic P2Y receptors

PAGE- Polyacrylamide gel electrophoresis

PAP- Prostatic acid phosphatase

PBS- Phosphate buffered saline medium

PLA_2- Phospholipase A2

PLC- Phospholipase C

POMs- Polyoxometalates

PP- Perforant pathway

PS- Population spike

PS1- Presenilin 1

PSD-95- Postsynaptic density protein 95

PSV- Pre-synaptic volley

RNA- Ribonucleic acid

SAH- S-Adenosyl-L-homocysteine

SCH 58261- 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

SDS- Sodium dodecyl sulfate

SEM- Standard error of the mean

***sh*RNA**- Small hairpin RNA

siRNA- Small interfering RNA

SNAP-25- Synaptosomal-associated protein 25

SSV- Small synaptic vesicles

TA- Temporoammonic pathway

TBS- Tris buffered saline medium

TBS-T- TBS with 0.1% Tween-20

TNAP- Tissue-nonspecific alkaline phosphatase

UDP- Uridine-5´-diphosphate

US- Unconditioned stimulus

UTP- Uridine-5´-triphosphate

vGluT- Glutamate vesicular transporters

vNUT- Vesicular nucleotide transporters

Publications List

The following papers were **published** in peer-reviewed international scientific journals during the development of this thesis:

Rial D, Castro AA, Machado N, Garção P, **Gonçalves FQ**, Silva HB, Tomé AR, Köfalvi A, Corti O, Raisman-Vozari R, Cunha RA and Prediger RD. **(2014)** Behavioral phenotyping of Parkin-deficient mice: looking for early preclinical features of Parkinson's disease. **PLoS One.** Dec 8;9(12):e114216. doi: 10.1371/journal.pone.0114216.

Ferreira SG, **Gonçalves FQ**, Marques JM, Tomé ÂR, Rodrigues RJ, Nunes-Correia I, Ledent C, Harkany T, Venance L, Cunha RA and Köfalvi A. **(2015)** Presynaptic adenosine A_{2A} receptors dampen cannabinoid CB1 receptor-mediated inhibition of corticostriatal glutamatergic transmission. **Br J Pharmacol.** Feb 172(4):1074-86. doi: 10.1111/bph.12970.

Gonçalves FQ, Pires J, Pliassova A, Beleza R, Lemos C, Marques JM, Rodrigues RJ, Canas PM, Köfalvi A, Cunha RA and Rial D. **(2015)** Adenosine A_{2B} receptors control A_1 receptor-mediated inhibition of synaptic transmission in the mouse hippocampus. **Eur J Neurosci.** Apr;41(7):878-88. doi: 10.1111/ejn.12851. Epub 2015 Feb 19.

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Viana da Silva S, Haberl MG, Zhang P, Bethge P, Lemos C, Gonçalves N, Gorlewicz A, Malezieux M, **Gonçalves FQ**, Grosjean N, Blanchet C, Frick A, Nägerl UV, Cunha RA and Mulle C. **(2016)** Early synaptic deficits in the APP/PS1 mouse model of Alzheimer's disease involve neuronal adenosine A_{2A} receptors. **Nat Commun.** Jun 17;7:11915. doi: 10.1038/ncomms11915.

Rial D, Morató X, Real JI, **Gonçalves FQ**, Stagljar I, Pereira FC, Fernández-Dueñas V, Cunha RA and Ciruela F. **(2017)** Parkinson's disease-associated GPR37 receptor regulates cocaine-mediated synaptic depression in corticostriatal synapses. **Neurosci Lett.** Jan 18;638:162-166. doi: 10.1016/j.neulet.2016.12.040.

Rodrigues-Neves AC, Aires ID, Vindeirinho J, Boia R, Madeira MH, **Gonçalves FQ**, Cunha RA, Santos PF, Ambrósio AF and Santiago AR. **(2018)** Elevated Pressure Changes the Purinergic System of Microglial Cells. **Front. Pharmacol.** Jan 24; doi: 10.3389/fphar.2018.00016

The following papers are **submitted** to peer-reviewed international scientific journals:

Gonçalves FQ, Almeida T, Oses J, Matos MA, Rodrigues RJ, Tomé AR and Cunha RA.; The pattern of ATP release from brain cortical nerve terminals is mostly different from that of classical neurotransmitters

Gonçalves FQ, Lopes JP, Silva HB, Lemos C, Silva AC, Gonçalves N, Tomé AR, Ferreira SG, Canas PM, Rial D, Agostinho P and Cunha RA.; Increased ATP-derived adenosine activating A_{2A} receptors mediates synaptic and memory dysfunction by β-amyloid peptides

RESUMO

A adenosina é um neuromodulador no sistema nervoso central (CNS) que inibe a transmissão sináptica basal, através da ativação dos recetores A_1 (A_1R), durante baixos níveis de estimulação e facilita a plasticidade sináptica, através da ativação de recetores A_{2A} ($A_{2A}R$), durante altos níveis de estimulação. Isto resulta do facto de após estímulos de alta intensidade existir uma libertação preferencial de ATP, embora ainda não se saiba o porquê de esta libertação ocorrer nestas circunstâncias.

No cérebro, o ATP é armazenado em vesículas sinápticas e libertado de uma forma dependente da atividade. Quando libertado no espaço extracelular, o ATP é catabolizado, através da ação de ecto-nucleotidases, nos seus metabolitos ADP, AMP e adenosina. O ultimo passo deste processo metabólico é promovido pela ecto-5'-nucleotidase (CD73), que se apresenta como o principal controlador da taxa de catabolismo do AMP em adenosina. Embora já existam algumas evidências que mostram uma relação direta entre a função da CD73 e a ativação dos $A_{2A}R$, a importância fisiológica desta relação continua praticamente desconhecida.

Clarificamos agora, não só em que circunstâncias, o ATP é preferencialmente libertado, mas também o papel fisiopatológico da CD73, uma vez que, tal como os $A_{2A}R$, apresentou um aumento de densidade em situações patológicas, conferindo assim à adenosina proveniente do ATP um papel fundamental na formação de alterações plásticas anormais nos circuitos cerebrais.

Foi observado que o perfil de libertação de ATP em terminais nervosos é distinto quando comparado com os neurotransmissores clássicos, sendo semelhante ao perfil de libertação de peptídeos. O ATP é preferencialmente libertado de vesículas sinápticas de alta densidade (LDCV), estando essa libertação dependente da ativação de canais de Ca^{2+} tipo L que são precisamente aqueles que são recrutados durante níveis de estimulação elevados.

No hipocampo de ratinho observou-se que a adenosina proveniente do ATP e da atividade da CD73, ativa seletivamente os $A_{2A}R$ neuronais controlando assim a plasticidade sináptica. Similarmente ao que acontece com os $A_{2A}R$, também a CD73 apresentou um aumento de densidade num modelo animal da doença de Alzheimer, após a administração intracerebroventricular de $A\beta_{1-42}$, um peptídeo neurotóxico. Este aumento na densidade e atividade da CD73 parece ser fundamental para promover o prejuízo na plasticidade sináptica e na memória tendo como consequência diretas, défices sinápticos e alterações comportamentais. Consequências essas, que foram totalmente prevenidas em animais onde a CD73 foi geneticamente removida ou farmacologicamente bloqueada.

Finalmente, e face a estas observações, foi atribuído à CD73 um novo papel fisiológico. Em determinadas circunstâncias, a CD73 pode ser olhada como um novo alvo modulador do comportamento de ratinhos uma vez que foi observado pela primeira vez que, sendo a principal fonte de adenosina proveniente do ATP no cérebro, controla processos de memória relacionados com o medo.

Em suma, as nossas observações ajudam a compreender porque é que o ATP é preferencialmente libertado em circunstâncias de estimulação de alta intensidade, sustentando assim processos de plasticidade sináptica, no hipocampo de ratinho, através da sua conversão em adenosina. Estabelecemos assim um novo papel regulador da atividade da CD73, implicando-a como um novo alvo para controlar os efeitos terapêuticos dos $A_{2A}R$ na doença de Alzheimer.

ABSTRACT

Adenosine is a neuromodulator in the central nervous system (CNS) that inhibits basal synaptic transmission through the action of adenosine A_1 receptors (A_1R) under low levels of stimulation and facilitates synaptic plasticity through the activation of adenosine A_{2A} receptors ($A_{2A}R$) upon higher levels of stimulation. This results from the fact that under high stimulation levels there is a preferential release of ATP, although it is not known why this release occurs under these circumstances. In the brain, ATP is stored in synaptic vesicles and released in an activity-dependent manner. Once in the extracellular side, it is catabolised through the action of ecto-nucleotidases to its metabolites ADP, AMP and adenosine. The final step of this catabolism is performed by ecto-5'-nucleotidase (CD73), the rate-limiting player controlling the formation of ATP-derived adenosine from AMP. Although there are some evidences showing a direct relationship between CD73 function and $A_{2A}R$ activation, this link remains unclear.

We now show in which circumstances the main source of adenosine, ATP, is preferential released and why. Furthermore, we tested the physiopathological role of CD73, since there is a parallel upregulation of $A_{2A}R$ and CD73 in brain damage situations, thus suggesting a key role for ATP-derived adenosine in formatting abnormal plastic changes in brain circuits.

It was found that the pattern of ATP release from nerve terminals is different from that of classical neurotransmitters and follows a peptidergic-like pattern. ATP is mostly released from large dense core vesicles (LDCV) in a manner sensitive to L-type channels that are typically recruited at higher intensities of nerve stimulation.

In mouse hippocampus, ATP-derived adenosine resulting from CD73 activity selectively activates neuronal $A_{2A}R$ to control synaptic plasticity. Similarly to $A_{2A}R$ CD73 is up regulated in a mouse model of early Alzheimer's disease (AD) based on

the intracerebroventricular administration of the neurotoxic peptide $A\beta_{1-42}$. This increased CD73 activity appears to be critically required for the impairment of synaptic plasticity and memory with the synaptic deficits and behavioural changes being fully prevented in mice where CD73 is deleted or pharmacologically blocked.

Finally, it was detected a new physiological role for CD73. Under certain circumstances, CD73 can be looked upon as a new target to modulate mouse behaviour since it was observed for the first time that CD73, being the main source of ATP-derived adenosine in the brain, controls fear-related processes.

Overall, our observations help understanding why ATP is preferentially released under high stimulation levels, sustaining hippocampal synaptic plasticity processes through its conversion to adenosine. This establishes a new regulatory role of CD73 activity over neuronal $A_{2A}R$ and implying CD73 as a novel target for $A_{2A}R$ modulation in AD.

CHAPTER 1

Introduction

The nervous system is a huge and complex web of cells that inter-communicate between them mainly through specialized junctions called synapses. In 1911, Santiago Ramón y Cajal described these junctions, demonstrating experimentally that the relationship between nerve cells was not continuous, but contiguous. This finding supported the "neuron doctrine", as it was known at the time, and is widely considered a hallmark in the history of neuroscience.

Indeed, synapses are considered "the heart" of synaptic transmission where nervous cells (neuron cells and glia cells) interact electrically and chemically to exchange information through the release of neurotransmitters and neuromodulators. This thesis is focused on a specific form of extracellular signalling mediated by purine nucleotides and nucleosides such as adenosine-5'-triphosphate (ATP) and adenosine, involving the activation of purinergic receptors – Purinergic Signalling.

The exchange of information between neuronal cells depends on the brain region and the cell type involved and is the genesis of the complexity of the nervous system. It is in this complexity that neuroscientists have been looking not only for answers to explain cognitive and behavioural characteristics between species, but also for attempts to control some neurodegenerative disorders that are far from being fully understood.

1.1. Purinergic Signalling

Purinergic signalling is a scientific concept that refers to a form of extracellular signalling mediated by purine nucleotides and nucleosides, involving the extracellular activation of specific receptors, named purinergic receptors that regulate cellular functions (Ralevic and Burnstock, 1998). Purines (ATP, adenosine-5'-diphosphate (ADP), adenosine) and pyrimidines (uridine-5'-triphosphate (UTP),

uridine-5'-diphosphate (UDP)) are extracellular messengers widely distributed throughout the body, promoting their effects through activation of purinergic receptors (Ralevic and Burnstock, 1998).

Purinergic receptors (P1 receptors and P2 receptors) are among the most abundant receptors in living organisms and appeared early in evolution (Burnstock, 1976), implying that purines are one of the most primitive and widespread chemical messengers in the animal and plant kingdoms, being present in the living beings since very early (Burnstock and Verkhratsky, 2009). As a scientific concept this has attracted the attention of scientific community in the last 40 years. However the burst of interest in this area occurred in the last years, with the validation of some drugs used to manipulate purinergic signalling Regadenoson®, a vasodilator that is an agonist of adenosine $A_{2A}R$ which was approved by the United States Food and Drug Administration (FDA) in 2008 and is currently widely used in the field of cardiology (Chen *et al.*, 2013; Palani and Ananthasubramaniam, 2013and recently, the foundation of a scientific journal ("Purinergic Signalling" – the official journal of the) that is strictly dedicated to the divulgation of new scientific facts in this growing area.

This dissertation is focused on the role of the extracellular ATP-derived adenosine in the control of specific cellular mechanisms and its impact on physiological responses. This will follow the interest in purinergic neuromodulation that was recently bolstered by the recognition of ATP as a multi-target danger signal in the brain (Rodrigues *et al.*, 2015) and the neuroprotective effect afforded by chronic caffeine consumption in a diversity of brain disorders that are mediated by adenosine A_{2A} receptors ($A_{2A}R$) (Cunha and Agostinho, 2010).

1.1.1. Extracellular ATP neurotransmission

ATP is a multifunctional nucleotide discovered in 1929 (Lohmann, 1929). Its integration in the "purines family" is related to the presence of a purine (adenine), attached to a pentose (ribose), to which three phosphate groups are linked (Fig 1.1).

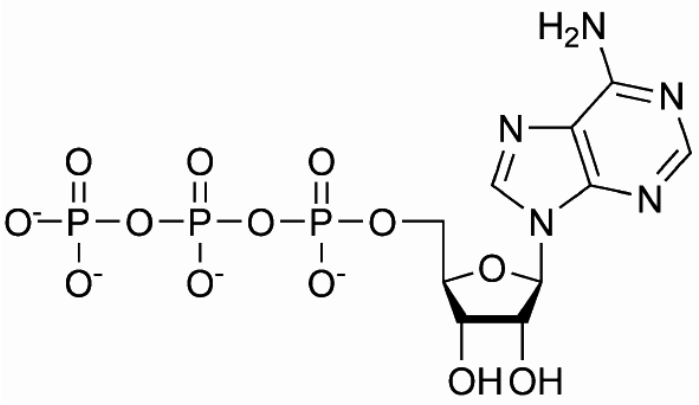

Figure 1.1: Chemical structure of adenosine 5´-triphosphate (ATP).

In 1929, Drury and Szent-Györgyi showed for the first time the extracellular actions of purine nucleotides and nucleosides in the cardiovascular system (Drury and Szent-Györgyi, 1929). However, it was only forty years later that Geoffrey Burnstock introduced the concept of "purinergic neurotransmission", in 70's, after showing that ATP was a transmitter in non-adrenergic and non-cholinergic inhibitory nerves (Burnstock, 1972), igniting decades of controversy. At the time, this proposal was provocative, mainly because ATP was known to have a canonical intracellular role in energy transfer as a metabolic source of phosphate (Lipmann, 1941). During the next 40 years Burnstock's concept was supported by the identification of mechanisms of ATP release the use of isolated CNS nerve terminalsallowed to start checking that adenine nucleotides are released upon stimulation (Kuroda and McIlwain, 1974and of ecto-enzymes metabolizing ATP, named ecto-nucleotidases (eNT).

In fact, as a chemical messenger, ATP is released from living cells via several physiological mechanisms, which include exocytosis, diffusion through membrane channels and via transporters (North and Verkhratsky, 2006; Pankratov *et al.*, 2006; Burnstock, 2007; Abbracchio *et al.*, 2009) (Fig 1.2), and is also known to be released from dying cells, being an early and universal indicator of cell damage (Burnstock, 2008; Rodrigues *et al.*, 2015).

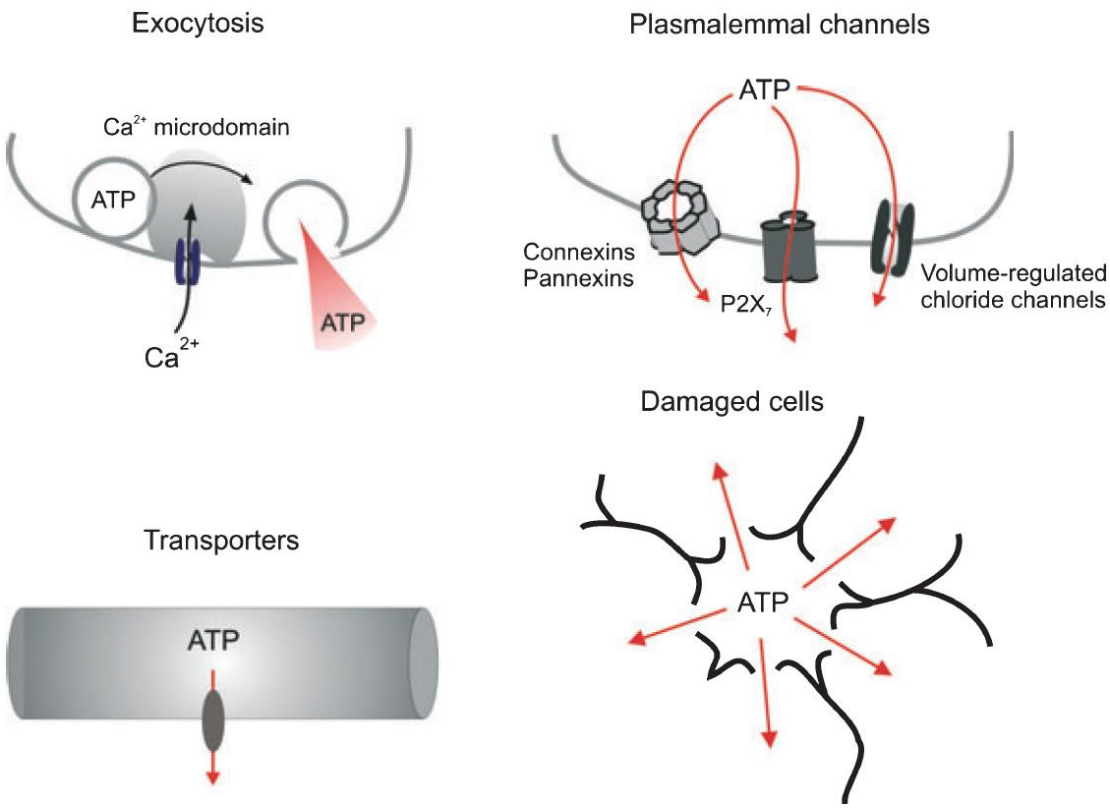

Figure 1.2: Schematic representation of pathways of ATP release from cells (copied from Burnstock and Verkhratsky, 2009)

As a neurotransmitter, ATP is accumulated in central presynaptic terminals and stored in synaptic and in astrocytic vesicles (Sperlágh *et al.*, 1998b; White, 1978; Richardson and Brown, 1987; Terrian *et al.*, 1989; Unsworth and Johnson, 1990; Zimmermann, 1994; Pankratov *et al.*, 2006; Larsson *et al.*, 2012) being released

and/or co-released via exocytosis (Pankratov *et al.*, 2006) to modulate biological processes through the direct activation of P2R (Ralevic and Burnstock, 1998). In addition, several works showing the release of ATP upon electrical stimulation from cortical (Wu and Phillis, 1978), hippocampal (Cunha *et al.*, 1996b), habenula (Sperlágh *et al.*, 1998a) and hypothalamic preparations (Sperlágh *et al.*, 1998b), as well as from other CNS cells types such as glial cells (Queiróz *et al.*, 1997) or post-synaptic structures (Hamann and Attwell, 1996), promoted ATP as the new "spotlight" within the neurotransmitters and principally within the neuromodulators.

Altogether, it is now well established that ATP acts as either sole transmitter (fast excitatory neurotransmitter), or as a co-transmitter in most nerves, both in the peripheral nervous system and CNS (Burnstock, 2007). ATP is considered since the 80's as the main adenine nucleotide released from nerve terminals upon stimulation, controlling pre- and post-synaptic responses (Potter and White, 1980).

The fact that the majority of cells are endowed with ecto-enzymes able to ATP in the extracellular space (), further highlights the role of the purinergic signalling in controlling different biological responses (among them CNS events). Furthermore, ATP is a direct source of other neuromodulators, since it is through the action of into different metabolites (ADP, adenosine-5'-monophosphate (AMP) and adenosine) that are endogenous ligands of different P2R and P1R (Fig 1.3 and Fig 1.4).

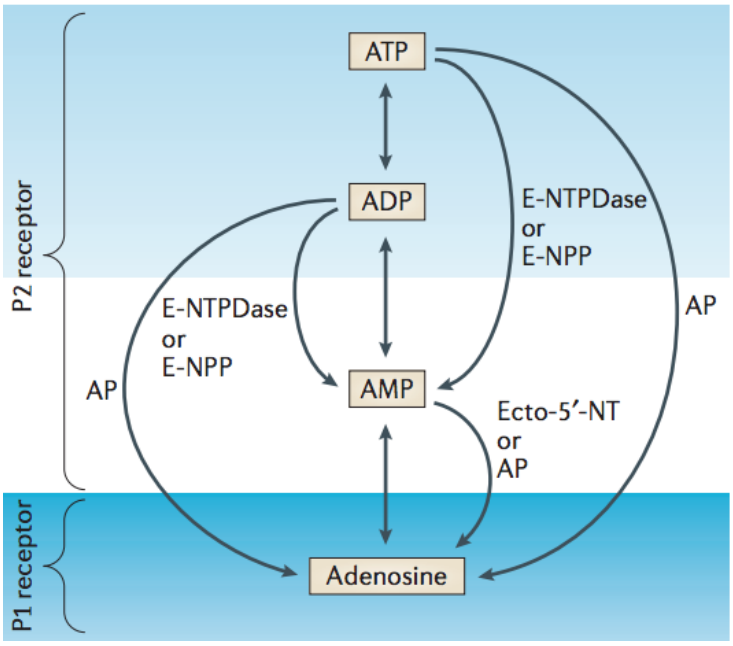

Figure 1.3: Extracellular catabolism of ATP. P2 receptors bind ATP and ADP, whereas P1 receptors bind adenosine. The extracellular ATP is regulated by several ecto-ATPases or ectonucleotidases, including members of the E-NTPDase (ectonucleoside triphosphate diphosphohydrolase) family and the E-NPP (ectonucleotide pyrophosphatase/phosphodiesterase) family. Ecto-5′-nucleotidase (ecto-5′-NT or CD73) and alkaline phosphatase (AP) catalyse the adenine nucleotides into adenosine (copied from Fields and Burnstock, 2006).

1.1.2. Adenosine neuromodulation

Adenosine is known to be a ubiquitous molecule that is directly involved in the key processes sustaining cellular viability and adaptability, namely the energy charge (ATP/ADP), redox control (nicotinamide adenine nucleotide (NADH)), deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)(ribose composed by adenosine) and epigenetic control (S-adenosyl-L-homocysteine (SAH)).

As a purine nucleoside, adenosine exists in all cells because all cells have an intracellular metabolism based on adenosine. Thus, adenosine is widely available to be used in a controlled manner as an extracellular signal. Indeed, all (known) mammalian cells in different tissues use adenosine as a paracrine signal to coordinate their cellular activities to produce an adequate output of different organs and systems such as the kidney, liver, lung, or inflammatory system to name a few.

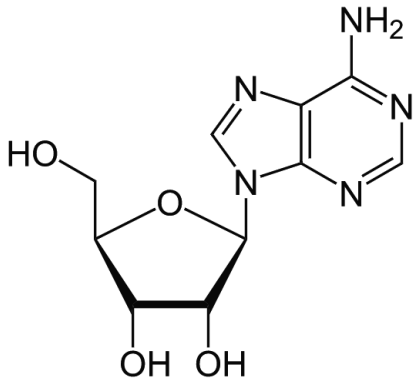

Figure 1.4: Chemical structure of adenosine.

Adenosine can exist either intra- or extracellularly and it is continuously produced under physiological conditions (Fredholm *et al.*, 2001). Adenosine can cross the plasma membrane through selective transporters responsible for equilibrating the levels of adenosine inside and outside of the cell (Choi *et al.*, 2004); alternatively adenosine can be produced extracellularly from membrane–anchored enzymes that catabolise AMP into adenosine (CD73) (Schubert *et al.*, 1979; Zimmermann *et al.*, 1998).

The particular interest for the role of adenosine in the brain arises from the fact that the sensors for adenosine – adenosine receptors – are far more abundant in the brain than in any other organ or cell type in mammals (Cunha *et al.*, 2008). In the CNS, adenosine can act as a neuromodulator or as a homoeostatic regulator (Sebastião and Ribeiro, 2000; Cunha *et al.*, 2008), controlling neurotransmitter

release and neuronal excitability, as well as information flow through neuronal circuits. Traditionally, adenosine is considered an inhibitory neuromodulator responsible for a feedback decrease in the activity of excitatory synapses (Dunwiddie and Masino, 2001).

Known as a prototypical neuromodulator, adenosine acts through a concerted action on facilitatory and inhibitory A_1 receptors (A_1R) (Cunha, 2005; Gomes *et al.*, 2011). It is also an endogenous ligand for adenosine A_{2B} receptors ($A_{2B}R$) and adenosine A_3 receptors (A_3R) (Fredholm *et al.*, 2011) although at this time it is mostly considered that A_1R and $A_{2A}R$ are the main adenosine receptors responsible for the effects of adenosine in the brain (Fredholm *et al.*, 2005).

Through the activation of its G-protein coupled receptors, adenosine is involved in diverse physiological and pathological processes in the brain parenchyma: 1- typically, as a synaptic neuromodulator, adenosine contributes to sharp information salience in neuronal circuits (Cunha *et al.*, 2008), which is probably its best established role in the brain; 2- as an astrocytic modulator, adenosine controls metabolism (Håberg *et al.*, 2000; Blood *et al.*, 2003; Lemos *et al.*, 2015; Duarte *et al.*, 2016), calcium waves (Kawamura and Kawamura, 2011; Kanno and Nishizaki, 2012) and the ability of astrocytes to uptake neurotransmitters (Nishizaki *et al.*, 2002; Matos *et al.*, 2012; Cristóvão-Ferreira *et al.*, 2013); 3- as an astrocyte-to-neuron signal, adenosine links astrocytic activation to heterosynaptic depression (Manzoni *et al.*, 1994; Serrano *et al.*, 2006; Andersson *et al.*, 2007); 4- as a controller of oligodendrocyte differentiation and functions (Rivkees and Wendler, 2011; Coppi *et al.*, 2015); 5- as a microglia modulator, controlling proliferation (Haselkorn *et al.*, 2010; Gomes *et al.*, 2013; George *et al.*, 2015), motility (Orr *et al.*, 2009; Choi *et al.*, 2004; Ohsawa *et al.*, 2012) and reactivity (Saura *et al.*, 2005; Dai *et al.*, 2010; Rebola

et al., 2011; Madeira *et al.*, 2015; Newell *et al.*, 2015); 6- as a microglia-to-neuron signal, controlling short-term synaptic plasticity upon microglia activation (Lauro *et al.*, 2010; George *et al.*, 2016); 7- as an endothelial modulator, controlling the vasodilation of brain capillaries (O'Regan, 2005; Ohata *et al.*, 2006; Kusano *et al.*, 2010; Paisansathan *et al.*, 2010; McClure *et al.*, 2011), contributing to the fine-tuning of the neurovascular coupling that critically impacts on the sustain ability of function of neuronal networks (Chen *et al.*, 2013; Kimbrough *et al.*, 2015).

All these adenosine functions have a well described physiological impact, such as the regulation of sleep, general arousal state and activity, local neuronal excitability, and coupling of the cerebral blood flow to the energy demand (Cunha, 2016). Moreover, manipulation of adenosine signalling may have therapeutic potential in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease, and in psychiatric diseases such as schizophrenia and autism (Ribeiro *et al.*, 2002). Therefore, alterations in the adenosine concentration could have dramatic effects in function and behaviour of the whole organism (Dunwiddie and Masino, 2001).

1.1.3. Purinergic Receptors

Purinergic receptors (Fig 1.4) are a family of plasma membrane receptors present in almost all mammalian tissues. Within the field of purinergic signalling they are the means to achieve an end. They are the tools through which nucleotides and nucleosides exert their physiological functions (Burnstock and Verkhratsky, 2009). However, not all of these functions have been fully characterized and the effect of the

extracellular microenvironment on their functions is also still incompletely understood.

The term purinergic receptor (or purinoceptor) was first introduced to describe classes of membrane receptors that, when activated by either neuronal or astrocytic released ATP (P2 purinoceptor) or its breakdown product adenosine (P1 purinoceptor), mediate relaxation of gut smooth muscle (Burnstock, 1978).

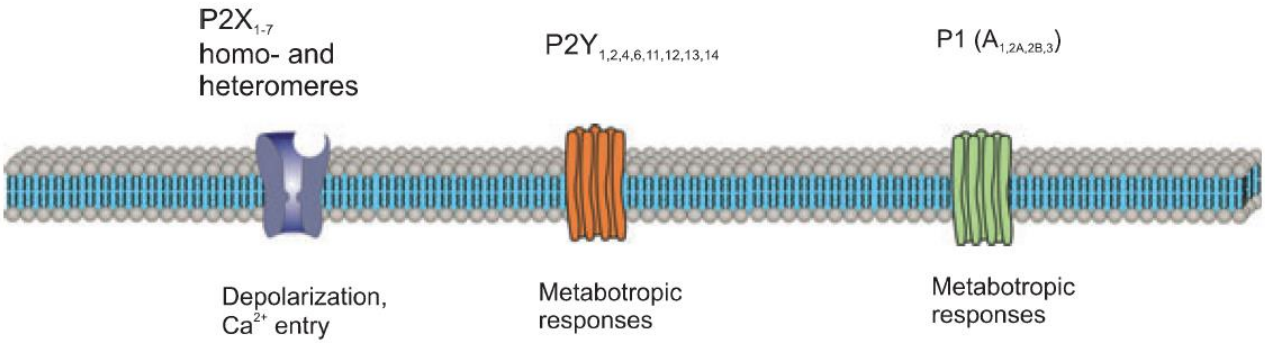

Figure 1.4: Schematic representation of (copied from Burnstock and Verkhratsky, 2009)

In the extracellular space, ATP and its metabolites can produce their biological effects through the activation of P2 receptors (ATP and ADP), or through the activation of P1 receptors (adenosine). This division in P1 and P2 receptors was in part based on the relative potencies and affinities of ATP, ADP, AMP and adenosine, but mainly by the selective antagonism of the effects of adenosine by methylxanthines, such as theophylline or caffeine (Sattin and Rall, 1970). Currently, this major division remains a fundamental part of purine receptor classification, although P1 and P2 receptors are now characterised primarily according to their distinct molecular structures supported by evidence of different effector systems and pharmacological profiles (Ralevic and Burnstock, 1998).

1.1.3.1. Purinergic eceptors type I P1

Adenosine receptors are far more abundant in the brain than in any other organ or cell type in mammals (Cunha, 2008). In the brain, it is mostly A_1R and $A_{2A}R$ that are responsible for the effects of adenosine (Fredholm *et al.*, 2005). The existence of A_1R and A_2R was first described in 1979 (van Calker *et al.*, 1979), showing that the activation of these receptors by adenosine inhibited, via A_1R, or facilitated, via A_2R, adenylate cyclase activity.

All adenosine receptors couple to G proteins and, in common with other G protein-coupled receptors, they have seven transmembrane domains, the N-terminal lying on the extracellular side and the C-terminal on the cytoplasmic side of the membrane. With respect to signal transduction, adenosine receptors have been traditionally divided into two broad groups: A_1R and A_3R, negatively coupled to adenylate cyclase via G_i protein; and $A_{2A}R$ and $A_{2B}R$, positively coupled to adenylate cyclase via G_s protein (Fredholm *et al.*, 2000). However, all adenosine receptors have been shown to couple to different G proteins, operating via different signal transducing pathways (Cunha, 2005). The existence of selective pharmacological tools, as well as the development of selective knock out (KO) mice for each receptor has greatly helped to improve not only the knowledge on adenosine receptor function, but also to determine the physiological and pathophysiological roles associated to adenosine receptors (Fredholm *et al.*, 2000; Fredholm *et al.*, 2003).

P1 receptors have been implicated in several biological functions, both physiological and pathological (Fredholm *et al.*, 2001; Fredholm *et al.*, 2011). These include cardiac rhythm and circulation (Eltzschig *et al.*, 2012; Eltzschig, 2009), lipolysis (Johansson *et al.*, 2007), renal blood flow (Grenz *et al.*, 2012; Sun *et al.*, 2001), immune function (Rosenberger *et al.*, 2009), sleep regulation (Huang *et al.*,

2005; Lazarus *et al.*, 2011) and angiogenesis (Liu *et al.*, 2010), as well as inflammatory diseases (Haskó *et al.*, 2008; Eltzschig and Eckle, 2011), ischaemia–reperfusion (Eltzschig and Eckle, 2011) and neurodegenerative disorders (Fredholm, 2007).

A_1R have a widespread distribution, being the second most abundant metabotropic receptor in the brain, after the cannabinoid CB_1 receptor. Similarly to $A_{2A}R$, A_1R are mostly located in synapses (Tetzlaff *et al.*, 1987; Rebola *et al.*, 2003; Rebola *et al.*, 2005b), in particular in excitatory (glutamatergic) synapses (Rebola *et al.*, 2005b) although both receptors are also present in GABAergic (Cunha and Ribeiro, 2000; Shindou *et al.*, 2002; Rombo *et al.*, 2015), cholinergic (Cunha *et al.*, 1995; Rodrigues *et al.*, 2008), dopaminergic (Borycz *et al.*, 2007; Garção *et al.*, 2013; Pandolfo *et al.*, 2013) serotoninergic (Barraco *et al.*, 1996; Okada *et al.*, 1999) or noradrenergic synapses (Jackisch *et al.*, 1985; Barraco *et al.*, 1995). In excitatory synapses (glutamatergic), under conditions of basal synaptic transmission (low frequency stimulation levels), it is mainly through A_1R that adenosine exerts its role. Briefly, adenosine activates presynaptic (Thompson *et al.*, 1992) A_1R to inhibit basal synaptic transmission (Dunwiddie and Masino, 2001) working as gatekeepers of excessive excitatory transmission since synaptic adenosine levels are increased even by a single pulse of stimulation (Mitchell *et al.*, 1993).

Being less abundant than A_1R, $A_{2A}R$ are more abundant in the basal ganglia and the striatum (Fredholm *et al.*, 2005). $A_{2A}R$ contrast with A_1R and are not engaged under basal conditions, being only recruited upon higher frequencies of nerve stimulation, triggering and sustaining plastic changes of synaptic efficiency (*e.g.* (Rebola *et al.*, 2008; Costenla *et al.*, 2011). $A_{2A}R$ have attracted recent attention because of their robust impact on inflammation and vascular reactivity in the

periphery and on synaptic plasticity and neurodegeneration in the brain (Cunha, 2005; Gomes *et al.*, 2011). Accordingly, $A_{2A}R$ agonists are potent anti-inflammatory compounds and $A_{2A}R$ antagonists are looked upon as novel brain neuroprotective drugs (Cunha, 2005 and Gomes *et al.*, 2011).

$A_{2B}R$ are also expressed in the brain, mainly in neurons and glial cells (Fredholm *et al.*, 2005). However, $A_{2B}R$ function in the brain is largely unknown. Recently, it was described that, in mouse hippocampus, $A_{2B}R$ control A_1R -mediated inhibition of synaptic transmission (Gonçalves *et al.*, 2015).

A_3R are expressed in low levels in the brain (Dixon *et al.*, 1996) and, like $A_{2B}R$, their role is not well described.

1.1.3.2. Purinergic eceptors type II P2R

Although this dissertation is focus more on the role of P1 receptors in controlling plasticity phenomena, it is important to highlight the presence of P2 receptors in the CNS.

In the CNS, we can find two main classes of P2 receptors, (Table I), P2X and P2Y receptors, a nomenclature originally used in a subdivision of P2 receptors based on pharmacological criteria (Burnstock and Kennedy, 1985). P2X receptors are ligand-gated cation channels and P2Y receptors are metabotropic receptors, belonging to the superfamily of G protein-coupled receptors with seven transmembrane domains (Abbracchio and Burnstock, 1994; Fredholm *et al.*, 1994). Briefly, P2X receptors allow the passage of cations, while the P2Y receptors, via G proteins, activate second messengers triggering transduction systems such as phospholipase C (PLC) and phospholipase A_2 (PLA_2).

Through the activation of P2X receptors, which can be constituted of seven different subtypes, ATP mediates rapid (< 10 ms) opening of a nonselective cation pore, with almost equal permeability to Na^+ and K^+ and significant permeability to Ca^{2+} (North, 2002).

P2Y metabotropic receptors are divided in 8 receptor subtypes ($P2Y_{1,2,4,6,11,12,13,14}$) (Abbracchio and Verderio, 2006). P2Y receptor subtypes show a low level of sequence homology between them (19-55% identical) and, consequently, not only show significant differences in the signal transduction mechanisms through which they operate, but also in their pharmacological profiles (Abbracchio and Verderio, 2006). P2Y receptors binds to a single heterotrimeric G protein, tipically $G_{q/11}$, although $P2Y_{11}$ can couple to both $G_{q/11}$ and G_s, whereas $P2Y_{12,13,14}$ couple to G_i solely. $P2Y_{1,2,4,6,11}$ receptors couple to members of the $G_{q/11}$ family and stimulate phospholipase C (PLC) resulting in the formation of inositol-(1,4,5)- triphosphate (IP3) and diacylglycerol, with subsequent mobilization of Ca^{2+} from intracellular stores (Abbracchio and Verderio, 2006). In addition, it has been reported that native P2Y receptors may activate phospholipases A2 and D, mitogen-activated protein kinases (MAPK), tyrosine kinase and the serine-threonine kinase Akt (Lazarowski *et al.*, 2003). In contrast to P2X receptors, P2Y receptors not only bind to ATP, but ADP (Webb *et al.*, 1993; Communi *et al.*, 2001; Hollopeter *et al.*, 2001; Lazarowski *et al.*, 2003) and the pyrimidine nucleotides UTP (Lustig *et al.*, 1993; Bogdanov *et al.*, 1998), UDP (Chang *et al.*, 1995) and UDP-glucose (Abbracchio *et al.*, 2003) are also endogenous ligands of some P2Y receptors.

1.1.4. Sources of Adenosine

Adenosine causes different actions by activating different receptors inhibiting basal synaptic transmission through the activation of A_1R and facilitating synaptic plasticity through the activation of $A_{2A}R$ (Cunha, 2005). The conditions of activation of different adenosine receptors vary with the needs of the system (Cunha, 2005). It is described that A_1R and $A_{2A}R$ have similar affinities to adenosine (20 nM) (Fredholm *et al.*, 2005) suggesting that there might be different ways of generating adenosine to control the activation of these receptors (Cunha, 2001).

In the CNS, adenosine exists intracellularly in concentrations of *circa* 10-50 nM (Cunha, 2001) and extracellularly concentrations with an average of *circa* 200 nM and due to the role that it plays in most of the physiological processes, it is continuously produced (Fredholm *et al.*, 2001).

Intracellular adenosine can be formed by the action of nucleotidases (AMP degradation) (Schubert *et al.*, 1979; Zimmermann *et al.*, 1998) or be formed SAH hydrolysis (Broch and Ueland, 1980) via a reversible reaction catalysed by SAH hydrolase (E.C.3.3.1.1). This pathway is limited by the availability of L-homocysteine, which has very low concentration in brain tissue (Reddington and Pusch, 1983; Hack and Christie, 2003).

Extracellular adenosine can also have different sources: it can be originated from ATP through the action of ecto-nucleotidases (Zimmermann, 2000) or from cyclic adenosine-5'-monophosphate (cAMP) (Rosenberg and Li, 1995) although this pathway has been found to be of minor importance (Brundege *et al.*, 1997).

Intra- and extracellular levels of adenosine are also regulated by equilibrative nucleoside transporters (ENTENT1 and ENT2) (Dunwiddie and Masino, 2001).

Depending of the intra- and extracellular adenosine concentration ENT fulfil a dual role, since the blockade of adenosine transport can inhibit either adenosine release or adenosine uptake (Gu *et al.,* 1995). The importance of ENT1 in regulating adenosine levels was demonstrated in KO mice lacking this protein (Choi *et al.*, 2004).

Finally, some groups have suggested that adenosine can be released *per se* (Frenguelli *et al.,* 2007; Klyuch *et al.*, 2012), through mechanisms independent of ENT activity (Sperlágh *et al.*, 2003) and blocked by bafilomycin and modulated by metabotropic glutamate type 4 receptors (mGluR4) activation (Klyuch *et al.*, 2012). However, there is still some controversy about this question.

There are two enzymes that constitute the major pathways of adenosine removal: adenosine kinase (AK, EC 2.7.1.20) and adenosine deaminase (ADA, EC 3.5.4.4). Intracellular adenosine can be converted to AMP by phosphorylation by AK or degraded to inosine by ADA (Arch and Newsholme, 1978; Lloyd and Fredholm, 1995; Svenningsson *et al.*, 1999). AK is important at low levels of intracellular adenosine, with ADA coming into play only when large amounts of adenosine have to be cleared (Fredholm *et al.,* 2005). AK inhibitors are able to increase extracellular adenosine levels under physiological conditions, whereas ADA inhibitors are able to increase the extracellular adenosine levels during metabolic insults (Sciotti and Van Wylen, 1993; Lloyd and Fredholm, 1995).

1.1.5. Ecto-nucleotidases

By metabolising extracellular ATP into adenosine, ecto-nucleotidases play a double role in purinergic signalling: on one hand they terminate the signalling role of

extracellular ATP while, at the same time, they constitute a metabolic source of adenosine, formed through the selective action of ecto-5'-nucleotidase (e5'NT or CD73) (Cunha, 2008). In fact these enzymes are able to catabolise nucleotides with the particularity of being anchored to the cellular membranes. Their membrane localization/organization allow a cascade-like of (Zimmermann, 2000).

Originally it was assumed that single and defined enzymes existed for the hydrolysis of either ATP (ecto-ATPase), ADP (ecto-ADPase), or also ATP and ADP (ecto-ATP diphosphohydrolase, ecto-apyrase). Instead, and although this type of nomenclature is still in the current literature, this simplistic view had to be revised after the molecular and functional characterization of several novel enzyme families with overlapping substrate specificities and tissue distributions (Matsuoka and Ohkubo, 2004; Langer *et al.*, 2008).

The currently known ecto-nucleotidases are ecto-nucleoside triphosphate diphosphohydrolases (NTPDases), ectopyrophosphatase/phosphodiesterases (NPPs), ecto-5'-nucleotidase (CD73) and ecto-alkaline phosphatase (AP) (Table I).

Ecto-enzymes (alternative name)*	Catalyzing reaction#
NTPDase1 (ecto-apyrase, CD39)	ATP→ADP→AMP
NTPDase2 (ecto-ATPase, CD39L1)	ATP→ADP
NTPDase3 (HB6, CD39L3)	ATP→ADP→AMP
NPP1 (PC-1)	ATP→AMP
NPP2 (autotaxin, PD-Iα)	ATP→AMP (LPC→LPA)
NPP3 (gp130^{RB13-6}, B10, PD-Iβ)	ATP→AMP
Ecto-5'-nucleotidase (CD73)	AMP→Adenosine
Ecto-alkaline phosphatase	ATP→ADP→AMP→Adenosine

Table I: Ectonucleotidases and extracellular ATP catabolism (copied from Matsuoka and Ohkubo, 2004)

Among the NTPDase family, NTPDase 1–3 are plasma membrane bound enzymes. NTPDase1 (ecto-apyrase, CD39) hydrolyses ATP and ADP with similar rates, whereas NTPDase2 (ecto-ATPase or CD39L1) hydrolyses ATP with high selectivity over ADP. NTPDase3 (CD39L3 or HB6) hydrolyses ATP with some selectivity over ADP. Other NTPDase members (NTPDase4–6) are less selective for adenine nucleotides and located in intracellular organelles, such as the Golgi apparatus (NTPDase4), or secreted as soluble enzymes (NTPDase5, 6). The E-NPP family is composed of NPP1 (PC-1), NPP2 (autotaxin or PD-I, recently identified as lysophospholipase D to hydrolyse lysophosphatidylcholine into lysophosphatidic acid); and NPP3 (gp130^{RB13-6}, B10, or PD-Iβ). These enzymes hydrolyse ATP directly to AMP. CD73 considered to play a principal role in conversion of AMP into adenosine. Ecto-AP is also able to hydrolyse AMP to adenosine, but less attention has been paid to the role of this enzyme in the ATP-induced response and extracellular nucleotide metabolism (Matsuoka and Ohkubo, 2004). Besides the ATP metabolism promoted by ecto-nucleotidases, ATP can also be metabolied by other nucleotidases that can be in the cytosol.

The physiological function of nucleotides and of their metabolites displays great variance between individual tissues. the important cellular responses attributed to the action of these extracellular nucleotides are blood clotting, inflammation, immune reactions, pain perception, smooth muscle contraction, cell proliferation, synaptic activity and cancer. Inhibition of the catabolism of extracellular nucleotides makes it possible to interfere with nucleotide signalling pathway and therefore provides a means by which these cellular responses can be modulated. Consequently, the functional implication of the ectonucleotidase pathway can be expected to vary with the physiological mechanism it is governing and, therefore, selective

ectonucleotidase inhibitors are highly sought after as therapeutic agents and have much anticipated therapeutic applications.

An understanding of how these ectonucleotidases are involved in regulating cellular responses is crucial for the development of potent and selective ectonucleotidase inhibitors. A few examples have come to light, which highlight the role of these enzymes in tissue function.

1.1.5.1. CD73 – A source of extracellular adenosine

Ecto-5′-nucleotidase (ecto-5′-NT, eN, CD73, EC 3.1.3.5) is an ecto-nucleotidase that dephosphorylates extracellular nucleotides and is a member of a large superfamily of metallophosphatases that contain a dinuclear metal centre at the C-terminal end of two sandwiched babab-motifs (Fig 1.5). This superfamily comprises phosphatases with remarkably diverse substrate specificities, including protein phosphatases, nucleotidases, and nucleases (Zimmermann, 1992).

CD73 is one of the several enzymes showing 5'-nucleotidase activity and is a Zn^{2+}-binding glycosylphosphatidylinositol- (GPI-) anchored protein, with its catalytic domain facing the extracellular medium. It can be cleaved off the anchor and released as a soluble protein (Sträter, 2006) (Fig 1.5).

CD73 hydrolyses ribonucleoside monophosphates having higher affinity to 5'-, and not to 2'- or 3'-monophophates. 5'-AMP is assumed to be the best substrate, with K_M values around 3-6 μM (Burger and Lowenstein, 1975; Naito and Lowenstein, 1981).

In vertebrates, CD73 emerges as the major control point for extracellular adenosine levels (Knöfel and Sträter, 2001) catalysing the hydrolysis of extracellular AMP to adenosine.

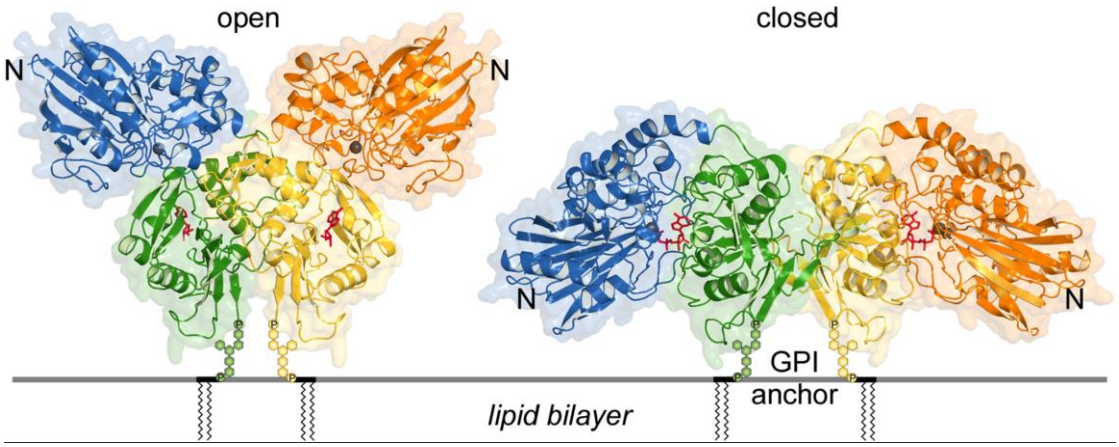

Figure 1.5: Human CD73 in an open conformation (crystal form I), and a closed conformation (crystal form II). The N- and C-terminal domains of one subunit of the dimer are shown in blue and green, respectively. The N- and C-terminal domains of the adjacent subunit are shown in orange and yellow, respectively. Ligands are shown as red sticks while the metal ions (Zn^{2+}) are shown as grey spheres. Schematic representations of GPI anchors and the cell membrane are also shown (copied from Knapp *et al.*, 2012).

As one of the main regulators of extracellular adenosine levels, CD73 manipulation has emerged as a fundamental tool for the interpretation of the physiological and pathological functions of the purinergic system/signalling, not only in CNS but also in peripheric systems. It is possible to find in the literature at least three ways to direct manipulate CD73 and consequently manipulate the pathway that converts ATP into adenosine. The first one is the use of CD73 inhibitors where it should be pointed out the inhibitor (AOPCP (Fig 1.6) (Bhattarai *et al.*, 2015) that is a stable analogue of ADP and is one of the most potent competitive inhibitors of CD73 known to date (Freundlieb *et al.*, 2014). ADP and ATP are also competitive inhibitors of the enzyme, with inhibition constants in the micromolar range (Grondal and Zimmermann, 1987; Zimmermann, 1992; Zimmermann *et al.*, 2012). However, these

inhibitors can also be subjected to enzymatic degradation by NTPDase, NPP or (Yegutkin *et al.*, 2011; Yegutkin, 2014). In addition to this nucleotide analogue (AOPCP), anthraquinones (Baqi *et al.*, 2010), sulphonamides (Ripphausen *et al.*, 2012), various polyphenols (Braganhol *et al.*, 2007) and some polyoxometalates (POMs) (Lee *et al.*, 2015) are currently known to potently inhibit CD73. Anthraquinones and sulfonamides were found to display a competitive mechanism of inhibition whereas polyphenols and POMs were shown to be non-competitive inhibitors.

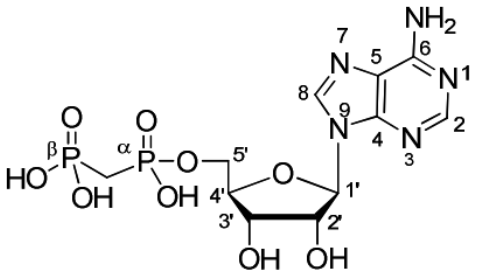

Figure 1.6: Chemical structure of α,β-methylene-ADP (AOPCP)

Besides the pharmacological tools, it is also possible to manipulate CD73 using genetic tools. At this moment there is a well-characterized CD73 KO mouse model (Thompson *et al.*, 2004) and another genetic tool that consists in the use of lentivirus (LV)-mediated small hairpin RNA (*sh*RNA) to downregulate CD73 gene expression (Ena *et al.*, 2013).

1.1.5.1.1 CD73 in peripheral systems (outside the CNS)

Although we focused on the role of CD73 in controlling physiological actions in the CNS, its physiological role is better documented in peripheral systems, where it became an appealing drug target with potential applications in the treatment of

inflammation (Ohtsuka *et al.*, 2010), chronic pain (Sowa *et al.*, 2010), and hypoxia (Thompson *et al.*, 2004; Li *et al.*, 2006).

In fact, this enzyme is also used as a marker of lymphocyte differentiation (T-Cell and B-Cell) and has been widely considered pivotal in the generation of immunosuppressive microenvironments through adenosine production (Antonioli *et al.*, 2013; de Oliveira Bravo *et al.*, 2016) a deficiency of CD73 occurs in a variety of immunodeficiency diseases.

CD73 activity contributes to decrease mortality and organ injury during sepsis episodes (Haskó *et al.*, 2011), and modulates the innate immune response to influenza infection, but is not required for development of influenza-induced acute lung injury (Aeffner *et al.*, 2015). Another evidence showing a key role of CD73 in non-CNS disorders is the fact that the hydrolytic cascade from extracellular ATP to adenosine has been considered one of the most important immunosuppressive regulatory pathways in the tumour microenvironment (Sitkovsky and Ohta, 2013). That is why CD73 has been implicated in the promotion and metastasis of cancer (Stagg and Smyth, 2010; Salmi and Jalkanen, 2012; Stagg, 2012; Zhang, 2012). CD73, which is overexpressed in a multitude of cancer cell types, impairs adaptive antitumor immune responses, and enhances tumour growth and metastasis (Niemelä *et al.*, 2004; Sadej *et al.*, 2006; Braganhol *et al.*, 2007; Zhang, 2010; Zhang, 2012). Consequently, control CD73 activity emerged during the last years as a possible an effective adjuvant therapy against tumour growth. ecreasing CD73 activity with monoclonal antibodies, siRNA, and small molecule inhibitors including AOPCP, attenuates the growth and metastasis of tumours (Zhi *et al.*, 2007; Zhi *et al.*, 2010; Zhou *et al.*, 2007; Stagg and Smyth, 2010). CD73-deficient mice also showed a resistance to tumour growth (Wang *et al.*, 2011; Stagg, 2012; Yegutkin *et al.*, 2011) and it has been established

that these effects are largely mediated by the decrease of adenosine production in these animals (Jin *et al.*, 2010; Stagg, 2012; Yegutkin *et al.*, 2011). These evidences suggest that targeting tumour-derived CD73 may constitute, alone or in combination with other therapeutic strategies, a new way to fight tumour progression (Jin *et al.*, 2010; Stagg and Smyth, 2010; Yegutkin *et al.*, 2011; Stagg, 2012).

CD73 has also been looked as a novel regulator of carotid body sensory function and therefore, it has been suggested that CD73 may offer a new target for reducing carotid body activity in selected cardiovascular diseases (Holmes *et al.*, 2017).

Finally, CD73 is considered essential in the maintenance of the circulatory system homoeostasis. It is very well described that in the heart, adenosine acts on multiple cells (Peart and Headrick, 2007 and Donato and Gelpi, 2003), playing a role in ischaemic preconditioning (Baxter *et al.*, 1994). There are recent evidences showing that the pharmacological activation of adenosine receptors or the treatment with inhibitors of the adenosine uptake (dipyridamole, that increases endogenous adenosine levels) can attenuate infarct-induced left ventricular remodelling (Wakeno *et al.*, 2006), protect against heart failure induced by pressure overload, attenuate hypertrophy, fibrosis, and heart failure (Liao *et al.*, 2003; Chung *et al.*, 1998). CD73 activity accounts for approximately 46% of total adenosine production in rat heart homogenates (Darvish *et al.*, 1996), whereas ATP-derived adenosine production is critical to ischaemic preconditioning, (Kitakaze *et al.*, 1995; Eckle *et al.*, 2007) implying CD73 on the contribution to extracellular myocardial adenosine production in pathological conditions. CD73 activity and endogenous extracellular adenosine also play a significant role in the protection against systolic overload–induced ventricular hypertrophy, fibrosis, and congestive heart failure and in the regulation of

pressure overload–induced ventricular remodelling (Xu *et al.*, 2008). As a consequence, the genetic deletion of CD73 (CD73 KO) (Thompson *et al.*, 2004) exacerbates systolic overload–induced ventricular hypertrophy, fibrosis, and dysfunction (Xu *et al.*, 2008). This indicates that increasing extracellular adenosine production or activation of specific adenosine receptors may be a therapeutic approach for treating the pressure-overloaded heart.

1.1.5.1.2 CD73 in CNS – ATP-derived adenosine source

Ecto-nucleotidases are widely expressed in the CNS. In the last years, physiological functions, including modulation of i) synaptic transmission, ii) ATP-mediated propagation of glial Ca^{2+} waves, iii) microglial function iv) adult neurogenesis, or the control of vascular tone, haemostasis and thromboregulation were proposed to be related with ecto-nucleotidases distribution and activity (Zimmermann, 2006).

Within these ecto-nucleotidases, CD73 is a key regulator of the extracellular nucleotide breakdown also in CNS. ATP is a neuron-glia signalling molecule and also plays a role as a danger signal controlling neuronal plasticity and phenotypic changes of astrocytes and microglia (Fields and Burnstock, 2006).

The distribution and characterization of CD73 in the CNS are ill defined due to the lack of tools to perform the studies. Recently, the generation of inhibitors, antibodies and the creation of a CD73 KO animal provided important breakthroughs in the characterization of the role of CD73 in the CNS.

The first reference that is easily found in the literature regarding the presence of CD73 in CNS appeared in 70's when CD73 was described to be present, as a plasma membrane enzyme, in CNS-derived cells (mainly astrocyte-derived cells)

(Trams *et al.*, 1976). In fact, CD73 was since the 80s classically know as marker of myelin (Cammer and Tansey, 1986; Kreutzberg and Barron, 1978), astrocytes and of activated microglial cells (Kreutzberg and Barron, 1978; Kreutzberg *et al.*, 1978; Kreutzberg and Hussain, 1982; Gehrmann *et al.*, 1991) in the mature nervous system.

However this pre-established idea was refuted when some studies showed the presence of CD73 in non-glial cells in the brain (Schoen *et al.*, 1999). Nowadays it is well established that CD73 (and ecto-nucleotidases generically) is present in different types of cells in CNS and is widely spread among diverse brain regions (Table II).

Within brain regions, CD73 is highly abundant in the basal ganglia (striatum) (Augusto *et al.*, 2013) when compared with other regions such as hippocampus or prefrontal cortex. It is expressed predominantly in the postsynaptic side in striatopallidal neurons (Ena *et al.*, 2013), controlling striatum-dependent learning processes.

Due to the lower expression of CD73 in other brain regions apart from the striatum, its role in controlling synaptic processes (*e.g.* synaptic plasticity) not yet fully revealed. However its activity was already noticed in other structures such as hippocampus (hippocampal synaptosomes), showing a greater contribution (together with others ecto-nucleotidase) for the formation of extracellular adenosine in nerve terminals (2000; Cunha *et al.*, 2001. Furthermore, some studies showed that ATP has to be extracellularly converted into adenosine to exert its inhibitory effects on synaptic transmission in the hippocampus, making ecto-nucleotidases, and in particular CD73, key players in purinergic signalling (Cunha *et al.*, 1998).

Recently, CD73 was also associated brain disorders. CD73 was found to be up regulated (as $A_{2A}R$) in hippocampal astrocytes of human patients with mesial temporal lobe epilepsy (MTLE) (Barros-Barbosa *et al.*, 2016). Hippocampal

astrogliosis observed in MTLE patients was accompanied by a proportionate increase in $A_{2A}R$ and CD73 immunoreactivities. It was consequently hypothesized that selective blockade of excessive activation of astrocytic $A_{2A}R$ and/or inhibition of surplus adenosine formation by membrane-bound CD73 may reduce neuronal excitability, thus providing a novel therapeutic target for drug-refractory seizures in MTLE patients (Barros-Barbosa *et al.*, 2016).

Finally, it is also important to mention the presence of CD73 in spinal cord, mainly because it is described to be involved in pain-related processes. In fact, CD73, together with TNAP and PAP , is responsible for the metabolism of extracellular metabolites in the somatosensory system (Street *et al.*, 2013), hydrolysing extracellular AMP to adenosine in dorsal root ganglia (DRG) neurons and in the dorsal spinal cord. ATP and its metabolites, including adenosine, are important pain mediators and modulators in the sensory nervous system (Hamilton and McMahon, 2000; Burnstock, 2009) and consequently activation of different purinergic receptors have multiple impacts in nociception. For instance, ATP usually activates P2X receptors to induce pain in peripheral nerves (Wirkner *et al.*, 2007) and also to sensitize in neuropathic pain (Fabbretti, 2013), while adenosine usually generates analgesia via activation of P1 receptors (Sawynok and Liu, 2003). Two types of ecto-nucleotidases (CD73 and PAP) have been identified as responsible for extracellular AMP hydrolysis in the spinal cord (Street and Zylka, 2011). Knockout of CD73 and/or PAP reduced adenosine generation and enhanced nociception in animal models following inflammation and nerve injury (Sowa *et al.*, 2010). These results indicate that endogenous adenosine plays a crucial role in hampering the pain signal transduction and transmission in nociceptive circuits (enriched with nociceptors that are a type of receptor at the end of a sensory neuron's axon that responds to damaging

or potentially damaging stimuli by sending pain signals to the spinal cord and the brain in a process is called nociception). It also suggests that manipulating extracellular AMP hydrolysis would provide an alternative mechanism to control the pain (Zylka, 2011). Recently CD73 was demonstrated to control extracellular adenosine generation in the trigeminal nociceptive nerves mitigating orofacial pain. Thus, potential drugs that alter this catabolic machinery of ATP to adenosine will provide an exciting opportunity for improved therapeutic management of dental orofacial pain (Liu *et al.*, 2017).

Brain region	5′-Nuc	ATP E-NTPDase	ADP E-NTPDase	TNAP	E-NPPase
Olfactory bulb: external plexiform layer	++	+	+	++	±
Olfactory bulb: internal plexiform layer	++	+	+	+	±
Rostral migratory stream	–	++[b]	+[b]	++	±
Olfactory bulb: glomeruli	±	±	±	±	±
Optic nerve	–	+	+	–	+
Caudoputamen	++	+	+	±	++
Olfactory tubercle	++	+	+	+	++
Hypothalamus	+	++[c]	±	++	+
Thalamus	+	±	±	+	+
Cortex	+	+	±	++	+
Superior + inferior colliculus	+	++	±	++	+
Cerebellum: molecular layer	+	+	–	±	–
Cerebellum: granule cell layer	–	–	–	–	+
Medulla oblongata	+	+	+	++	+
Hippocampus: stratum oriens	+	+[a]	±[a]	±	+
Hippocampus: stratum radiatum	+	+[a]	±[a]	±	+
Hippocampus: stratum lacunosum	+	++[a]	±[a]	±	+
Dentate gyrus: inner/outer mol. layer	+	+[a]	±[a]	+	+
Dentate gyrus: subgranular layer	–	++[b]	±	–	–
Fiber tracts	+	±	±	±	±
Blood vessels	–	++	++	++ or –	++
Microglia	–	++[a]	++[a]	–	++

Table II: Relative quantification of enzyme reactions. Note that the staining intensities are evaluated within a given enzyme reaction. The relative quantifications do not allow a direct comparison of the catalytic activity between enzyme species. Staining intensities are rated from very strong (++), strong (+), weak (±) to absent (–). In the case of TNAP, the enzyme reaction on blood vessels was either very strong (++) or absent (–) (copied from Langer *et al.*, 2008).

1.2. Hippocampus, spatial memory and adenosine

Among all the components that make up the brain, there is a region that differs from all others in terms of morphological structure and organization of the neuronal circuits. The hippocampus (Fig 1.7) (named after its resemblance to the seahorse) is a major component of the brain of humans and other vertebrates and is one of the most extensively studied areas of the mammalian CNS. It appears as an elongated, banana-shaped structure, with its longer axis extending in a C-shaped appearance. Humans and other mammals have two hippocampi, one in each brain hemisphere. The reasons that make the hippocampus one of the most studied regions in the brain are related to the fact that: 1- the hippocampus displays a very distinct laminated structure in which both neuronal cell bodies and zones of connectivity are arranged in organized layers; 2- the hippocampus plays a central role in learning and memory processes and its dysfunction is often related to neurodegenerative diseases such as Alzheimer's disease (Kandel, 2001). The study of the hippocampus has contributed enormously to our understanding of the operation of elemental brain circuits, showing a particular feature of storing long-term memory traces (Burgess *et al.*, 2002). In rodents, the hippocampus plays a crucial role in episodic and spatial information processing. A group of cells (known as *place cells*particularly localized in CA3 and CA1 region (Witter *et al.*, 2014)) exhibits an increased firing frequency when animals move across a specific location (new location or known location) allowing animals to acquire or recognize the environment (O'Keefe, 1993). Through its interactions with other brain structures associated with emotions (*e.g.* amygdala), the hippocampus has been associated emotional behaviour (ventral hippocampus) (Sahay and Hen, 2008).

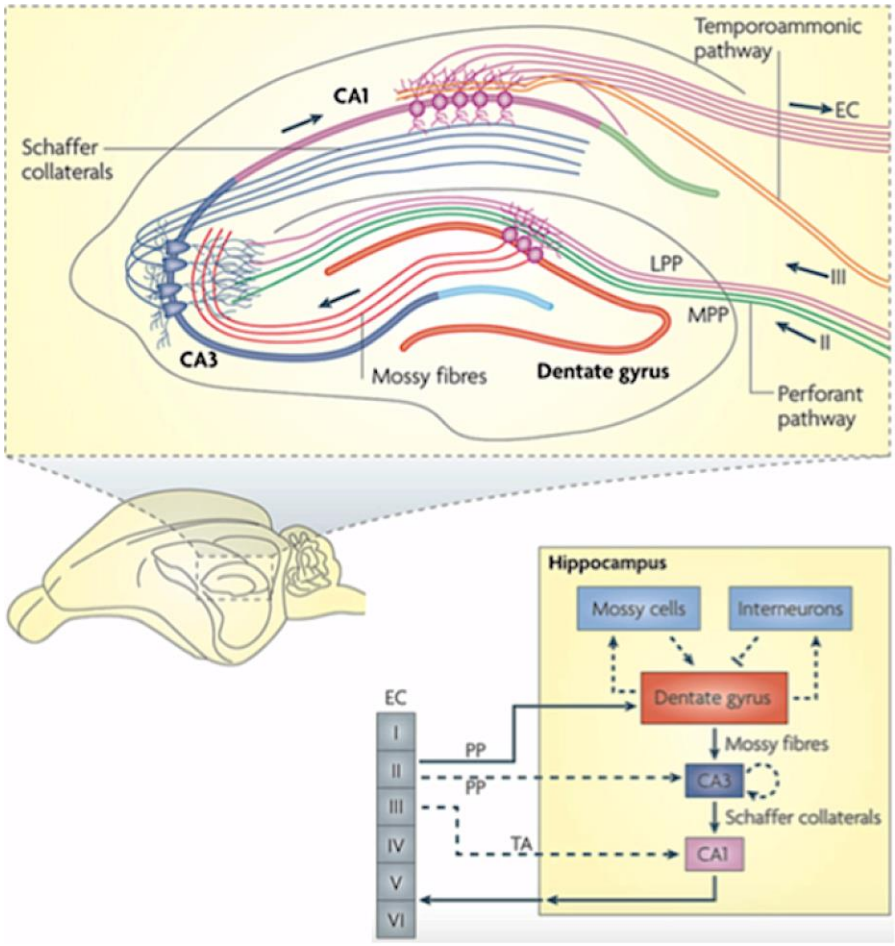

Figure 1.7: The neural circuitry in the mouse hippocampus: An illustration of the hippocampal circuitry and neural network from sagittal view of the mouse hippocampus, where it is possible to identify the relative locations of the dorsal and ventral hippocampus. The traditional excitatory pathway is depicted by solid arrows (entorhinal cortex (EC)–dentate gyrus (DG)-CA3–CA1–EC). The axons of layer II neurons in the EC project to the DG through the perforant pathway (PP), including the lateral perforant pathway (LPP) and medial perforant pathway (MPP). The DG sends projections to the pyramidal cells in CA3 through mossy fibres. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals (neuronal network that we used in this work to record long term potentiation processes). CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons through the PP. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway (TA). The dentate granule cells also project to the mossy cells in

the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells (adapted from Deng *et al.*, 2010).

The study of hippocampal circuits was facilitated through electrophysiological recordings of hippocampal neuronal responses. The association between hippocampal slices and electrophysiology allowed the possibility to better understand neuronal activity including action potential activity of the neurons. A major advantage of hippocampal slice preparations is that the cytoarchitecture and synaptic circuits of the hippocampus are largely retained and the pattern of synaptic connections within the slice is minimally altered relative to the *in vivo* patterns (Fig. 1.8). Through the recording of extracellular synaptic potentials of pyramidal cells from CA1 region from hippocampus slices, it is possible to "read" the activity of many local cells as the synaptic transmission generated by a simultaneous stimulation of many neurons (Schaffer collaterals) (Fig1.8). It is important to identify the different type of that are in an extracellular potential: 1- the pre-synaptic volley (PSV) which is a biphasic deflection right after the stimulus artefact and before a field synaptic potential. It results from the sum of the action potentials of the Schaffer fibres stimulated (Andersen *et al.*, 1978); 2- the field synaptic potential: results from the sum of the inhibitory and excitatory potentials (Alger and Nicoll, 1982). It is composed by a descendent slope which reflects the field excitatory postsynaptic potentials (fEPSPs) of the neuronal population stimulated that represents a temporary depolarization of postsynaptic membrane potential caused by the flow of positively charged ions into the postsynaptic cell as a result of opening of ligand-gated channels (Andersen *et al.*, 1966). The ascendant slope reflects the inhibitory activity (Kamphuis *et al.*, 1988). This type of signal is obtained when the recording electrode is positioned in the CA1

stratum radiatum layer.; 3- the population spike (PS): 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 neighbourhood of the recording electrode (Andersen *et al.*, 1971). This type of signal is obtained when the recording electrode is positioned in the CA1 *stratum pyramidal* layer.

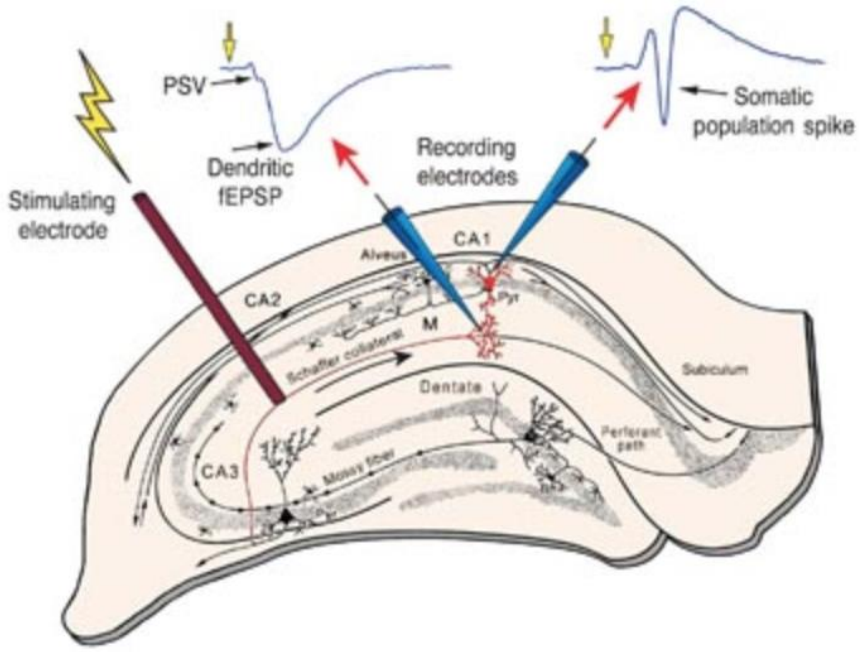

Figure 1.8: Electrophysiology recordings from CA1 region from hippocampal slices: Hippocampal slice and the positioning of stimulating and recording electrodes in CA1 region. Representative field potential recordings of somatic (population spike) and dendritic (fEPSP) post-synaptic responses generated in CA1 after stimulation of Schaffer collaterals are shown. Yellow arrows indicate the moment of electrical stimulation of Schaffer collateral fibres. The presynaptic volley (PSV), representing the summed activation of Schaffer collateral axons, is evident in the dendritic recording.

(Copied from *https://www.scripps.edu/news/scientificreports/sr2008/mind08gruol.html*)

In addition to basal synaptic transmission it is also possible to acquire plasticity phenomena that briefly correspond to an adaptation of the synapses in

response to increase or decrease in their activity (Hughes, 1958). Many synapses in the CNS show activity-dependent changes in synaptic strength and therefore modify neuronal circuit function. At pre-existing synapses, there can be increase or decrease in synaptic efficacy, in response to specific patterns of activation. Modifications can occur both pre- and post-synaptically and can endure for short (ms) but also long (hours or days) periods of time. One of these plasticity phenomena is the long-term potentiation (LTP), which is a persistent strengthening of synapses after the application of an increased stimulus that differs from the recent pattern of activity. Memories, for instance, are described to be encoded by modification of synaptic strength (Takeuchi *et al.*, 2014). Consequently, LTP is widely considered one of the major cellular mechanisms that underlie learning and memory (Takeuchi *et al.*, 2014). LTP occurs most prominently in the hippocampus, where consolidation of experience into long-term memory is thought to occur (Madison *et al.*, 1991).

Purinergic signalling is well described to affect these different synaptic events in the hippocampus. ATP is released upon stimulation and is a potent inhibitor of hippocampal neuronal excitability by activating hippocampal or by acting as a substrate for ecto-nucleotidases (Cunha, 2005). In fact, once in the extracellular , ATP is converted to adenosine, which inhibits synaptic transmission through A_1R activation and facilitates synaptic plasticity through the activation of $A_{2A}R$ (Cunha *et al.*, 1998). CD73 plays a pivotal role in these processes because it is responsible for the final conversion of into adenosine. The role of CD73 gains more importance in the control of synaptic plasticity since there is a preferential release of ATP (Cunha *et al.*, 1996b) and as a consequence a availability of its substrate, AMP to convert into adenosine (Augusto *et al.*, 2013). Accordingly, $A_{2A}R$ are preferably engaged during high stimulation levels to trigger synaptic plasticity events (*e.g.* LTP).

In the hippocampus $A_{2A}R$ are mainly expressed in neurons (Rebola *et al.*, 2003; Rebola *et al.*, 2005a; Rebola *et al.*, 2005b) being enriched in the active zone of glutamatergic (preferentially) synapses mainly in presynaptic terminals (Rebola *et al.*, 2005a; Rebola *et al.*, 2005b); $A_{2A}R$ are also present in astrocytes control glutamate uptake and release through modulation of the activity of glutamate transporters (Matos *et al.*, 2012; Matos *et al.*, 2013).

Despite of $A_{2A}R$ being preferentially located on the presynaptic side (in the hippocampus), there are also postsynaptic hippocampal $A_{2A}R$, that are able to modulate plasticity phenomena, being required for LTP (Rebola *et al.*, 2008; Fontinha *et al.*, 2009; Diógenes *et al.*, 2011). This control of long-term potentiation by $A_{2A}R$ has been explained based on two evidences: 1- the direct control of extracellular glutamate levels by $A_{2A}R$ as referred previously and 2- the preferential release of ATP and consequently the accumulation of ATP-derived adenosine, under high frequency neuronal firing, that preferential activates $A_{2A}R$ (Cunha *et al.*, 1996a; Cunha, 2005), attenuating A_1R function (Lopes *et al.*, 2002).

Genetic manipulation of $A_{2A}R$ provided direct evidence that $A_{2A}R$ play a central role in the control of memory performance. Global $A_{2A}R$ KO mice displayed an improved spatial memory and recognition the Y-maze (Wang *et al.*, 2006), the water maze (Zhou *et al.*, 2009) and the radial arm maze (Wei *et al.*, 2011)., transgenic rats over-expressing $A_{2A}R$ in the cortex exhibited impaired memory performance in several behavioural paradigms such as water maze, 6-arm radial maze and novel object recognition (Giménez-Llort *et al.*, 2007).

These findings, related to the adenosine neuromodulation role, supported the contention that the suppression of $A_{2A}R$ activity is pro-cognitive upholding the idea that adenosine modulates cognition, namely memory functions. In fact, it is described

that the consumption of caffeine (a non-selective adenosine receptor antagonist and the most psychoactive drug by humans) can improv by itself human cognition (Nehlig, 2010) .

The overall control of plasticity processes in the hippocampus may play crucial roles not only in physiological but also in pathological conditions affecting hippocampal functions, like memory, it has been reported an increase of $A_{2A}R$ density under situation (Gomes *et al.*, 2011). nimal models of Alzheimer's disease also show an increased density of $A_{2A}R$ in hippocampal synapses. These animals show a clear deficit on hippocampal-dependent memory that is prevented with the pharmacological blockade of $A_{2A}R$ (Canas *et al.*, 2009).

1.3. Alzheimer's disease and adenosine

Alzheimer's isease (AD) was described for the first time in 1906 by Alois Alzheimer. AD is an irreversible brain disorder characterized by a progressive loss of memory (particularly short-term memory at early stages), delusions, language impairment, personality changes and confusion (Alzheimer, 1906). AD progression has been associated with a gradual damage of the hippocampus and neocortex, two vulnerable brain areas involved in memory and cognition (Selkoe *et al.*, 2012). It is now recognized that the loss of episodic hippocampal-dependent memory is the earliest clinical sign of AD, consistent with reduced activation of hippocampal regions during memory encoding tasks in patients with mild cognitive impairment (Sperling, 2007).

Neuro-pathologically, AD is characterized by three main markers: amyloid plaque deposition (aggregates of amyloid-beta (Aβ) peptides), presence of neurofibrillary tangles (NFT- hyperphosphorylat tau protein) and selective synaptic

and neuronal loss (Selkoe, 2011). Although many studies have been done, the cause of AD is still poorly understood and until now there is no pharmacological tool to prevent or revert disease. However, epidemiological studies have proposed relationships between certain modifiable factors, such as diet, cardiovascular risk, pharmaceutical products, or intellectual activities, among others, and a population's likelihood of developing AD (Szekely *et al.*, 2007).

According to the World Health Organization (March 2015) AD accounts for 60% to 70% of the cases of dementia and is also the most prevalent neurodegenerative disease in the elderly population, affecting close to 40 million people worldwide, making it an alarming health care problem, with a vast social and economic impact. Therefore, it becomes necessary to invest in AD research, and during the last years many research groups have been focused on this issue. One of the tools that facilitated the study of this disease was the creation of AD animal models. Among these models, it can be highlighted those where there is an acute application of micro aggregates (oligomers) of Aβ peptides (*e.g.* Canas *et al.*, 2009) and those that use transgenic mice expressing familial AD (FAD) mutations of amyloid precursor protein (APP) or presenilin 1 (PS1) or both over long periods of time, resulting in a chronic Aβ production (Sasaguri *et al.*, 2017). All these models show a synaptic toxicity mediated by soluble micro aggregates of Aβ, leading to synaptic dysfunction and synapse loss (Canas *et al.*, 2009; Sasaguri *et al.*, 2017).

As it happens in other neurodegenerative disorders, $A_{2A}R$ were found to be up-regulated in cortical and hippocampal regions, both in animal models (Arendash *et al.*, 2006; Canas *et al.*, 2009; Viana da Silva *et al.*, 2016), as well as in cortical tissue collected from patients with AD in either early or advanced stages of this disease (Angulo *et al.*, 2003; Albasanz *et al.*, 2008). Retrospective epidemiological studies

showed that the incidence of AD was inversely associated with the consumption of coffee (Maia and de Mendonça, 2002) and indicated that regular intake of caffeine (non-selective antagonist of $A_{2A}R$) attenuates memory decline during ageing (Ritchie *et al.*, 2007). In animal models of AD, chronic caffeine intake prevents memory deterioration, an effect mimicked by the selective inhibition of $A_{2A}R$ (Dall'Igna *et al.*, 2007; Canas *et al.*, 2009; Viana da Silva *et al.*, 2016). The mechanisms by which the blockade of $A_{2A}R$ restores memory impairment are not fully understood and the role of adenosine and its receptors in regulating the pathogenesis of this neurodegenerative disease remains . However it is already recognized that the impact of $A_{2A}R$ may depend on the stage of progression of the disease, with a role for astrocytic $A_{2A}R$ at late stages (Orr *et al.*, 2015). Conversely, the over activation of hippocampal $A_{2A}R$ is sufficient to disrupt memory performance (Li *et al.*, 2015; Pagnussat *et al.*, 2015).

In conclusion, the blockade of $A_{2A}R$ is considered as a novel promising prophylactic and/or therapeutic option to manage the phases (synaptic loss and cognitive impairment) of AD (Cunha and Agostinho, 2010). Furthermore, the ability of caffeine and $A_{2A}R$ antagonists to control memory dysfunction under different pathological conditions indicates that $A_{2A}R$ may impact on memory preservation in general rather than affecting selectively pathogenic mechanisms of AD.

1.4. Challenges and new perspectives

Distinct sources of adenosine, as well as different sources of ATP, can participate in the activation of different adenosine receptors, having different impact in brain functions. Therefore, determining the adenosine source and consequently the receptor that is activated becomes crucial in order to understand how to manipulate brain functions and to develop new therapeutic strategies for brain disorders.

CD73 is the last member of the enzyme cascade converting extracellular ATP into adenosine (Cunha, 2001), as a crucial go-between of two purinergic systems: the one dependent on adenosine and the other one dependent on ATP. However the importance of CD73 still remains to be characterised both in terms of its location and physio-pathological roles.

Recently, CD73 emerged as the source of ATP-derived adenosine that selective engage $A_{2A}R$. This selective engagement of $A_{2A}R$ results from particular source of adenosine, *i.e.* adenosine formed by synaptic ecto-nucleotidases upon release of ATP from nerve terminals high-frequency stimulation is required to trigger a disproportional release of ATP (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996a) that significantly contributes to the formation of extracellular adenosine (Cunha *et al.*, 1996a) near $A_{2A}R$, in view of the physical association between CD73 and $A_{2A}R$ (Augusto *et al.*, 2013). These $A_{2A}R$ enhance the release of glutamate (Lopes *et al.*, 2002; Marchi *et al.*, 2002; Rodrigues *et al.*, 2005; Shen *et al.*, 2013; Matsumoto *et al.*, 2014; Machado *et al.*, 2017) and the activation of NMDA receptors (Wirkner *et al.*, 2004; Guntz *et al.*, 2008; Rebola *et al.*, 2008; Azdad *et al.*, 2009; Higley and Sabatini 2010; Scianni *et al.*, 2013; Sarantis *et al.*, 2015), effectively bolstering the implementation of LTP (D'Alcantara *et al.*, 2001; Rebola *et al.*, 2008; Fontinha *et al.*, 2009; Costenla *et al.*, 2011; Li *et al.*, 2015; Viana da Silva *et al.*, 2016).

Although this scenario has never been explored in the context of brain disorders, this evidence prompts considering the hypothesis that CD73 might generate adenosine to selective activate $A_{2A}R$ in physiological and pathological conditions. However, two major limitations: 1) it is difficult to disentangle peripheral and central effects of $A_{2A}R$; 2) there are different pools of brain $A_{2A}R$ with different sensitivity to different antagonists (Orru *et al.*, 2011). One possibility to circumvent these

limitations would be to target the source of adenosine activating $A_{2A}R$ rather than directly targeting $A_{2A}R$. This might become realistic in view of previous observations that suggest that $A_{2A}R$ are selectively activated by ATP-derived adenosine (Cunha *et al.*, 1996a; Rebola *et al.*, 2008). This would make CD73, the only enzyme able to adenosine from extracellular adenine nucleotides (Lovatt *et al.*, 2012), a new target to control neurodegeneration (Baqi *et al.*, 2010).

However, it is still required to provide a solid experimental support of the direct functional association of CD73 activity with $A_{2A}R$, given that some reports implied that the extracellular catabolism of ATP released from astrocytes might also be associated with the activation of neuronal A_1R (Cunha *et al.*, 1998; Pascual *et al.*, 2005). Thus, there is a clear need to define the levels of extracellular adenosine that activate the different adenosine receptors. Likewise, the eventual extrapolation of the role of CD73 from physiology to pathological conditions needs to be carefully tested since ageing or brain insults trigger an up-regulation of CD73 together with a gain of function of $A_{2A}R$ and a decreased expression and function of A_1R (Cunha, 2005).

CHAPTER 2

Objectives

This work was fully done at the "Purines at CNC" group of the Center for Neuroscience and Cell Biology the University of Coimbra under the supervision of Professor Rodrigo Cunha, with the continuous assistance of Professor Ângelo Tomé. "Purines at CNC" has been on the frontline of characterizing the localization, kinetic properties and functional role of CD73 in central synapses (Cunha, 2008; Rebola *et al.*, 2008) as well as the role of $A_{2A}R$ in brain disease (Cunha, 2005; Gomes *et al.*, 2011). All together, this means we have gathered a unique set of knowledge and underlying expertise to realistically define if CD73 provide a source of adenosine selectively activating $A_{2A}R$ in specific brain structures (mainly in the hippocampus) and if the manipulation of CD73 might constitute a more robust strategy to manipulate $A_{2A}R$-mediated control of synaptic plasticity and of neurodegeneration.

We now hypothesize that CD73 might generate adenosine activating $A_{2A}R$ in physiological and pathological conditions since: 1- ATP is preferentially released under high stimulation levels and is released as a danger signal upon brain damage; 2- coincidentally, $A_{2A}R$ are preferentially activated under high stimulation levels, for instance during plasticity events; 3- brain dysfunction triggers an up-regulation of $A_{2A}R$ (Cunha, 2005). This would make CD73 a new target to control neurodegeneration, as recently proposed by one of our collaborators (Baqi *et al.*, 2010)the case the immunological control of cancer elimination (Stagg, 2012).

These premises placed us in a comfort zone allowing us to define the following objectives:

1- hich conditions allow the release of the main source of adenosine (ATP) hy ATP is preferentially released under high stimulations levels the levels of ATP affected by CD73 or other ecto-nucleotidases;

2- efine the physiological role of CD73, by combining electrophysiological, and neurochemical (neurotransmitter release) assays with pharmacological manipulation of CD73 (*ex vivo* and *in vivo*) to probe its role in the activation of $A_{2A}R$;

3- haracterise CD73 KO mice, defining the selectivity of the genetic elimination and eventual adaptive changes of the purinergic system (ATP release);

4- ombine the use of this CD73 KO with pharmacological tools to define the role of CD73 in animal models of brain disease, namely Alzheimer's disease, typified by β-amyloid-induced synaptotoxicity and memory impairment (Canas *et al.*, 2009).

CHAPTER 3

The pattern of ATP release from brain cortical nerve terminals is different from that of classical neurotransmitters

3.1. Abstract

ATP is released from nerve terminals but it is unclear if ATP is co-released with classical neurotransmitters or is released from independent vesicles, a question relevant to understand purinergic modulation of synaptic transmission and plasticity. Once in the extracellular milieu, ATP can act as a neurotransmitter and/or as a presynaptic neuromodulator, either by direct activation of P2 receptors or through the indirect activation of P1 receptors upon its extracellular catabolism by ecto-nucleotidases into adenosine. Throughout this chapter we proposed to finish a work previously started in group characterizing the pattern of ATP release from purified nerve terminals of the rat hippocampus and cerebral cortex.

The high potassium-evoked release of ATP was mostly vesicular, based on its sensitivity to extracellular calcium, bafilomycin A1 and botulinum toxin E. contrast to glutamate, GABA or release, the evoked release of ATP was disproportionately larger at higher potassium-evoked depolarization. Extracellular ATP accumulation limited the ATP release. The evoked ATP release was mostly sensitive to the L-type calcium channel inhibitors nitrendipine or nifedipine, contrast to the evoked release of classical neurotransmitters, which was insensitive to nifedipine and was instead attenuated by N-type (ω-conotoxin GVIA) or P-type (ω-agatoxin IVA) calcium channel inhibitors. The fractionation of the synaptic vesicles (present in purified synaptosomes) showed a higher content of ATP in segretogranin II-positive compared to vesicular transporter type 1 (vGluT1)-positive vesicles. These results indicate that the pattern of ATP release from nerve terminals is different from that of classical neurotransmitters and follows a peptidergic-like

pattern, ATP being mostly released in a manner sensitive to L-type calcium channels that are typically recruited at higher intensities of nerve stimulation.

3.2. Introduction

ATP is one of the most primitive extracellular signals for communication between cells (Burnstock and Verkhratsky, 2010) and it fulfils a variety of signalling roles in the central nervous system, namely as a neurotransmitter, as a neuromodulator, as an astrocyte-neuron communication signal and as an activator of astrocytes and of microglia (Burnstock *et al.*, 2011; Khakh and North, 2012; Koizumi *et al.*, 2013). ATP can be released from the different brain cell types such as neurons, astrocytes, oligodendrocytes and microglia (Pankratov *et al.*, 2006; Koizumi, 2010; Franke *et al.*, 2012). Extracellular ATP directly signals through P2 receptors (Burnstock, 2007) and indirectly through adenosine receptors (Fredholm *et al.*, 2005) after the efficient extracellular catabolism of ATP into adenosine by ecto-nucleotidases (Cunha, 2001). The interest in extracellular ATP was bolstered by the recognition that it fulfils an important role as a danger signal in the brain (Di Virgilio, 2000; Rodrigues *et al.*, 2015), as heralded by the observations that noxious brain stimuli cause a sustained increase of the extracellular levels of ATP (Davalos *et al.*, 2005; Frenguelli *et al.*, 2007; Melani *et al.*, 2012) and the blockade of different P2 receptors affords neuroprotection (Franke *et al.*, 2006; Skaper *et al.*, 2010; Carmo *et al.*, 2014) and because it is a main source of adenosine in the brain, (Cunha, 2001) responsible for the P1 receptors and, under brain danger conditions, responsible for the exacerbation of the neurodegenerative phenomena (Gomes *et al.*, 2011). In this context, the synaptic release and functions of ATP merit particular attention in view of the increased recognition that synaptic dysfunction and damage is one of the earliest features of different neurodegenerative and neuropsychiatric diseases

(Coleman *et al.*, 2004; van Spronsen and Hoogenraad, 2010; Duman and Aghajanian, 2012).

It is well established that ATP is stored in synaptic vesicles (Zimmermann, 1994) and is released from stimulated brain nerve terminals in a calcium-dependent manner (White, 1978). Albeit it was found the presence of pre- and post-synaptic P2 receptors in central synapses (Rodrigues *et al.*, 2005), their physiological roles still remain elusive. In contrast, we have collected robust evidence suggesting that synaptically released ATP is degraded by ecto-nucleotidases to generate the particular pool of adenosine that selectively activates adenosine A_{2A} receptors (Cunha *et al.*, 1996a; Rebola *et al.*, 2008; Augusto *et al.*, 2013). Interestingly these A_{2A} receptors are selectively engaged in conditions of synaptic plasticity in the hippocampus (Rebola *et al.*, 2008; Costenla *et al.*, 2011). This would require that presynaptic ATP release should mainly be evident at higher frequencies of nerve stimulation, which was indeed found to occur in hippocampal slices (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996a). However, these observations were made in slice preparations, where it is not currently possible to isolate the vesicular release of ATP from nerve terminals, given that different cell types can release ATP (Bodin and Burnstock, 2001; Corriden and Insel, 2010; Li *et al.*, 2011). Furthermore, these observations also cast doubts on whether ATP is co-released with glutamate or if instead ATP is released from nerve terminals in a manner different from that of classical neurotransmitters, as has been proposed to occur in different systems (Rabasseda *et al.*, 1987; Trachte *et al.*, 1989; Ellis and Burnstock, 1990; Fariñas *et al.*, 1992; Gonçalves *et al.*, 1996; Todorov *et al.*, 1996).

During this chapter, we took advantage of synaptosomes to allow isolating the release of ATP from nerve terminals (Raiteri and Raiteri, 2000), which could be

directly compared to that of classical neurotransmitter like glutamate, GABA or. This allowed to define that: 1) ATP release displays a sensitivity to the intensity of stimulation different from classical neurotransmitters and depends on the extracellular availability of the ATP itself; 2) ATP release mostly depends on the recruitment of L-type voltage-sensitive calcium channels in contrast to classical neurotransmitters; 3) ATP is more abundant in large dense core vesicles rather than in small synaptic vesicles.

All together, these results show that ATP release from nerve terminals occurs in a vesicular manner, but follows a pattern different from the release of classical neurotransmitter, occurring mainly with more intense depolarizations, in a peptidergic like-manner.

3.3. Materials and methods

3.3.1. Animals and preparation of synaptosomes

Wistar rats (8-10 weeks, males) were purchased from Charles River. Animals were maintained under controlled environment (23 ± 2°C; 12 h light/dark cycle and *ad libitum* access to food and water) and handled in accordance with the European Community guidelines (Directive 2010/63/EU) and the Portuguese law on animal care (1005/92), and approved by the Animal's Ethics Committee of the Center for Neuroscience and Cell Biology (Orbea 78-201) and the Portuguese Veterinarian Office. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort, following ARRIVE guidelines (Kilkenny *et al.*, 2010).

The preparation of synaptosomes (purified nerve terminals) was carried out from the cerebral cortex and hippocampus using sucrose/Percoll differential centrifugations, as previously described (Cunha *et al.*, 1992). Briefly, rats were deeply anaesthetized with halothane before being killed by decapitation. The cerebral cortex was quickly removed into ice-cold 0.32 M sucrose solution containing 1 mM EDTA, 1 mg/ml bovine serum albumin, and 5 mM HEPES buffered to pH 7.4 and homogenized with a homogenizer. After centrifugation at 3,000 *g* for 10 min, the supernatant was collected and centrifuged at 14,000 *g* for 12 min. The pellet was resuspended in 1 ml of a solution of 45% (v/v) Percoll in Krebs-HEPES solution (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$,10 mM glucose, and 10 mM HEPES, pH 7.4) adjusted to 115 mM NaCl with 1 M NaCl and buffered to pH 7.4 with 1 M HEPES/NaOH. After centrifugation at 14,000 *g* for 2 min, the top layer (synaptosomal fraction) was removed, washed once with ice-cold Krebs solution, down, resuspended in 1 ml ice-cold Krebs solution and maintained on ice. We confirmed our previous Western blot characterization showing that thcortical synaptosome enriched in presynaptic markers (synaptophysin, SNAP-25, syntaxin-I) and display a contamination <15% with post-synaptic density markers (PSD-95, MAP-2) and <2% with astrocytic markers (GFAP, GLT-I) (Cunha *et al.*, 1992; Rodrigues *et al.*, 2008; Matos *et al.*, 2012).

When we tested the impact of botulinum toxin E (Sigma), the cortical brain tissue (half a hemisphere) was homogenized in 2 ml of sucrose-HEPES solution containing 100 nM of the toxin, to ensure its effective incorporation inside the nerve terminals (Raiteri and Raiteri., 2000). As expected from the ability of botulinum toxin E to proteolytically remove the C-terminal 26 amino acids from SNAP-25 (Binz *et al.*, 1994), we confirmed by Western blot analysis of synaptosomes collected after an

equilibration period of 12 min at 25 ºC, that this treatment with botulinum toxin E caused a 71±9% (n=3) decrease of SNAP-25 immunoreactivity in treated compared to non-treated synaptosomes (data not shown) using an antibody directed against the C-terminus of SNAP-25 (AB1762 from Chemicon).

3.3.. Evoked release of ATP

The release of ATP was measured on-line using the luciferin-luciferase assay (Cunha *et al.*, 1996b). This suspension was equilibrated at 25 ºC during 3 min (for stimulus-dependent evoked release of ATP) up to 10 min (when testing the impact of most drugs) to ensure the functional recovery of the synaptosomes (Ferreira *et al.*, 2015). The suspension was the transferred to a well of a white 96-well plate, which was maintained for 1 min at 25 ºC inside a luminometer (Perkin Elmer Victor3 or Wallac 1250) before initiating the recording of the electrical signal generated by the photomultiplier. We first measured the basal outflow of ATP during 60 sec, before triggering the evoked release of ATP with a chemical stimulus consisting of a Krebs-HEPES solution with an isomolar substitution of NaCl by 32 mM of KCl (or with the amount of KCl to attain the required final concentration of between 10-60 mM). After this chemical stimulation, the light levels were recorded for an additional 200 sec. The evoked release of ATP was calculated by integration of the area of the peak upon subtraction of the estimated basal ATP outflow and the levels of ATP were estimated using a calibration curve for ATP, which were linear between $2x10^{-12}$ and $8x10^{-5}$ M (Cunha *et al.*, 1996b). To test the impact of different drugs on ATP release, these drugs were added to the synaptosomes during the 10 min equilibration period at 25 ºC and were present throughout the assays. None of the tested drugs modified the light

emission by the luciferin-luciferase assay when testing different concentrations of ATP standards.

3.3.3. Evoked release of glutamate, GABA or

The release of [^{3}H]glutamate, [^{3}H]GABA and [^{3}H] was carried out as previously described (Cunha and Ribeiro, 2000; Rebola *et al.*, 2003; Rodrigues *et al.*, 2005). Briefly, the synaptosomes were pre-labelled with 0.2 µM [^{3}H]glutamate (specific activity 45 Ci/mmol; GE Healthcare) for 5 min or with 1.875 nM γ-amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]GABA, specific activity 74.0-88.6 Ci/mmol; GE Healthcare), together with 6 nM unlabelled GABA (Ascent) for 20 min or with 0.125 µM [methyl-^{3}H]choline (specific activity 41.3 Ci/mmol; GE Healthcare) for 10 min at 37ºC. After washing, the synaptosomes were placed over Whatman® GF/C filters and superfused (flow rate: 0.8 ml/min for glutamate, 0.6 ml/min for GABA or) with Krebs solution (124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM glucose) gassed with a 95% O_2 and 5% CO_2 mixture. The synaptosomes were stimulated during 1 min with 10-60 mM K^+ (isomolar substitution of Na^+ by K^+ in the Krebs superfusion solution) at 3 and 12 min (for glutamate) or 4 and 22 min (for GABA or) after starting sample collection (S_1 and S_2). All tested drugs (except botulinum toxin E, which was previously entrapped inside the synaptosomes during their preparation) were added 7-10 min before S_2 onwards. Samples were collected every minute and radioactivity was determined in these effluent samples. The amount of radioactivity recovered was mostly , glutamate or GABA, since enzymatic (Rebola *et al.*, 2003) or HPLC of the effluent samples (Cunha and Ribeiro, 2000; Lopes *et al.*, 2002) showed that 83-91% of total

radioactivity in superfusate samples upon K^+ stimulation was [^{3}H], [^{3}H]glutamate or [^{3}H]GABA. The release of tritium evoked by each potassium pulse, i.e. the evoked release, was calculated by integration of the area of the peak upon subtraction of the estimated basal tritium outflow from the total outflow of tritium due to stimulation. The effect of drugs was evaluated by alterations of the ratio between the evoked release caused by the second stimulation period and the evoked release caused by the first stimulation period (S_2/S_1 ratio), as previously described (Cunha and Ribeiro, 2000; Rebola *et al.*, 2003; Rodrigues *et al.*, 2005).

3.3.4. Analysis of the integrity of synaptosomes

The integrity of the synaptosomes during the ATP release assays was estimated by comparing lactate dehydrogenase activity in the incubation medium with that found in the synaptosomal pellet upon its solubilization with 2% (v/v) Triton X-100, as previously described (Cunha *et al.*, 1992). Apart this well-established marker of synaptosomal integrity, we also quantified the amount of glutamine, a low molecular weight metabolite that is not released in an activity-dependent manner from nerve terminals. This was carried out by fluorescent detection upon HPLC separation of the synaptosomal superfusate after derivatization with o-phthaldialdehyde (Lindroth and Mopper, 1979), as previously described (Cunha *et al.*, 1997).

3.3.5. Fractionation and analysis of synaptic vesicles

The purification and separation of synaptic vesicles was carried out as previously described (Whittaker, 1984). Briefly, after the preparation of the cortical

synaptosomes from 3 rats as described above, the synaptosomes were osmotically lysed by resuspension in 10 ml of an ice-cold solution with 5 mM Tris (pH 7.4) with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin and 10 μg/ml aprotinin) and this suspension was centrifuged at 120,000 *g* for 2 h. The pellet was resuspended into 2 ml of ice-cold sucrose solution and layered over a continuous sucrose gradient between 0.2 and 1.4 M in 10 mM HEPES and 0.5 mM EGTA (pH 7.4). After centrifugation at 65,000 *g* for 4 h, 1 ml fractions of this equilibrium density sucrose gradient were collected and analysed by luminometry (to quantify their ATP content, as described above) and Western blot analysis (to determine markers of the different types of synaptic vesicles).

Western blot analysis was carried out after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separation as previously described (Rodrigues *et al.*, 2005) to detect a protein present at different densities in all types of synaptic vesicles (Winkler, 1997), a marker of large dense-core peptidergic vesicles (Rosa *et al.*, 1985; Weiler *et al.*, 1990) and a marker expected to be present in small synaptic vesicles (Takamori, 2006). Incubation with the primary antibodies directed against synaptophysin (1:10,000 AB9272 from Millipore), chromogranin C (1:1,000 ab20245 from Abcam), or vGluT1 (1:5,000, clone 217D5 from Synaptic Systems), diluted in Tris-buffered saline (137 mM NaCl and 20 mM Tris–HCl, pH 7.6) with 0.1% Tween (TBS-T) and 5% (fatty acid free), was carried out overnight at 4ºC. After washing twice with TBS-T, the membranes were incubated with secondary antibody conjugated with alkaline phosphatase (GE Healthcare) for 2 hours at room temperature. After washing, the membranes were revealed using an ECF kit (Amersham) and visualized with an imaging system (VersaDoc 3000, Bio-Rad)he

densitometric analysis of protein bands was performed using the Quantity One software (Bio-Rad).

3.3.6. Drugs

Botulinum toxin E was supplied as a 1 mg/ml in 0.2 M sodium chloride and 0.05 M sodium acetate, pH 6.0 from Sigma. ARL 67156 (6-N,N-diethyl-D-β,γ-dibromomethylene ATP; Tocris) was made up as a 5 mM stock solution in water. Nifedipine and nitrendipine (Sigma) were made up into 5 mM stock solutions in dimethylsulfoxide. Bafilomycin A1 (Ascent) was made up into a 0.1 mM stock solution in . ω-Agatoxin-IVA and ω-conotoxin-GVIA were from Peptide Institute and were prepared as a 0.1 mM stock solutions in water. α,β-Methylene ADP (AOPCP; from Sigma, Sintra, Portugal) was prepared to a 10 mM stock solution and used at a supra-maximal and selective concentration of 100 µM (Cunha *et al.*, 1992).

Drug solutions were stocked at -20 ºC and aqueous dilution of these stock solutions was made daily.

3.3.7. Statistics

The values are mean ± SEM of *n* experiments. To test the significance of the effect of a drug *versus* control, a paired Student's *t* test was used. When making comparisons from different set of experiments with control, a one way variance analysis (ANOVA) was used, followed by a Dunnett's test. $P < 0.05$ was considered to represent a significant difference.

3.4. Results

The group of results graphed on Fig 3.1, Fig 3.3 and Fig 3.4 were collected and previously described by Teresa Almeida, Jean Oses and Ricardo Rodrigues under the supervision of Professor Rodrigo Cunha. these results , they were seen as further evidence support the that ATP is released in a like manner

3.4.1. Different intensity-dependent evoked release of ATP and of classical neurotransmitters

Purified nerve terminals (synaptosomes) have the unique advantage allow isolatithe specific contribution of the presynaptic component for neurochemical processes (Raiteri and Raiteri, 2000). However, due to the small size of presynaptic component, which only comprise *circa* 2% of cortical volume (Rusakov *et al.*, 1998), the release of neurotransmitters can only be reliably triggered with chemically induced depolarization (Nicholls, 1989). It has already been detailed the impact of high extracellular K^+-induced depolarization to trigger neurotransmitter release, which causes a concentration-dependent depolarization in the range of 5-60 mM (Blaunstein and Goldring, 1975; McMahon and Nicholls, 1990; Meder *et al.*, 1997). Accordingly, as shown in Fig 3.1, the chemical depolarization of cortical synaptosomes with high extracellular K^+ in the range of 10-60 mM triggered a concentration-dependent enhancement of classical neurotransmitters such as glutamate, GABA or ; thus, moderate depolarization of nerve terminals achieved with the lower high extracellular K^+ concentrations (10-30 mM) was sufficient to effectively release most of the classical neurotransmitters stored in the synaptic vesicles forming the ready-releasable pool (Rizzoli and Betz, 2005).

Strikingly, the K^+-evoked release of ATP displayed a different profile: the release of ATP was lower when using lower extracellular K^+ concentrations (10-30 mM) and was disproportionately larger when using a higher extracellular concentration of K^+.

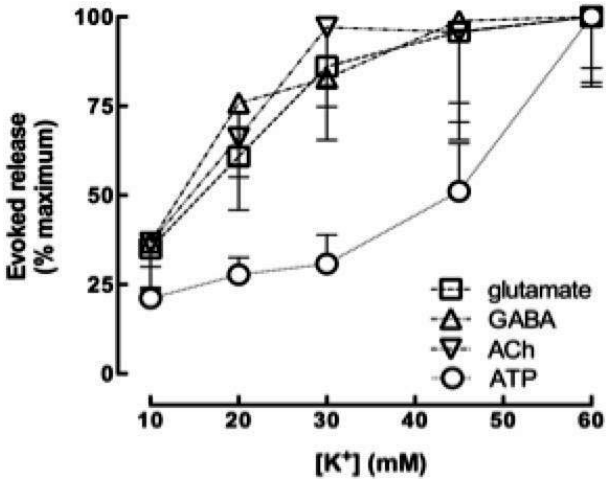

Figure 3.1: The concentration-dependence of K^+-evoked release of ATP from nerve terminals is different from that of the classical neurotransmitters glutamate, GABA and . The release of ATP (O) was evaluated with a luciferin-luciferase enzymatic assay, whereas the release of glutamate (□), GABA (Δ) and ACh ∇) was evaluated as tritium outflow from superfused synaptosomes previously labelled with either [^{3}H]glutamate, [^{3}H]GABA or [^{3}H]. The evoked release of these transmitters was triggered by exposure to different concentrations of K^+, with isomolar substitution of Na^+ by K^+. The evoked release was calculated by subtraction of the basal outflow from the total release on K^+-stimulation. It was confirmed either by enzymatic assay (ACh) or by HPLC separation that the K^+-evoked tritium release essentially corresponded to the release of the neurotransmitters. The evoked release was expressed as % of the release evoked by 60 mM K^+, which was 4.32±0.62%, 0.87±0.17% and 2.07±0.38% of total tritium retained for [^{3}H]glutamate, [^{3}H]GABA and [^{3}H], respectively and 71.8±10.9 mol/mg protein for ATP. The values are mean±SEM of 4-6 experiments.

This non-monotonous dependency of ATP release with the intensity of stimulation could be due to a balanced outflow and metabolism of ATP by synaptic ecto-nucleotidases (Cunha, 2001), which are known to display a high catalytic efficiency (Dunwiddie *et al.*, 1997; Cunha *et al.*, 1998). This was tested by comparing the pattern of ATP release in the absence and in the presence of an inhibitor of ecto-nucleotide diphosphohydrolases, ARL67156 in an effective concentration of 50 μM (Rebola *et al.*, 2008) and the CD73 inhibitor AOPCP in an effective concentration of 100 μM (Cunha *et al.*, 1992). As shown in Fig 3.2 (a and b), ARL67156 increased the basal outflow of ATP from nerve terminals by 102.6 ± 18.21 luminescence levels/mg protein (n=4; $t_{3,792}$= 3.209; P<0.05 *vs* Control) whereas the evoked release of ATP, caused by 30 mM K^+, was decreased by 171.6±29.43 % when compared to the control.

It was observed that CD73, an ecto-nucleotidase that selective converts AMP into adenosine, does not control extracellular accumulation of ATP that decrease K^+-evoked release of ATP from synaptosomesncubation of synaptosomes with 100 μM AOPCP did not change the basal levels of ATP nor the K^+-evoked release of ATP (Fig 3.2 and).

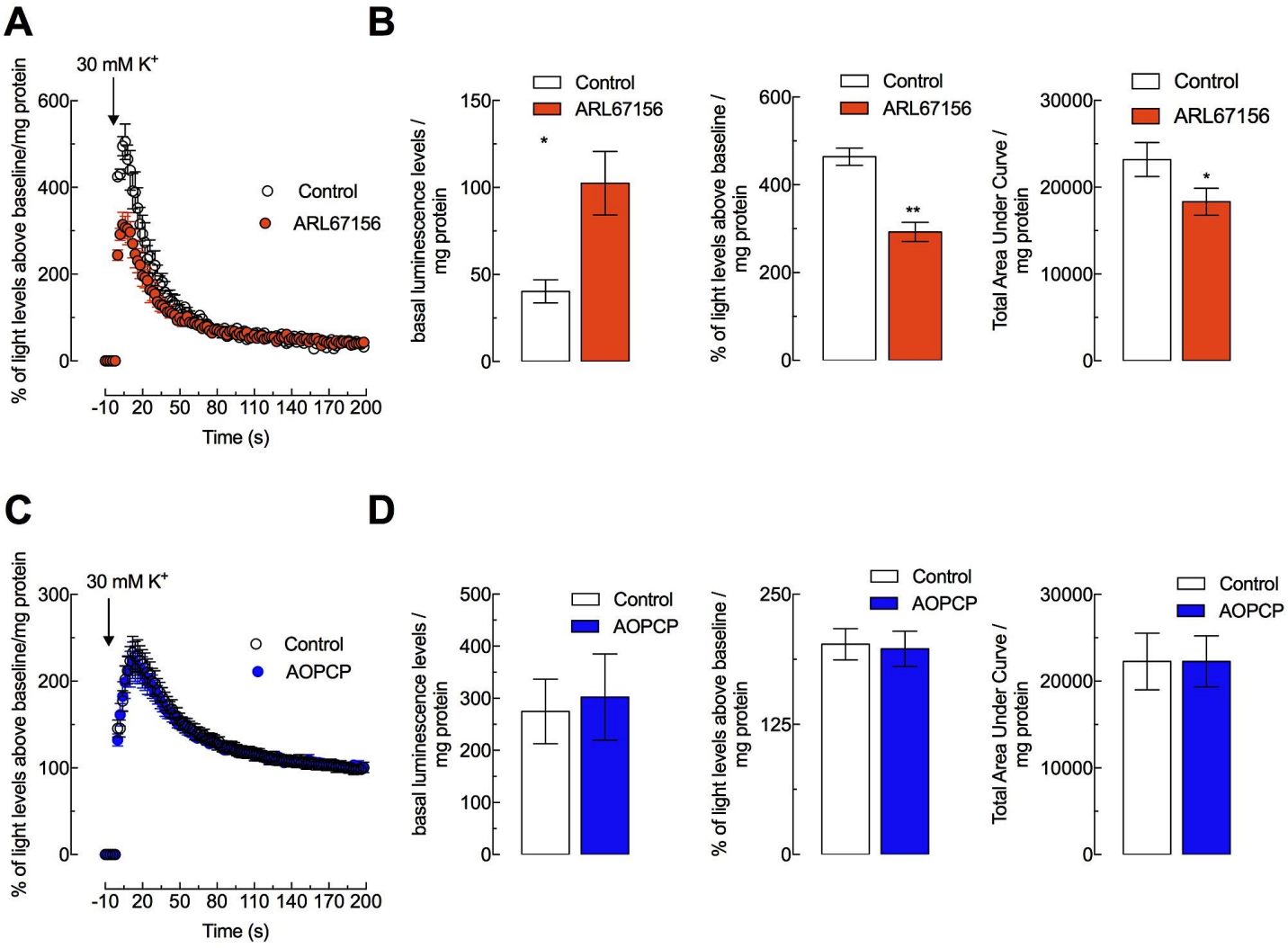

Figure 3.2: extracellular levels of ATP decrease K^+-evoked ATP release. () Comparison of the time course variation of the extracellular levels of ATP in cortical synaptosomes subject to K^+ stimulation in the absence () or in the presence of the ecto-ATPase inhibitor ARL67156 (50 μM, orange). **()** Ecto-ATPase inhibition enhanced basal ATP levels and decreased K^+- evoked levels of luminescence, *i.e.* ATP levels. **()** Surprisingly the inhibition of CD73 with AOPCP did affect neither the basal outflow of ATP nor the K^+-evoked release of ATP. The values are mean±SEM. **P*<0.05 using a two-tailed *t* test *vs* Control.

We also excluded that the enhanced ATP extracellular levels measured upon exposure of synaptosomes to higher extracellular K^+ concentrations could result from

the leakage of intracellular ATP due to the loss of integrity of synaptosomes, since: 1) the exposure to higher extracellular K^+ caused a peak of ATP release that returned to baseline (Fig. 3.2 and) indicative of a transmitter-like release of ATP rather than an outflow of ATP resulting from irreparable loss of plasma membrane integrity; 2) there was no augmentation of the activity of lactate dehydrogenase, an intracellular marker (Johnson and Whittaker, 1963), in the medium after a 3-min incubation period with Krebs containing 60 mM K^+ (n=4); 3) likewise, the extracellular levels of another low molecular weight molecule, glutamine, that is not expected to be released from nerve terminals in an activity-dependent manner, were similar in the absence (0.28±0.04 μM, n=4) and in the presence of 60 mM K^+ for 3 min (0.26±0.03 μM, n=4); 4) finally, there was no significant additional increase of the evoked release of ^{3}H-glutamate upon exposure to 60 mM compared to 30 mM extracellular K^+ (Fig. 3.1), in spite of the intracellular abundance of glutamate, in particular within nerve terminals.

3.4.2. Both ATP and classical neurotransmitters are released in a vesicular manner from synaptosomes

The evoked release of ATP from cortical nerve terminals displayed all the expected characteristics of a vesicular release of neurotransmitters (Fig. 3.2). Thus, both the evoked release of classical neurotransmitter such as ACh as well as the evoked release of ATP were strictly dependent on extracellular calcium since the absence of extracellularly added calcium essentially abolished the evoked release of both ACh (Fig. 3.3) and of ATP (Fig. 3.3). Likewise, the presence of 100 nM bafilomycin, an inhibitor of the proton pump that energizes the intra-vesicular

accumulation of substances utilizing vesicular transporters (Moriyama *et al.*, 1992), similarly inhibited the evoked release of (60.0±16.5%, n=4; $P<0.05$ compared to control) (Fig. 3.2) and of ATP (60.7±25.1%, n=4; $P<0.05$ compared to control) (Fig. 3.3). Finally, the intra-synaptosomal trapping of botulinum toxin E, which cleaves SNARE proteins responsible for the vesicular release of neurotransmitters (Binz *et al.*, 1994), caused a similar inhibition of the evoked release of (56.0±14.9%, n=3; $P<0.05$ compared to control) (Fig. 3.2) and of ATP (79.7±21.3%, n=3; $P<0.05$ compared to control) (Fig. 3.2). As expected from the ability of botulinum toxin E to proteolytically remove the C-terminal 26 amino acids from SNAP-25 (Binz *et al.*, 1994), we confirmed by Western blot analysis of synaptosomes collected after an equilibration period of 12 min at 25 ºC, that this treatment with botulinum toxin E caused a 71±9% (n=3) decrease of SNAP-25 immunoreactivity in treated compared to non-treated synaptosomes (data not shown) using an antibody directed against the C-terminus of SNAP-25.

Thus, it appears that the evoked release of ATP and of classical neurotransmitters occurs through a similar vesicular-like process, but the calcium entry into nerve terminals has a different impact on the evoked release of ATP and on that of classical neurotransmitters. This led us to test if calcium entry through different calcium channels was differentially involved in controlling the evoked release of classical neurotransmitter and of ATP.

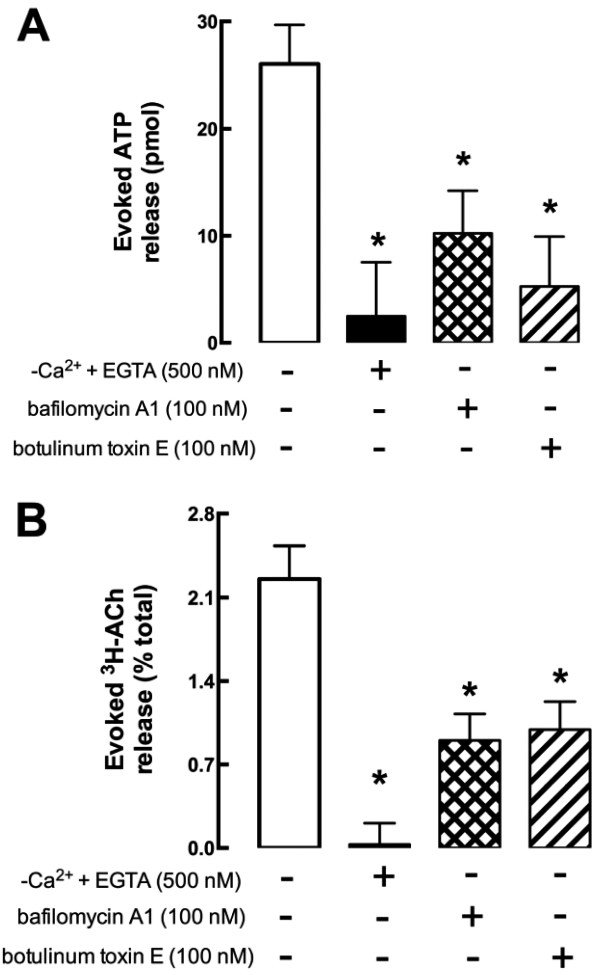

Figure 3.3.: The evoked release of ATP from nerve terminals occurs in a vesicular-like manner, similar to the evoked release of . The removal of extracellular calcium (reduced to 200 nM) together with the presence of EGTA (500 nM), decreased the K^+ (30 mM)-evoked release of ATP **()** and of ACh **()** from cortical nerve terminals (black bars), compared to the evoked release in control Krebs solution (open bars). Likewise, the inhibition of the vesicular proton pump with bafilomycin (100 nM, dark grey bars) similarly decreased the evoked release of ATP **()** and of ACh **()**. Additionally, the cleavage of SNAP-25 by the entrapment of botulinum toxin E (100 nM, light grey dashed bars) inside nerve terminals also similarly decreased the evoked release of ATP **()** and of ACh **()**. The values are mean±SEM of 3-5 experiments. **P*<0.05 compared to control (i.e. absence of added drugs, open bar), using a Dunnett's test.

3.4.3. ATP released from synaptosomes is mostly dependent on L-type calcium channels in contrast to classical neurotransmitters

Previous studies have already characterised the different role of different classes of calcium channels on the evoked release of classical neurotransmitter such as glutamate or GABA from hippocampal nerve terminals (Ambrósio *et al.*, 1997; Cunha and Ribeiro, 2000). These studies showed that the evoked release of glutamate or of GABA from hippocampal nerve terminals was mainly dependent on calcium entry through P- and N-type channels, whereas L-type channel inhibitors were essentially devoid of effects (Ambrósio *et al.*, 1997; Cunha and Ribeiro, 2000), in agreement with the impact of the different calcium channels on excitatory and inhibitory transmission in the CNS (Takahashi and Momiyama, 1993). Likewise, we now report (Fig. 3.4) that the evoked release of another classical neurotransmitter, , was also inhibited by the P-type channel inhibitor, ω-agatoxin IVA (200 nM, 43±6% inhibition, n=4, $P<0.05$) and by the N-type channel inhibitor, ω-conotoxin GVIA (500 nM, 33±6% inhibition, n=4, $P<0.05$), and was insensitive to the L-type channel blocker, nifedipine (10 μM, n=4). By contrast, as shown in Figure 3.4 , the evoked release of ATP from cortical synaptosomes was insensitive to N-type channel inhibit ω-conotoxin GVIA (n=4), slightly inhibited (24±4%, n=4, $P<0.05$) by the P-type channel inhibitor, ω-agatoxin IVA (200 nM), and mostly sensitive to the L-type channel inhibitors nitrendipine (10 μM) and nifedipine (10 μM) (46±9% and 51±12% inhibition, n=4, respectively).

These observations suggest that the evoked release of ATP from nerve terminals follows a pattern more similar to the release of neuropeptides, which mainly occurs at

high intensities or frequencies of nerve stimulation and also involves calcium entry through L-type calcium channels (Verhage *et al.*, 1994).

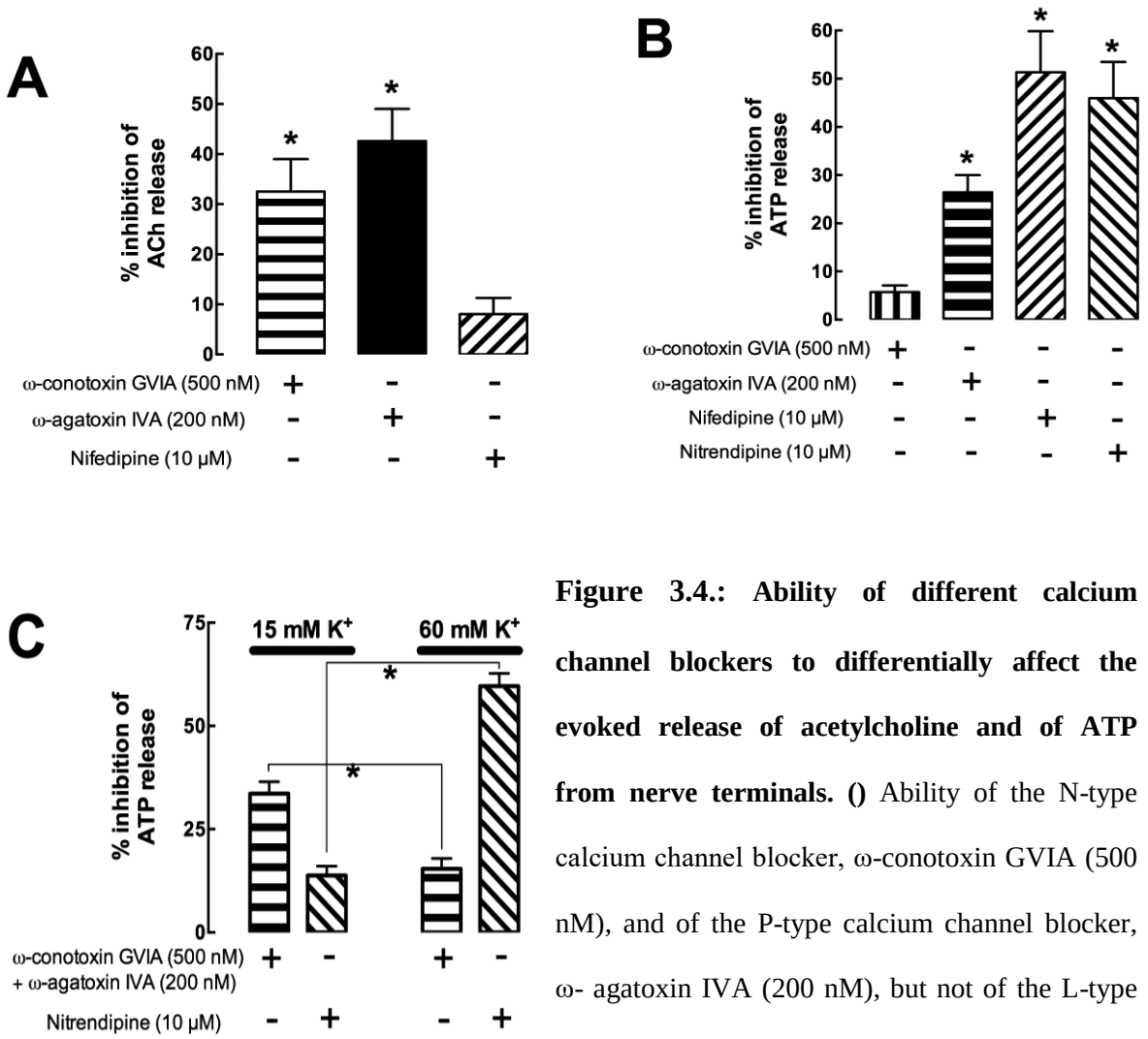

Figure 3.4.: Ability of different calcium channel blockers to differentially affect the evoked release of acetylcholine and of ATP from nerve terminals. () Ability of the N-type calcium channel blocker, ω-conotoxin GVIA (500 nM), and of the P-type calcium channel blocker, ω- agatoxin IVA (200 nM), but not of the L-type calcium channel blocker, nifedipine (10 μM), to inhibit release triggered by 30 mM K^+. The results are mean±SEM of 4 experiments. * $P<0.05$ versus 0%, i.e. to the S2/S1 ratio of evoked tritium release in the absence of any added drug. By contrast, **()** shows that the K^+ (30 mM)-evoked release of ATP is mostly inhibited by blockade of L-type rather than N- or P-type calcium channels. The results are mean±SEM of 4 experiments. *$P<0.05$ versus 0%, i.e. to the evoked release of ATP in the absence of added drugs (22.14±5.80 pmol/mg protein, n=4). Panel **()** illustrates the different ability of L-type calcium channels blockade to mostly inhibit ATP release at higher intensity stimulation (60 mM K^+), whereas the blockade of N-/P-type calcium channels mostly affects

ATP release at lower intensity of stimulation (15 mM K^+). The results are mean±SEM of 4-6 experiments. *P<0.05

3.4.4. Large dense core vesicles store larger amounts of ATP than small synaptic vesicles

Since neuropeptides are mainly stored in a type of vesicles with different biophysical properties compared to the that mostly store neurotransmitters (Winkler, 1997), we next took advantage of fractionation protocols designed to separate these different types of vesicle (Whittaker, 1984) to gauge the relative abundance of ATP in

As shown in Figure 3.5 , the fractionation by size and density of different types of synaptic vesicles in an equilibrium density sucrose gradient, confirmed the presence of two main types of vesicles (Coco *et al.*, 2003; Morciano *et al.*, 2005), as gauged by the relative distribution of markers characteristic of synaptic vesicles (*e.g.* synaptophysin; Winkler, 1997), present mostly in SSV (*e.g.* vGluT1; Takamori, 2006) and characteristic markers of LDCV (secretogranin II/chromogranin C; Rosa *et al.*, 1985; Weiler *et al.*, 1990). Importantly, the vesicle pool with higher size and density and displaying an enrichment of secretogranin II (LDCV) also displayed higher levels of ATP, compared to the vesicle pool with small size and density and displaying an enrichment of vGluT1 (SSV) (Fig. 3.5).This larger amount of ATP levels in LDCV fractions was not due to mitochondria contamination since the levels of the mitochondria marker Tom20 were actually higher in the SSV fractions than in the LDCV fractions (Fig. 3.5). that even though we have used as biological preparation cortical synaptosomes from Wistar rats, it is clear that we did not obtain a very high yield in terms of final protein amount (synaptosomes) to look for mitochondrial

densities and consequently, the Western blot revealed a light reactivity of the Tom20 band.

Using synaptophysin immunoreactivity to normalise the vesicular content of SSV and LDCV fractions, we calculated the ratio of Tom20/synaptophysin immunoreactivities between LDCV/SSV (Fig. 3.5 c), which was 0.37±0.16 (n=4) confirming a lower relative amount of mitochondria contaminants in LDCV compared to SSV.

This suggests that ATP is most abundantly located in , although it is also present in SSV typically storing classical neurotransmitters in nerve terminals. Accordingly, vesicular nucleoside transporters (vNUT) immunoreactivity was found in both LDCV and SSV fractions (Fig. 3.5), further arguing for the ability of both LDCV and SSV to store and release ATP. When we calculated the ratio of vNUT/synaptophysin immunoreactivities between LDCV/SSV (Fig. 3.5), we obtained a value of 0.86±0.16 (n=4), which indicates that both LDCV and SSV are endowed with a similar potential ability to store ATP.

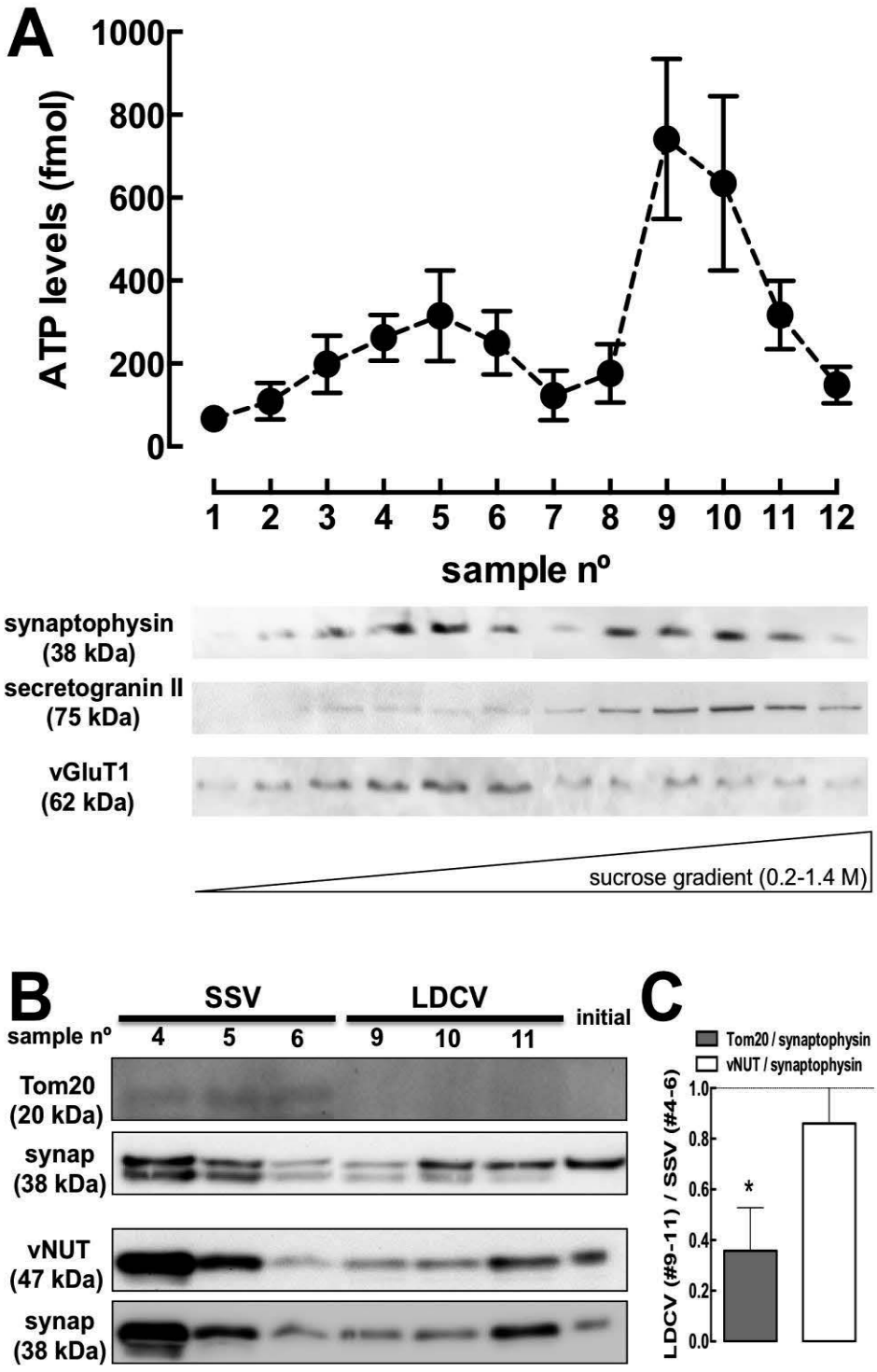

Figure 3.5.: The separation of synaptic vesicles reveals that ATP levels are higher in large dense core vesicles than in small synaptic vesicles. The fractionation in an equilibrium density sucrose gradient (between 0.2 and 1.4 M of sucrose) of synaptic vesicles

from cortical nerve terminals allowed the separation of two main pools of synaptic vesicles: **()** as shown in the Western blots, both pools were labelled with synaptophysin, but one pool was enriched in secretogranin II, a marker of large dense core vesicles (LDCV), and the other pool was enriched in vesicular glutamate transporters type I (vGluT1), a marker of small synaptic vesicles (SSV). Surprisingly, vGlut1 was present in all vesicular fractions, suggesting that either there are minor contaminants of SSV in the LDCV fractions or LDCV are also endowed with the ability to release glutamate. The quantification of ATP using the luciferin-luciferase assay revealed higher levels of ATP in large dense core vesicles than in small synaptic vesicles. ATP levels are presented as mean±SEM of 3 determinations. **()** The localization of ATP in both fractions of synaptic vesicles is in accordance with the localization of the vesicular nucleotide transporter (vNUT) in both vesicle fractions; also the higher levels of ATP in LDCV are unlikely to result from mitochondria contaminants, since the immunoreactivity of Tom20, a mitochondria marker is actually greater in SSV than LDCV fractions. **()** Indeed, the quantification of the ratio of either Tom20 or vNUT immunoreactivities in LDCV and SSV, normalised by the synaptophysin immunoreactivity, showed that LDCV contained lower levels of Tom20 than SSV, whereas vNUT is present in nearly equal amounts in SSV and LDCV fractions. Data are mean±SEM of 4 experiments. *P<0.05 using a one-sample *t* test versus a ratio of 1.

3.4.5. The depletion of the readily-releasable pool of vesicles differentially affects ATP release triggered by low and high K^+

It is well defined that an hyperosmotic stimulus can trigger a vesicular release of neurotransmitters, causing a rapid exhaustion of the readily-releasable pool of vesicles; indeed, previous studies have shown that the exposure of synaptosomes to Krebs-like medium supplemented with 100 mM sucrose triggers a rapid release of classical neurotransmitters such as glutamate and GABA which takes several minutes

to be replenished (Ashton and Ushkaryov, 2005). Fig 3.6 shows that the exposure of synaptosomes to 100 mM sucrose also triggered the release of ATP.

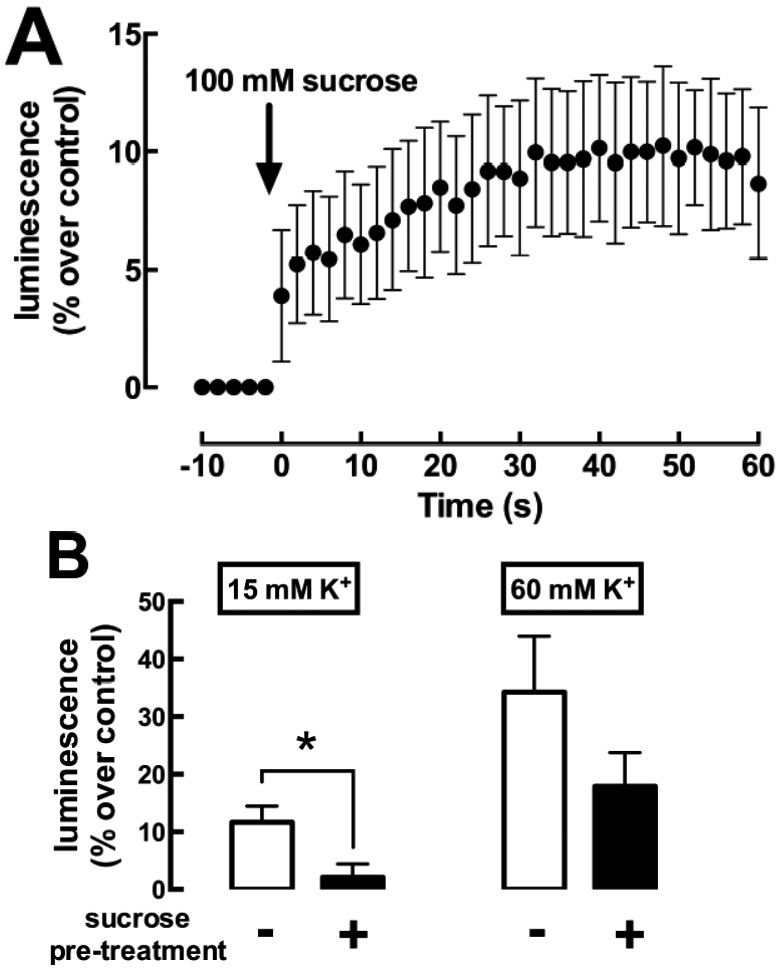

Figure 3.6: A hyperosmotic challenge with 100 mM sucrose, that rapidly exhausts the readily-releasable pool of small synaptic vesicles, triggers a release of ATP and abrogates the subsequent ATP release triggered by low intensity, but not by high intensity stimulation. () Time course of the increased extracellular levels of ATP in cortical synaptosomes upon exposure to a hyperosmotic medium with 100 mM sucrose, as evaluated by the enhanced luminescence with a luciferin-luciferase enzymatic assay. The sucrose-induced ATP release was 24% of ATP release triggered by 60 mM K^+, as shown in panel (). Data are mean±SEM of 5 experiments. **()** The exhaustion of the readily-releasable pool upon exposure to 100 mM sucrose for 1 min largely abrogated the subsequent (within 2 min) ATP release induced by 15 mM K^+, indicating that the low intensity stimulation of nerve terminals

mostly triggers an ATP release originated from the readily-releasable pool of small synaptic vesicles. By contrast, a pre-exposure to 100 mM sucrose only tended to attenuate the subsequent ATP release triggered by 60 mM K^+, indicating that the high intensity stimulation of nerve terminals mostly triggers an ATP release originated from a pool of vesicles different from the readily-releasable pool of small synaptic vesicles. Data are mean±SEM of 5-6 experiments. *P<0.05

However, this sucrose-induced ATP release corresponded to an increase of luminescence above baseline in the luciferin-luciferase assay of 8.4±2.9% (n=5), whereas 60 mM K^+ triggered a significantly (P<0.05) larger increase of 34.3±9.7% (n=6). This shows that while ATP release can be originated from the readily-releasable pool of SSV, the high K^+-induced ATP release is mostly originated from a population of vesicles distinct from the readily-releasable pool of SSV. We next tested the impact of the depletion of the readily-releasable pool of SSV on the ability of low and high K^+ to trigger ATP release. Thus, synaptosomes were first exposed to 100 mM sucrose for 1 min and were then exposed 1 min later to either low (15 mM K^+) or high intensity (60 mM K^+) stimulation. As shown in Fig 3.6 (), the depletion of the readily-releasable pool of SSV largely abrogated the ability of 15 mM K^+ to trigger ATP release (15 mM K^+ increased luminescence above baseline by 11.7±2.8%, n=5, without and by 2.1±1.3%, n=5, exposure to 100 mM sucrose); in contrast, the depletion of the readily-releasable pool of SSV only tended to attenuate (P= 0.20) the ability of 60 mM K^+ to trigger ATP release (60 mM K^+ increased luminescence above baseline by 34.3±9.7%, n=6, without and by 17.9±5.8%, n=5, exposure to 100 mM sucrose) (Fig. 3.6 ()). This further indicates that low intensity stimulation of nerve terminals triggers a release of ATP mostly from the readily-

releasable pool of SSV, whereas high intensity stimulation of nerve terminals mostly triggers a release of ATP from vesicles other than the readily-releasable SSV.

3.5. Discussion

The present results indicate that the evoked release of ATP from nerve terminals occurs in a vesicular-manner but follows a pattern different from classical neurotransmitters, requiring higher intensities of stimulation and mainly depending on calcium entry through L-type calcium channels. Accordingly, higher amounts of ATP were present in LDCV than in SSV. Overall, this prompts the new conclusion that ATP release from nerve terminals mostly occurs in a peptidergic-like manner.

The vesicular nature of ATP release from nerve terminals (Zimmermann, 1994) is heralded by the observations that it is dependent on: 1) extracellular calcium and its entry through voltage-calcium channels, since it is dampened by removing extracellular calcium or by inhibiting voltage-sensitive calcium channels (Fig. 3.3 and Fig. 3.4); 2) the maintenance of a proton-gradient in synaptic vesicles, since it is inhibited by bafilomycin (Fig. 3.3); 3) the integrity of SNARE proteins, since it is abolished by botulinum toxin (Fig. 3.3). This is in agreement with similar observation made in brain (White, 1978; Richardson and Brown, 1987) or amacrine-like retina terminals (Santos *et al.*, 1999), in cholinergic terminals of the Torpedo electric organs (Morel and Meunier, 1981; Unsworth and Johnson, 1990), or neuromuscular junctions (Silinsky and Redman, 1996; Magalhães-Cardoso *et al.*, 2003) and in sympathetic nerve terminals (Todorov *et al.*, 1996). This is also in line with the presence of ATP in synaptic vesicles (Zimmermann, 1994), the identification of vNUT in synaptic vesicles (Larsson *et al.*, 2012) and by the electrophysiological evidence of a quantal release of ATP in preparations such as brain slices (Edwards *et al.*, 1992; Bardoni *et*

al., 1997; Pankratov *et al.*, 2006; Pankratov *et al.*, 2007), neuromuscular junction (Silinsky and Redman, 1996), adrenergic nerves (Stjärne *et al.*, 1994), pancreatic β-cells (Karanauskaite *et al.*, 2009) or PC12 cells (Fabbro *et al.*, 2004). Thus, irrespective of the existence of different pathways for a constitutive release of ATP from different cell types (Bodin and Burnstock, 2001; Corriden and Insel, 2010; Li *et al.*, 2011), it seems that the bulk of activity-dependent release of ATP from nerve terminals occurs in a vesicular manner.

The results reported in the present study do not refute, and even further support, the possible co-release of ATP with classical neurotransmitters. Indeed, the present results show that ATP is located in SSV in agreement with the partial co-localization of vGluT1 and vNUT in synaptic vesicles (Larsson *et al.*, 2012). This would justify a co-release of ATP with classical neurotransmitters such as GABA (Jo and Schlichter, 1999) or , based on the observed ratio of released ATP/ACh similar to that found in synaptic vesicles (Richardson and Brown, 1987). However, it still remains to be established if this putative co-release of ATP with classical neurotransmitters results from the release of co-stored ATP and neurotransmitters or from different synaptic vesicles enriched in either classical neurotransmitters and ATP, as suggested by electrophysiological recordings of quantal activity (Edwards *et al.*, 1992; Pankratov *et al.*, 2006; Pankratov *et al.*, 2007).

The main finding of the present study is that the pattern of ATP release from nerve terminals seems to be mainly different from that of classical neurotransmitters. Thus, ATP release mostly depended on L-type calcium channels (Fig. 3.4) rather than on calcium entry through N- and/or P-type calcium channels that are mostly responsible for triggering the release of classical neurotransmitters in the CNS (Takahashi and Momiyama, 1993). Previous studies of ATP release from other

preparations also noted a predominant impact of L-type calcium channels to control the evoked release of ATP (Santos *et al.*, 1999; Magalhães-Cardoso *et al.*, 2003). Although a significant role of L-type channels to control neurotransmitter release has been difficult to demonstrate, L-type channels are activated by more intense/prolonged stimuli (Miller, 1987) and are mostly responsible for the release of neuropeptides (Verhage *et al.*, 1994). This is in agreement with the requirement of higher intensities (Fig. 3.1) or higher frequencies (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996b) of stimulation of nerve terminals to trigger a robust release of ATP, which is also required to trigger the release of neuropeptides from nerve terminals (Verhage *et al.*, 1994). Furthermore, the fractionation of synaptic vesicles revealed that secretogranin II/chromogranin C-immunopositive vesicles, which correspond to the pool of responsible for the storage of neuropeptides (Winkler, 1997), displayed ATP levels larger that present in the pool of identified as being vGluT1-positive (Fig. 3.5). This suggests that ATP is mostly stored in LDCV and is released from nerve terminals in a peptidergic-like manner. This is in notable agreement with the identification of different vesicles in synaptic processes labelled with vGluT1 and vNUT in the brain parenchyma (Larsson *et al.*, 2012) and with the localization of ATP in LDCV chromaffin cells, pancreatic β cells, blood platelets adrenergic terminals(Zimmermann, 2008). This localization of ATP in LDCV provides a rationale for the temporal and pharmacological dissociation of the release of ATP from the release of classical neurotransmitters in different preparations (*et al.*, 1987; Trachte *et al.*, 1989; Ellis and Burnstock, 1990; Fariñas *et al.*, 1992; Gonçalves *et al.*, 1996; Todorov *et al.*, 1996).

This predominant release of ATP at higher intensities of stimulation of nerve terminals is in line with the previously reported disproportional greater release of

ATP at higher frequencies of nerve stimulation in hippocampal slices (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996b) and at motor nerve endings of the rat diaphragm (Magalhães-Cardoso *et al.*, 2003). Importantly, this pattern of ATP release different from classical neurotransmitter supports the previous suggestion that synaptically originated ATP-derived adenosine is selectively associated with the activation of adenosine A_{2A} receptors controlling synaptic plasticity processes under high stimulation levels (Cunha, 2008). During plasticity processes ATP is preferentially released and its extracellular availability increase. According to the results collected in this chapter, we can speculate that, if it is not catabolised at least into AMP (Fig 3.2), it blocks the evoked released of more ATP, although more evidences are needed to actually draw this conclusion.

Others and we have shown that A_{2A} receptors seem to be selectively engaged in the control of frequency-dependent plastic changes (D'Alcantara *et al.*, 2001; Flajolet *et al.*, 2008; Rebola *et al.*, 2008; Costenla *et al.*, 2011). The activation of A_{2A} receptors depends on ATP-derived adenosine (Cunha *et al.*, 1996a; Ena *et al.*, 2013), based on a physical association between A_{2A} receptors and ecto-5'-nucleotidase (Augusto *et al.*, 2013), the ecto-nucleotidase with rate-limiting activity in nerve terminals (Cunha, 2001) does not control the evoked release of ATP (Fig 3.2 and). Thus, the presently reported disproportionally larger release of ATP from nerve terminals upon intense stimulation provides the missing rationale to understand the selective activation of A_{2A} receptors specifically in 'activated' synapses, *i.e.* in synapses undergoing activity-dependent changes of synaptic efficiency. It is important to emphasize that this synaptically localized release of ATP and formation of adenosine does not conflict with the reported association of ATP release from astrocytes as a source of adenosine activating inhibitory A_1 receptors (Zhang *et al.*,

2003; Pascual *et al.*, 2005; Serrano *et al.*, 2006; Klyuch *et al.*, 2012; Wall and Dale, 2013). In fact, astrocytic-derived ATP is rapidly converted into adenosine, which reaches synaptic A_1 receptors to decrease synaptic transmission to implement processes of heterosynaptic (Zhang *et al.*, 2003; Serrano *et al.*, 2006) or volume transmission (Pascual *et al.*, 2005; Wall and Dale, 2013). Therefore, there is a parallel contribution of extracellular ATP released from different cell types, such as neurons (in particular nerve terminals) and astrocytes, with each particular source dedicated to different signalling goals. Thus, astrocytic ATP-derived adenosine sets a global inhibitory tonus through A_1 receptor activation, whereas nerve-terminal ATP-derived adenosine locally activates A_{2A} receptors selectively in the 'activated' synapses to shutdown A_1 receptor inhibition (Lopes *et al.*, 1999) and assist implementing synaptic plastic changes selectively in the 'activated' synapse. This coordinated global release of ATP from astrocyt and local release of ATP from nerve terminals allows a parallel recruitment of A_1 and A_{2A} receptors in different synapses to assist encoding salience of information in brain circuits (Cunha, 2008). Another layer of complexity of purinergic modulation of brain circuits resides in the possible direct effects of ATP through the engagement of P2 receptors (Pankratov *et al.*, 2009), which autocrine role has been established in astrocytes (Koizumi *et al.*, 2013), but has not been explored in the control of synaptic plasticity processes. , other sources of extracellular ATP, such as axonal release (Hamann and Attwell, 1996; Zhang *et al.*, 2007; Fields, 2011), may be associated with additional non-synaptic regulatory roles of extracellular ATP.

In summary, the present study provides evidence for the preferential release of ATP at higher frequencies of nerve stimulation and this peptidergic-like release of ATP from nerve terminals is proposed to play a role in assisting the encoding of information salience in neuronal circuits. During this thesis we will constantly look to

ATP as the main source of adenosine and consequently we will address the role of its metabolism in plasticity phenomena under high stimulation levels.

CHAPTER 4

ATP-derived adenosine is crucial to sustain LTP on mouse hippocampus through activation of neuronal $A_{2A}R$

4.1. Abstract

ATP is stored in synaptic vesicles and released in an activity-dependent manner in the brain. Extracellular ATP is converted to adenosine in a process mediated by ecto-nucleotidases. CD73 is the rate-limiting player controlling the formation of ATP-derived adenosine. Adenosine is a homoeostatic modulator of excitatory synaptic transmission through activation of inhibitory A_1 receptors, and is also required to assist implementing synaptic plasticity through adenosine A_{2A} receptors. However, in the hippocampus, where the main physiological role of $A_{2A}R$ is the control of synaptic plasticity, the link between CD73 activity and $A_{2A}R$ function remains. We now show that CD73-mediated ATP-derived extracellular adenosine is mandatory for the selective activation of hippocampal $A_{2A}R$ controlling synaptic plasticity since the impairment of hippocampal slice LTP induced by the inhibition of CD73 with α,β-Methylene ADP (AOPCP) is occluded upon a previous superfusion of the slice with the $A_{2A}R$ antagonist SCH58261. AOPCP was also devoid of effects on LTP amplitude in global $A_{2A}R$ KO mice and in forebrain neuron-selective $A_{2A}R$ KO (FB $A_{2A}R$ KO), whereas the inhibition of CD73 significantly decreased LTP amplitude in astrocyte-selective $A_{2A}R$ KO (GFAP $A_{2A}R$ KO) mice, indicating that CD73-derived adenosine solely acts on neuronal $A_{2A}R$.

4.2. Introduction

ATP and adenosine are essential components of all living cells, mediating energy conversion and nucleic acid synthesis. In addition to these functions, both molecules play a pivotal role in extracellular signalling (Burnstock, 2014). Whereas

ATP may function as a neurotransmitter, co-transmitter, gliotransmitter, or even a neuromodulator at specific synapses (Burnstock, 2014; Cunha and Ribeiro, 2000; Butt, 2011; Fields and Stevens, 2000), adenosine fulfils neuromodulatory actions, being produced as function of brain activity (Fredholm *et al.*, 2005). The multiple effects of ATP are not only by purinergic receptors but are also controlled by extracellular ecto-nucleotidases capable of balancing the extracellular concentrations of ATP, ADP, AMP and adenosine (Zimmermann *et al.*, 2012). The most notable of these enzymes is ecto-5'-nucleotidase or CD73, responsible for the final step in the chain: the extracellular formation of adenosine from AMP (Cunha *et al.*, 1996b). Physiologically, adenosine is responsible for assisting in defining the salience of information encoding in brain circuits, mainly acting at the synaptic level by inhibiting the release of glutamate and postsynaptic responsiveness and predominantly modulating excitatory rather than inhibitory synapses (Fredholm *et al.*, 2005). This is achieved through a parallel control of basal excitatory synaptic transmission through inhibitory A_1 receptors and of synaptic plasticity through facilitatory A_{2A} receptors. In excitatory synapses there seems to be a segregation of the role of A_1R and $A_{2A}R$ that depends on the source of extracellular adenosine, with A_1R tonic activation being controlled by astrocytic-derived ATP and post-synaptic adenosine release, whereas $A_{2A}R$ are suggested to be activated by adenosine resulting from a robust vesicular ATP release (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996b), then extracellularly converted to adenosine by ecto-nucleotidases (Zimmermann *et al.*, 2012) to engage $A_{2A}R$, shutting-down A_1R and bolstering NMDA receptors to assist implementing LTP selectively in the activated synapse (Rebola *et al.*, 2008; Cunha, 2008).

Although there are some evidences showing a direct relationship between $A_{2A}R$ and CD73, mainly in brain structures where both are more abundant (striatum) (Augusto *et al.*, 2013), there is still a gap of knowledge to support the direct association between CD73 activity and $A_{2A}R$ function to control synaptic plasticity processes in the hippocampus.

4.3. Material and methods

4.3.1. Animals

We used male \mice with 8-12 weeks of age, obtained from Charles River (Barcelona, Spain), as well as male mice with the same age range from our in-house colonies of $A_{2A}R$ KO, CD73 KO, FB $A_{2A}R$ KO and GFAP $A_{2A}R$ KO mice together with their respective control littermates. The animals were housed in groups of up to five mice per cage in a 12-h light/dark cycle (lights on at 7:00 AM), under controlled temperature (23±2°C), with free access to food and water. Animal procedures were in accordance with the European Community guidelines (Directive 2010/63/EU) and the Portuguese law on animal care (1005/92), and approved by the Animal's Ethics Committee of the Center for Neuroscience and Cell Biology (Orbea 78-201) and the Portuguese Veterinarian Office. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort, following ARRIVE guidelines (Kilkenny *et al.*, 2010). Thus, the animals were anaesthetized in halothane atmosphere before decapitation and, whereas the hippocampus was used in this study, other tissues from these animals were collected for use in different projects at our research center.

4.3.2. Drugs

α,β-Methylene ADP (AOPCP; from Sigma, Sintra, Portugal) was a 10 mM stock solution and used at a supra-maximal and selective concentration of 100 µM (Cunha *et al.*, 1992). SCH58261 (from Tocris, Bristol, UK) was a 5 mM stock solution in and diluted in physiological solutions at a supra-maximal and selective concentration of 50 nM (Lopes *et al.*, 2004) and controll for the impact of the residual amount of DMSO.

4.3.3. Electrophysiological recordings

Extracellular recordings of synaptic transmission and plasticity were carried out in hippocampal slices, as previously described (Kaster *et al.*, 2015). After deep anaesthesia under halothane atmosphere, mice were killed by decapitation; the brain was quickly removed and placed in ice-cold, oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF; in mM: 124.0 NaCl, KCl, 1. $NaHPO_4$, .0 $NaHCO_3$, 2.0 $CaCl_2$, .0 Mg, 10.0 glucose). Using a McIlwain tissue chopper (Brinkmann Instruments, NY, USA), 400 µm-thick slices were cut transverse to the long axis of the hippocampus. edial hippocampal slices were collected and placed in a holding chamber with oxygenated ACSF at 32°C. Slices were allowed to recover for at least 1 h prior to recording, when they were transferred to a submerged recording chamber and continuously superfused with oxygenated ACSF at 3 ml/min kept at 30.5ºC.

A stimulation electrode was placed in the proximal CA1 *stratum radiatum* to stimulate the afferent Schaffer fibres every 15 s using either a Grass S44 or Grass S48 square pulse stimulator (Grass Technologies; Bionic Iberica, Barcelona, Spain). The

evoked field excitatory postsynaptic potentials (fEPSP) were recorded through a glass microelectrode filled with 4 M NaCl (1-2 MΩ resistance) that was coupled to an ISO-80 amplifier (World Precision Instruments, Berlin, Germany). Averages of consecutive responses were acquired with the winLTP software (Anderson and Collingridge, 2001) to quantify the initial slope of the averaged fEPSPs, used to estimate the effect of drugs added to the superfusion solution. We first determined the input/output (I/O) curves to choose a fEPSP with circa 40% of maximal response. Long-term potentiation (LTP) was induced by high-frequency stimulation (HFS - one train of 100 pulses at 100 Hz, for 1 s). LTP was quantified as the percentage change between the average slope of the five taken between 50 and 60 min after LTP induction in relation to the average slope of the fEPSP measured during the 10 min that preceded the HFS. To gauge the impact of a drug on LTP, LTP amplitude was compared in different slices from the same animal in the absence and in the presence of the drug.

4.3.4. Preparation of synaptosomes

Synaptosomes were prepared as previously described (Cunha *et al.*, 1992; Canas *et al.*, 2009). After deep anaesthesia under halothane atmosphere, mice were killed by decapitation. The hippocampi were quickly removed into ice-cold sucrose (0.32 M) solution containing 1 mM EDTA, 10 mM HEPES, 1 mg/ml BSA, pH 7.4 at 4 ºC, supplemented with a cocktail of inhibitors of proteases (CLAP 1%, Sigma) and the antioxidant dithiothreitol (DTT) (1 µM) and homogenized with a teflon Potter-Elvehjem. The homogenates were centrifuged at 3,000 *g* for 10 min at 4 °C and the supernatant was centrifuged at 14,000 *g* for 12 min at 4 ºC. The resulting pellet (P2 fraction) was resuspended in 1 ml of a 45% (v/v) Percoll solution in HEPES buffer

(140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose; pH 7.4), centrifuged at 14,000 *g* for 2 min at 4 ºC and the white top layer (synaptosomal fraction) was collected and washed once in 1 ml Krebs-HEPES buffer (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). After centrifugation, the synaptosomal pellet (which we have previously validated to correspond to synaptic contacts with less than 4% of glia contaminants) was resuspended in appropriate solutions for each assay.

4.3.5. Extracellular catabolism of AMP

The catabolism of extracellular AMP was performed as previously described (Cunha *et al.*, 1992). Briefly, synaptosomes were resuspended in Krebs-HEPES buffer and a 100 µl aliquot was added to 150 µl of Krebs-HEPES buffer kept at 30.5°C. After 10 min of incubation, 250 µl of Krebs-HEPES buffer (at 30.5°C) supplemented with AMP (final concentration of 10 or 30 µM), without or with AOPCP (final concentration of 100 µM), were added at time zero. The kinetics of catabolism of 10 µM AMP was analysed with 392-476 µg of synaptosomal protein by collecting 60 µl aliquots at 0, 1, 2.5, 5, 7.5 and 10 min, whereas the inhibition of the extracellular catabolism of 30 µM AMP was analysed with 47-65 µg of synaptosomal protein by collecting 60 µl aliquots at 0, 2, 5, 10, 15 and 30 min. Each aliquot was immediately centrifuged at 4ºC at 14000 *g* for 15 sec, and the supernatant was stored at -20 ºC for HPLC quantification of AMP and adenosine. The remaining synaptosomes were pelleted by centrifugation (14,000 *g* for 15 sec at 4 °C), homogenized in 2% (v/v) Triton X-100, and used for protein quantification with the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Oeiras, Portugal).

4.3.6. Statistics

Results are given as mean ± SEM of n animals, and significance was considered at $P<0.05$ using Student's *t* test for comparison between two groups and one-way ANOVA (followed by a Bonferroni's post hoc test) for comparison of multiple groups. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, CA, USA).

4.4. Results

4.4.1. CD73 activity is required for the control of hippocampal LTP and depend on $A_{2A}R$ activity

$A_{2A}R$ are responsible for the control of synaptic plasticity in the hippocampus and it is well documented that the blockade of these receptors significantly impairs long-term potentiation (LTP) (Costenla *et al.*, 2011; Fontinha *et al.*, 2009).

We first confirmed that (100 µM) effectively inhibited CD73 activity. Indeed HPLC analysis showed that AOPCP blunted extracellular adenosine formation upon addition of AMP (10 µM) to mouse hippocampal synaptosomes (purified synapses) (Fig. 4.1 a; n=3). CD73 knockout (CD73 KO) mice ensured the selectivity of AOPCP as a CD73 inhibitor, since there was no formation of extracellular adenosine upon AMP addition to hippocampal synaptosomes (Fig. 4.1b; n=3).

In mouse hippocampal slices, AOPCP (100 µM) did not affect basal excitatory synaptic transmission in Schaffer fib-CA1 pyramid synapses (104.7±4.729%; n=5, t_4=1.067, P=0.346 *vs* 100%), excluding an association of ATP-derived adenosine with tonic A_1R activation (Cunha *et al.*, 1998; Costenla *et al.*, 2011).

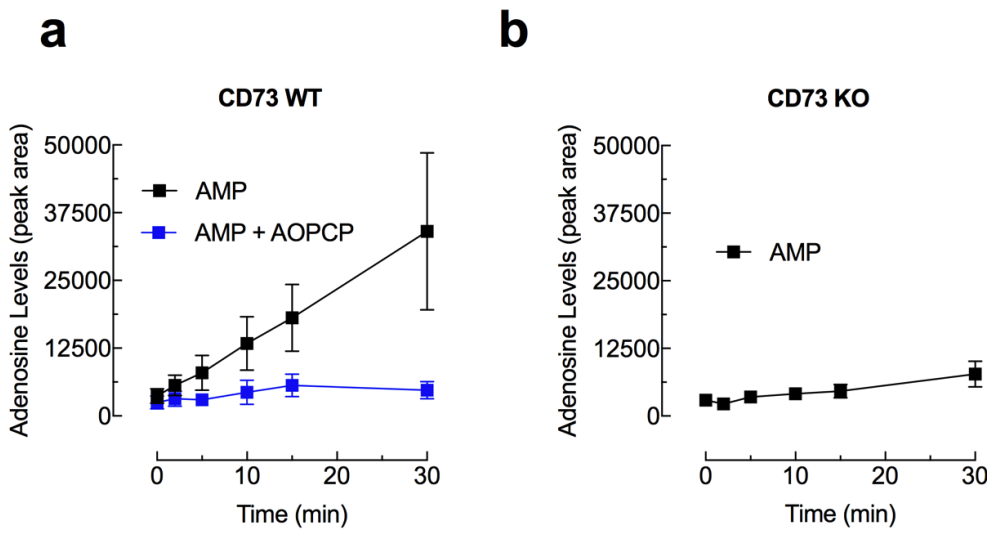

Figure 4.1: CD73 activity is effectively inhibited by AOPCP. (a) The formation of extracellular adenosine after addition of µM AMP to mouse hippocampal synaptosomes is prevented by 100µM AOPCP. **(b)** AOPCP selectively inhibits CD73 since in CD73 KO mice there is no extracellular adenosine formation on addition of AMP.

A first indication that CD73 activity impacts on $A_{2A}R$ function, was collected in electrophysiological recordings in Schaffer fib-CA1 pyramid synapses from hippocampal slices of WT and CD73 KO mice superfused with either the $A_{2A}R$ antagonist SCH58261 (50 nM, 20 min) or the CD73 inhibitor AOPCP 100 µM, 20min). AOPCP selectively decreased LTP amplitude, which was 54.7±4.9% over baseline without and 29.8±7.9% with AOPCP (n=5 t_{11}=4.622, P<0.001) (Fig. 4.2).

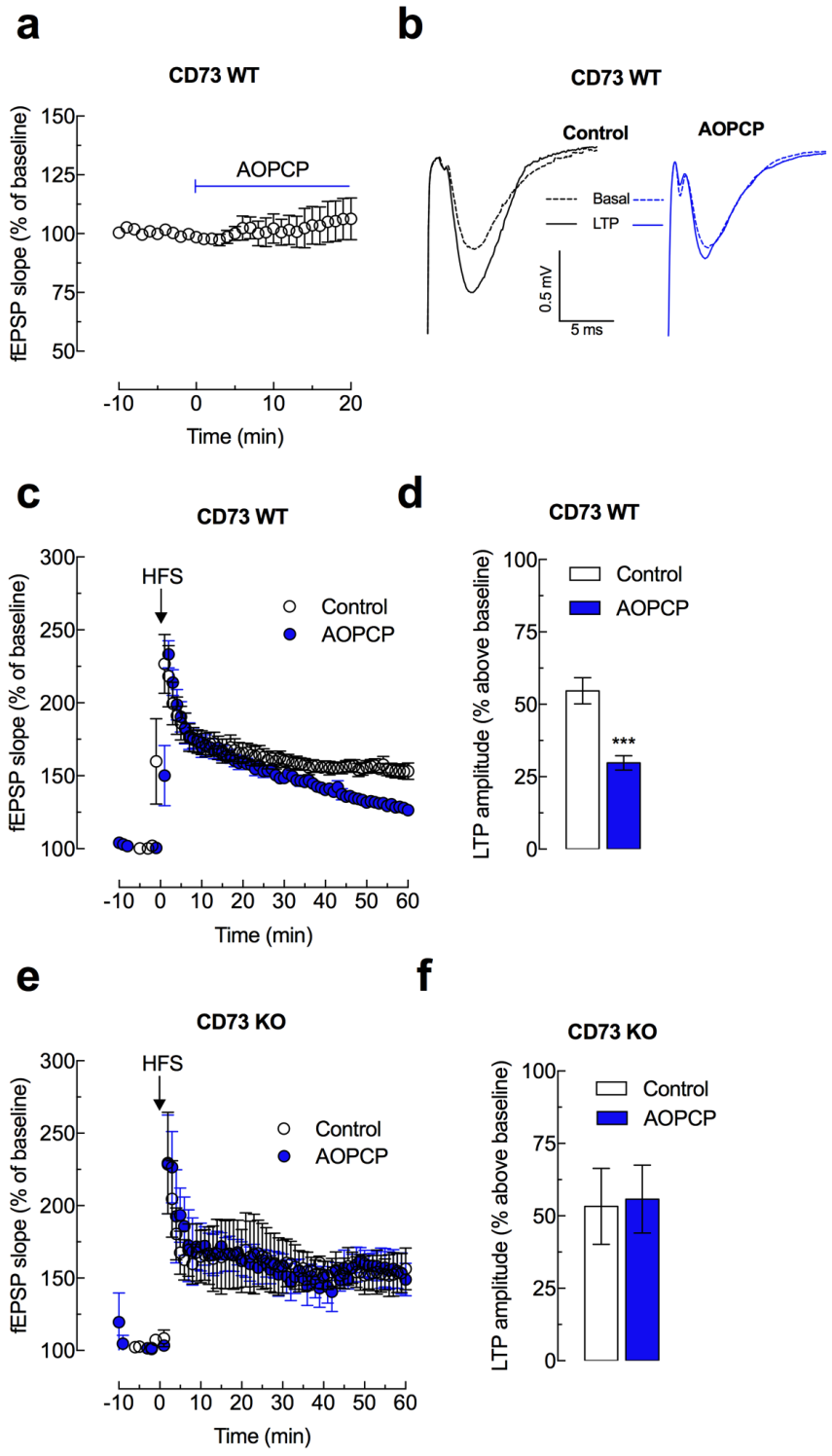

Figure 4.2: ATP-derived adenosine controls LTP in the mousse hippocampus. (a) AOPCP (100 µM) did not affect basal excitatory synaptic transmission in Schaffer fibre-CA1 pyramid synapses; **(b)** fEPSPs recorded CA1 pyramids before (thinner lines) and 60 min after a high-frequency train (HFS) applied to afferent Schaffer fibres (thicker lines) in the absence (black traces) or presence (blue traces) of 100 µM AOPCP, indicating that AOPCP

decreased LTP amplitude, **(c)** as shown in the time course of fEPSP recordings; and quantified in **(d); (e, f)** the use of CD73 KO mice ensured the selectivity of AOPCP to inhibit LTP nce the application of CD73 inhibitor in the mouse hippocampus did not change LTP in slices from CD73 KO mice.

AOPCP was devoid of effects on LTP in CD73 KO mice (Fig. 4.2. e and Fig. 4.2. f). Th effect of AOPCP involve ATP-derived adenosine activating $A_{2A}R$, since the $A_{2A}R$ antagonist SCH58261 (50 nM) phenocopied the AOPCP effect on LTP (n= 5, 65.8±6.7% without, 33.5±11.2% with SCH58261, $F_{2,14}$=4.007, P=0.042); Fig. 4.3 a and occluded further effects of AOPCP on LTP (n=5, 38.6±10.8% with SCH58261+AOPCP, $F_{2,14}$=4.007, P=0.945 vs. SCH58261 alone; Fig. 4.3 b).

Remarkably, the impairment of LTP triggered by SCH58261 was lost in hippocampal slices from CD73 KO mice, despite an apparent impact in magnitude (not statistically significant) when compared with control slices (Fig 4.3 c). Likewise, AOPCP was devoid of effects in LTP amplitude in hippocampal slices from global $A_{2A}R$ KO mice (n=5, 64.8±7.5% without, 55.3±12.5% with AOPCP, t_8=0.703, P=0.502; Fig. 4.3 d).

These results suggest that ATP-derived adenosine generated by CD73 is required for the control of hippocampal LTP through $A_{2A}R$ activation.

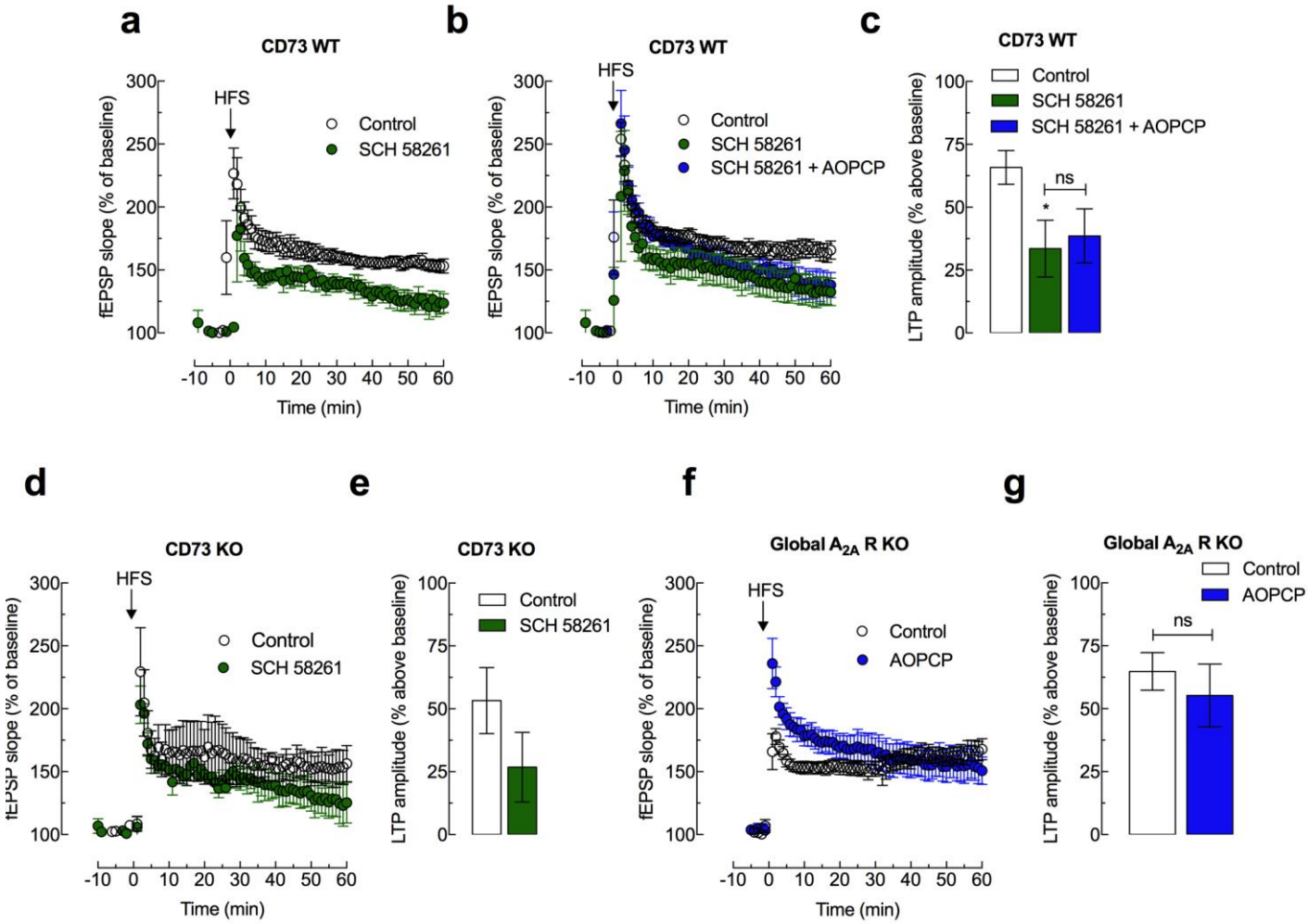

Figure 4.3: ATP-derived adenosine selectively activates $A_{2A}R$ to facilitate long-term potentiation in the CA1 region of mouse hippocampus. (a) $A_{2A}R$ blockade with 50 nM SCH58261 phenocopies the effects of AOPCP on hippocampal LTP and **(b, c)** AOPCP is devoid of effects in the presence of SCH58261; **(d, e)** SCH58261 loose the ability to decrease LTP in CD73 KO mice and **(f, g)** AOPCP in global $A_{2A}R$-KO mice.*P<005, 1

4.4.2. ATP-derived adenosine is preferentially coupled to neuronal $A_{2A}R$ to modulate long-term potentiation

Since $A_{2A}R$ can be found in different brain cell types, such as neurons, astrocytes and microglia (Burnstock *et al.*, 2011) and the two most relevant

populations, neuronal $A_{2A}R$ (Rebola *et al.*, 2008; Viana da Silva *et al.*, 2016) and astrocytic $A_{2A}R$ (Orr *et al.*, 2015; Matos *et al.*, 2015), have been demonstrated to work differently at the synapse (Costenla *et al.*, 2011; Canas *et al.*, 2009; Matos *et al.*, 2012) and perform opposite neuromodulatory effects (Matos *et al.*, 2015), we aimed at further uncovering which cell type $A_{2A}R$ are selectively triggered by CD73-generated adenosine to control LTP. For that we used mice with $A_{2A}R$ deletions either in forebrain neurons (FB $A_{2A}R$ KO) or in astrocytes (GFAP $A_{2A}R$ KO) to test which $A_{2A}R$ population was targeted by ATP-derived adenosine.

In hippocampal slices from mice where the genetic deletion of $A_{2A}R$ was performed only in forebrain neurons (forebrain-selective $A_{2A}R$ KO or FB $A_{2A}R$ KO), and similarly to what was recorded upon pharmacologic inhibition with SCH58261 (Fig 4.3) and in global $A_{2A}R$ KO mice (Fig 4.3), we did not observe any alterations of LTP amplitude upon superfusion with AOPCP (100 µM, 20 min) when compared with control slices (Fig 4.4 a and b). Thus, AOPCP did not affect LTP in FB $A_{2A}R$ KO mice (n=6, 44.4±9.4% without, 40.0±10.8% with AOPCP, t_{17}=0.296, P=0.771; Fig. 4.4). However, in an astrocyte-selective $A_{2A}R$ knockout mouse model (GFAP $A_{2A}R$ KO) the exposure of hippocampal slices to 100 µM AOPCP significantly decreased the magnitude of LTP (Fig 4.4 c and d). Thus, its effect is maintained in GFAP $A_{2A}R$ KO mice (n=6, 58.7±13.6% without, 18.1±5.3% with AOPCP, t_6=2.774, P=0.032) (Fig. 4.4). Such observations suggest that the adenosine derived from ATP metabolism by ecto-nucleotidases, and particularly CD73, is crucial for the modulation of LTP by neuronal, but not astrocytic $A_{2A}R$.

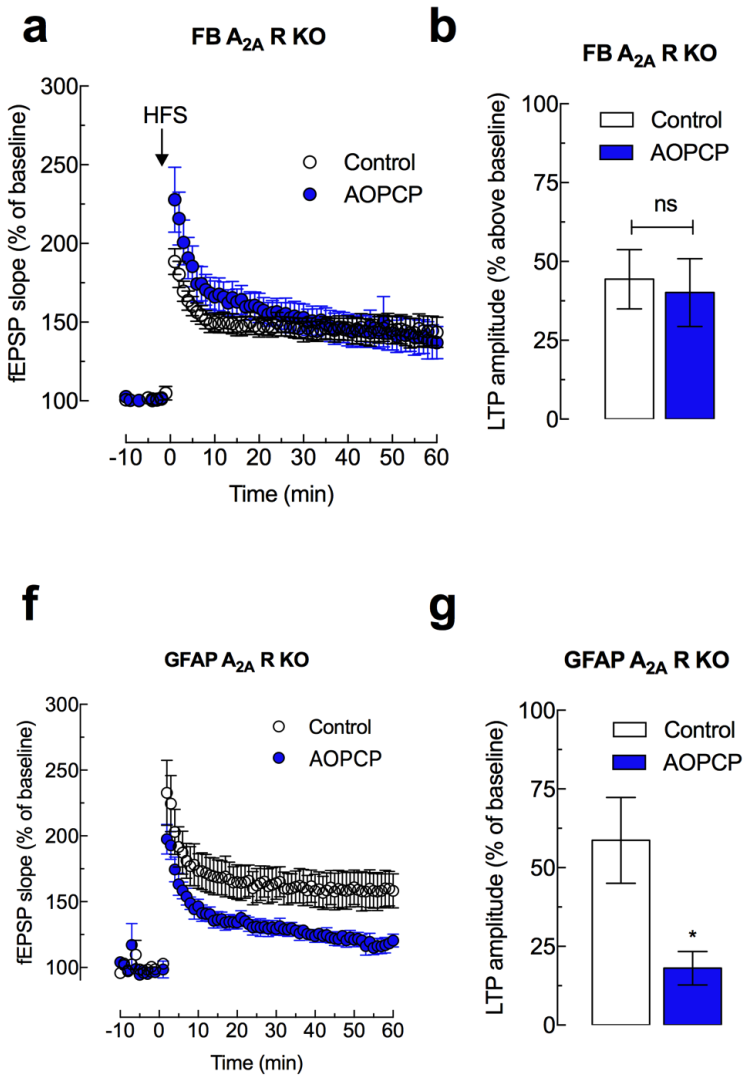

Figure 4.4: ATP-derived adenosine formed by CD73 selectively activates neuronal adenosine A_{2A}R controlling LTP in the mouse hippocampus. (a,b) The effects of AOPCP are blunted in FB A_{2A}RKO but **(c,d)** preserved in GFAP A_{2A}R-KO mice**.**

4.5. Discussion

The present study showed that CD73, through the regulation of ATP catabolism to adenosine, controls the activation of neuronal A_{2A}R, thus impacting on hippocampal LTP. We primarily established that CD73 is needed for eliciting LTP in

the hippocampus, with the blockade of CD73 activity by the inhibitor AOPCP triggering an impairment of hippocampal LTP. Strikingly, the magnitude of this decrease is identical one observed when the antagonist SCH58261 abrogates $A_{2A}R$ function. Notably the effect of SCH58261 was blunted by the genetic deletion of CD73, thus giving a first hint that, in mouse hippocampus, the control of LTP by CD73 depends on the modulation of $A_{2A}R$ activation. The most likely candidate through which this modulation could be exerted is adenosine, since CD73 is responsible for the dephosphorylation of AMP, the final and rate-limiting step of ATP catabolism to adenosine (Cunha, 2001; Kulesskaya *et al.*, 2013), the neuromodulator required for $A_{2A}R$ activation. Indeed, such a key role of adenosine is reinforced by the observation that both the genetic and the pharmacologic of CD73 blunt adenosine production. In the mouse striatum there are also evidences that CD73-mediated formation of extracellular adenosine is responsible for the engagement of $A_{2A}R$ function (Augusto *et al.*, 2013), although there was no assessment of synaptic plasticity, which is the main mechanism controlled by $A_{2A}R$ in the hippocampus (Cunha, 2016).

Basal synaptic transmission, a mechanism controlled by adenosine A1 receptors (A_1R), was also assessed. was revealed an absence of effect of the genetic deletion of CD73, which is not exclusive to physiological conditions but is also observed in situations of brain damage (Zhang *et al.*, 2012). Such observation reinforces the assumption that the activation of A_1R and $A_{2A}R$ is performed by adenosine from different origins (Cunha *et al.*, 2008). Since A_1R activity seems to depend only on tonic adenosine, we assessed if ATP-derived adenosine is targeted towards $A_{2A}R$ activation in hippocampal slices. The pharmacologic and genetic elimination of $A_{2A}R$ fully abrogated the effect on LTP of CD73 inhibition with

AOPCP, demonstrating that the adenosine generated by the catabolism of ATP is fully directed to the control of synaptic plasticity by $A_{2A}R$ in the hippocampus (Fig 4.3). However, $A_{2A}R$ can be found in diverse cell types (Burnstock *et al.*, 2011), from which neuronal $A_{2A}R$ and astrocytic $A_{2A}R$ are the two most studied and are involved in different mechanisms (Costenla *et al.*, 2011; Canas *et al.*, 2009; Matos *et al.*, 2012; Matos *et al.*, 2015). Therefore, it was important to detail if CD73-derived adenosine is specifically targeted towards the activation of a cell-specific $A_{2A}R$ population, an objective achieved through the use of two mouse models where $A_{2A}R$ are eliminated either on forebrain neurons (FB $A_{2A}R$ KO) or in astrocytes (GFAP driven $A_{2A}RKO$). The assessment of changes in LTP uncovered that the effect of CD73 inhibition with AOPCP was abrogated in forebrain $A_{2A}RKO$, similarly to what was observed in the pharmacologic blockade and full genetic deletion of $A_{2A}R$ (global $A_{2A}R$ KO), whereas in hippocampal slices from mice where astrocytic $A_{2A}R$ were eliminated, the effect of AOPCP was maintained. This observation provides the first evidence that adenosine generated from ATP catabolism mediated by CD73 is strictly directed towards the activation of neuronal $A_{2A}R$ and consequent control of synaptic plasticity.

CHAPTER 5

Memory and synaptic deficits present in an early AD mouse model are prevented with the blockade of CD73

5.1. Abstract

Previously (Chapter 4) we showed that adenosine generated from ATP catabolism mediated by CD73 is strictly directed towards the activation of neuronal $A_{2A}R$ and consequent control of synaptic plasticity. Furthermore, it is well described that neuronal $A_{2A}R$ overactivation triggers deficits of synaptic plasticity and memory in early Alzheimer disease. Thus, we posited that this resulted from a combined increased formation of ATP-derived adenosine and $A_{2A}R$ and CD73 upregulation.

In agreement with the concept that ATP is a danger signal in the brain, we report that ATP release from nerve terminals is increased in a mouse model of early AD based on the intracerebroventricular administration (icv) of the neurotoxic peptide $A\beta_{1-42}$, together with an upregulation of CD73 and $A_{2A}R$ in hippocampal synapses. Importantly, this increased CD73 activity appears to be critically required for $A\beta_{1-42}$ to impair synaptic plasticity and memory since the $A\beta_{1-42}$-induced synaptic and memory deficits were eliminated in CD73 KO mice. These observations imply CD73 as a novel target for modulation of AD.

5.2. Introduction

Although CD73-mediated formation of adenosine from ATP has been deemed critical for physiological $A_{2A}R$ functions (Augusto *et al., 2013*), there is increasing evidence that brain insults, such as thse associated with Parkinson's disease (Wang *et al.*, 2015), hypoxia/ischaemia (Melani *et al.*, 2005) or epilepsy-associated seizures (Dale and Frenguelli, 2009), trigger a sustained ATP release from nerve terminals, which acts as a danger signal (Rodrigues *et al.*, 2015). Besides ATP release, in situations of brain damage there is also an upregulation of CD73 and $A_{2A}R$ (Cunha,

2005), bolstering the susceptibility of neurons to injury. Strikingly, in pathologies where there is an exacerbated ATP release, both the pharmacologic and the genetic blockade of $A_{2A}R$ were shown to afford neuroprotection (Cunha *et al.*, 2008; Schwarzschild *et al.*, 2006).

Here we show how the control of adenosine formation from ATP by CD73 in the hippocampus regulates the modulation of synaptic plasticity by selective $A_{2A}R$ activation in both physiological and pathological conditions. Our results suggest that in physiological conditions, ATP-derived adenosine is strictly linked to the activation of neuronal $A_{2A}R$, whereas the induction of synaptic and behavioural dysfunction with Aβ is fully prevented/reverted by CD73 inactivation.

5.3 Material and Methods

5.3.1. Animals

Male C57\6 mice with 8-12 weeks of age, obtained from Charles River (Barcelona, Spain), were used as well as male mice with the same age range from our in-house colonies of $A_{2A}R$ KO, CD73 KO, FB $A_{2A}R$KO and GFAP $A_{2A}R$KO mice together with their respective control littermates. The animals were housed in groups of up to five mice per cage in a 12-h light/dark cycle (lights on at 7:00 AM), under controlled temperature (23±2°C), with free access to food and water. Animal procedures were in accordance with the European Community guidelines (Directive 2010/63/EU) and the Portuguese law on animal care (1005/92), and approved by the Animal's Ethics Committee of the Center for Neuroscience and Cell Biology (Orbea 78-201) and the Portuguese Veterinarian Office. All efforts were made to reduce the

number of animals used and to minimize their stress and discomfort, following ARRIVE guidelines (Kilkenny *et al.*, 2010). Thus, the animals were anaesthetized in halothane atmosphere before decapitation and, whereas the hippocampus was used in this study, other tissues from these animals were collected for use in different projects at our research centre.

5.3.2. Drugs

AOPCPSigma, Sintra, Portugal) wasa 10 mM stock solution and used at a supra-maximal and selective concentration of 100 µM (Cunha *et al.*, 1992). SCH58261 (from Tocris, Bristol, UK) wasa 5 mM stock solution in and diluted in physiological solutions at a supra-maximal and selective concentration of 50 nM (Lopes *et al.*, 2004) and controll for the impact of the residual amount of DMSO. The $A\beta_{1-42}$ peptide fragment was purchased from Bachem (Bubendorf, Switzerland) and dissolved in water at a final concentration 2.25 mg/ml in order to obtain a solution essentially composed of soluble forms of Aβ from monomers up to tetramers (Butt, 2011).

5.3.3. Intracerebroventricular injection

To model early AD (Canas *et al.*, 2009), mice were subjected to an intracerebroventricular (icv) injection (AP: -1.34 cm; ML: -2.13 cm; DV: -2.13 cm) of $A\beta_{1-42}$ or water (vehicle) as a control. The animals were anaesthetized with avertin (1.3% tribromoethanol, 0.8% tert-amyl alcohol) and placed in a stereotactic apparatus (Stoelting, Wood Dale, IL, USA). A single dose of 4 µl (2 nmol) of oligomeric $A\beta_{1-42}$

or a similar volume of water was applied to the left ventricle. The mice were left undisturbed during 14 days before carrying out their behavioural analysis.

5.3.4. Behavioural analysis

All behavioural tests were conducted as previously described (Kaster *et al.*, 2015), between 9:00 AM and 1:00 PM (light phase) in a sound attenuated room with 15 lux illumination, where the had been habituated for 1 h before beginning the tests. The apparatus and objects were cleaned with a 70% alcohol solution and rinsed with water after each session. The behaviour was video-monitored with ANY-maze^{TM} (Stoelting) and ranked by two observers blind to the treatments. Locomotion was evaluated in an open-field arena, measuring the distance travelled during a 10 min period.

On the same day, recognition memory was measured with the object recognition test using the same arena. first underwent a training session after 30 min, in which they were exposed to two identical objects (two 250 ml brown bottles) in the arena for 3 min. The test session was performed 90 min, where two dissimilar objects were presented (a familiar and a novel one, i.e., the 250 ml brown bottle and a 50 ml volumetric flask), and we measured the time spent exploring each object during 3 min, as previously described (Kaster *et al.*, 2015). Object exploration was defined as the orientation of the nose to the object at a distance ≤ 2 cm, touching with forepaws or nose, sniffing and biting the objects, but climbing on the objects was not considered. The preference for exploring the novel object (time exploring novel object/time exploring both objects) was calculated.

Hippocampal-dependent spatial reference memory was assessed the next day using two different tests. First, mice were in a modified Y-maze test: in the

behavioural room with visual cues on the walls, mice first explored the Y-maze during 8 min with one arm blocked and, 90 min, they re-explored for 8 min the Y-maze, now with all arms opened. We scored the number of entries and the time spent in the novel arm as a measure of spatial memory. The mice were then subjected to the object displacement test, where they were first exposed for 3 min to two identical objects (two 250 ml brown bottles) in a particular position in an arena placed in the behavioural room with visual cues. After 90 min, mice were re-exposed for 3 min to the same objects, but one of the objects. The preference for exploring the object that changed location (time exploring moved object/time exploring both objects) was calculated to assess spatial memory.

5.3.5. Electrophysiological recordings

Extracellular recordings of synaptic transmission and plasticity were carried out in hippocampal slices, as previously described (Kaster *et al.*, 2015). After deep anaesthesia under halothane atmosphere, mice were killed by decapitation; the brain was quickly removed and placed in ice-cold, oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF; in mM: 124.0 NaCl, KCl, 1. $NaHPO_4$, 2.0 $NaHCO_3$, 2.0 $CaCl_2$, .0 Mg, 10.0 glucose). Using a McIlwain tissue chopper (Brinkmann Instruments, NY, USA), 400 µm-thick slices were cut transverse to the long axis of the hippocampus. edial hippocampal slices were collected and placed in a holding chamber with oxygenated ACSF at 32°C. Slices were allowed to recover for at least 1 h prior to recording, when they were transferred to a submerged recording chamber and continuously superfused with oxygenated ACSF at 3 ml/min kept at 30.5ºC.

A bipolar electrode was placed in the proximal CA1 *stratum radiatum* to stimulate the afferent Schaffer fibres every s using either a Grass S44 or Grass S48

square pulse stimulator (Grass Technologies; Bionic Iberica, Barcelona, Spain). The evoked field excitatory postsynaptic potentials (fEPSP) were recorded through a glass microelectrode filled with 4 M NaCl (1-2 MΩ resistance) and placed in the *stratum radiatum* of the CA1 area . Averages of consecutive responses were acquired with the winLTP software (Anderson and Collingridge, 2001) to quantify the initial slope of the averaged fEPSP, used to estimate the effect of drugs added to the superfusion solution. We first determined the input/output (I/O) curves to choose a fEPSP with *circa* 40% of maximal response. Long-term potentiation (LTP) was induced by high-frequency stimulation (HFS - one train of 100 pulses of 1 Hz for 1 s). LTP was quantified as the percentage change between the average slope of the five potentials taken between 55 and 60 min after LTP induction in relation to the average slope of the fEPSP measured during the 10 min that preceded the HFS. To gauge the impact of a drug on LTP, LTP amplitude was compared in different slices from the same animal in the absence and in the presence of the drug.

5.3.6. Preparation of synaptosomes

Synaptosomes were prepared as previously described (Cunha *et al.*, 1992; Canas *et al.*, 2009). After deep anaesthesia under halothane atmosphere, mice were killed by decapitation. The hippocampi were quickly removed into ice-cold sucrose (0.32 M) solution containing 1 mM EDTA, 10 mM HEPES, 1 mg/ml BSA, pH 7.4 at 4 ºC, supplemented with a cocktail of inhibitors of proteases (CLAP 1%, Sigma) and the antioxidant dithiothreitol (1 µM) and homogenized with a teflon Potter-Elvehjem. The homogenates were centrifuged at 3,000 *g* for 10 min at 4 °C and the supernatant

was centrifuged at 14,000 *g* for 12 min at 4 ºC. The resulting pellet (P2 fraction) was resuspended in 1 ml of a 45% (v/v) Percoll solution in HEPES buffer (140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose; pH 7.4), centrifuged at 14,000 *g* for 2 min at 4 ºC and the white top layer (synaptosomal fraction) was collected and washed once in 1 ml Krebs-HEPES buffer (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). After centrifugation, the synaptosomal pellet (which we have previously validated to correspond to synaptic contacts with less than 4% of glia contaminants (Cunha *et al.*, 1992; Canas *et al.*, 2009) was resuspended in appropriate solutions for each assay.

5.3.7. Evoked ATP release

The release of ATP was measured on-line using the luciferin-luciferase assay, as previously described (Cunha *et al.*, 1996b; Ferreira *et al.*, 2015). Briefly, synaptosomes were resuspended in Krebs-HEPES buffer and a 35 µl aliquot (with *circa* 40 µg of protein) and 15 µl of ATP assay mix (containing luciferin and luciferase; from Sigma) were added to 150 µl of Krebs-HEPES buffer. This suspension was equilibrated at 25 ºC from 10 min to ensure the functional recovery of the synaptosomes and transferred to a well of a white 96-well plate, which was maintained for 1 min at 25 ºC inside a luminometer (Victor^3; Perkin Elmer, ILC, Lisbon, Portugal) before initiating the recording of the electrical signal generated by the photomultiplier. We first measured the basal outflow of ATP during 60 sec, before triggering the evoked release of ATP with a chemical stimulus consisting of a Krebs-HEPES solution with an isomolar substitution of NaCl by 32 mM of KCl. After this chemical stimulation, the light levels were recorded for an additional 200 sec. The

evoked release of ATP was calculated by integration of the area of the peak upon subtraction of the estimated basal ATP outflow and always discounting the (minor) variation of luminescence caused by possible mechanic or osmolarity alterations, which was evaluated in a parallel assay in the same batch of nerve terminals by adding medium with an amount of Krebs-HEPES solution equal to the amount of K^+-rich solution used in the test situation.

5.3.8. Extracellular catabolism of AMP

The catabolism of extracellular AMP was performed as previously described (Cunha *et al.*, 1992). Briefly, synaptosomes were resuspended in Krebs-HEPES buffer and a 100 µl aliquot was added to 150 µl of Krebs-HEPES buffer kept at 30.5°C. After 10 min of incubation, 250 µl of Krebs-HEPES buffer (at 30.5°C) supplemented with either AMP (final concentration of 10 or 30 µM), without or with AOPCP (final concentration of 100 µM), were added at time zero. The kinetics of catabolism of 10 µM AMP was analysed with 392-476 µg of synaptosomal protein by collecting 60 µl aliquots at 0, 1, 2.5, 5, 7.5 and 10 min, whereas the inhibition of the extracellular catabolism of 30 µM AMP was analysed with 47-65 µg of synaptosomal protein by collecting 60 µl aliquots at 0, 2, 5, 10, 15 and 30 min. Each aliquot was immediately centrifuged at 4ºC at 14000 *g* for 15 sec, and the supernatant was stored at -20 ºC for HPLC quantification of AMP and adenosine. The remaining synaptosomes were pelleted by centrifugation (14,000 *g* for 15 sec at 4 °C), homogenized in 2% (v/v) Triton X-100, and used for protein quantification with the assay (Thermo Fischer Scientific, Oeiras, Portugal).

5.3.9. Binding assay

The binding assays were performed as previously described (Lopes *et al.*, 2004). Briefly, synaptosomes were vigorously resuspended in a pre-incubation solution (containing 50 mM Tris, 1 mM EDTA, 2 mM EGTA, pH 7.4) at 4 ºC and centrifuged at 25,000 *g* for 20 min at 4 ºC; the supernatants were discarded and the pellet, corresponding to the synaptosomal membranes, was resuspended in the pre-incubation solution and a sample was collected to determine the protein concentration using the BCA assay. Adenosine deaminase (ADA, 2 U/ml; Roche, Amadora, Portugal) was added and the membranes were incubated for 30 min at 37 ºC to remove endogenous adenosine. The mixtures were centrifuged at 25,000 *g* for 20 min at 4 ºC, and the pelleted membranes were resuspended in Tris-Mg solution (containing 50 mM Tris and 10 mM $MgCl_2$, pH 7.4) with 4 U/ml of ADA. Binding with 3 nM of the selective $A_{2A}R$ antagonist, ^{3}H-SCH58261 (specific activity of 77 Ci/mmol; prepared by GE Healthcare and offered by Dr. E. Ongini, Schering-Plough, Italy) was performed for 1 h at room temperature with 187-223 μg of protein, with constant swirling. The binding reactions were stopped by addition of 4 ml of ice-cold Tris-Mg solution and filtration through Whatman® GF/C glass microfibre filters (GE Healthcare, , Portugal) in a filtration system (Millipore; , Portugal). The radioactivity was measured in a Tricarb 2900TR liquid scintillation counter (Perkin Elmer) at least 12 h after adding 2 ml of scintillation liquid (AquaSafe 500Plus, Zinsser Analytic, Frankfurt, Germany), with a counting efficiency of 55-60%. The specific binding was expressed as fmol/mg protein and was estimated by subtraction of the non-specific binding, which was measured in the presence of 12 μM of xanthine amine congener

(Sigma), an antagonist of adenosine receptors. All binding assays were performed in duplicate.

5.3.10. Western blot

Synaptosomal membranes were resuspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% IGEPAL® CA-630, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) plus (Roche), 0.1 mM DTT and 0.1 mM phenylmethylsulfonylfluoridehe amount of protein in synaptosomal membranes was determined using the BCA to carefully dilute all extracts to a final concentration of 2 μg protein/μl in SDS-PAGE buffer containing (in mM): Tris 83.3, DTT 100, 10.3% SDS, 30% glycerol, and 0.012% bromophenol blue. After denaturation at 95ºC for 10 min, equal amounts of protein of each sample and prestained precision protein standards (Bio-Rad, Alfragide, Portugal) were separated on a 10% SDS-PAGE and then electroblotted onto PVDF membranes. After incubation for 1 h at room temperature (RT) in TBS-T (in mM: NaCl 150, Tris–HCl 25, pH 7.6, with 0.1% Tween 20), containing 5% to block nonspecific binding, the membranes were incubated overnight at 4 ºC in TBS-T containing 1% BSA with the primary antibodies: rabbit polyclonal anti-CD73 (1:300), mouse monoclonal anti-synaptophysin (1:6000) and mouse monoclonal anti-SNAP25 (1:6000) or mouse monoclonal anti-syntaxin (1:2000). After washing, the membranes were incubated for 2 h at RT in TBS-T with 1% BSA and with the appropriate alkaline-phosphatase-conjugated anti-rabbit or anti-mouse secondary antibody (1:20,000; Amersham) before revealing the membranes with ECF (Amersham). All membranes were visualized with an imaging system (Chemidoc Plus, Bio-Rad) and quantified with the Quantity One software (Bio-Rad). The membranes were re-probed and tested for β-tubulin-III immunoreactivity (1:1,000; Abcam, Frilabo, Maia,

Portugal) to confirm that similar amounts of protein were applied to the gels (Kaster *et al.*, 2015).

5.3.11. Statistics

Results are given as mean ± SEM of n animals, and significance was considered at $P<0.05$ using Student's *t* test for comparison between two groups and one-way ANOVA (followed by a Bonferroni's post hoc test) or two-way ANOVA (followed by a Newman-Keuls post hoc test) for comparison of multiple groups. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, CA, USA).

5.4 Results

5.4.1. Intracerebroventricular $A\beta_{1-42}$ administration triggers an overfunctioning of the purinergic system.

Since neuronal $A_{2A}R$ overactivation triggers deficits of synaptic plasticity and memory in early AD (Pagnussat *et al.*, 2015; Viana da Silva *et al.*, 2016) we posited that this resulted from a combined increased formation of ATP-derived adenosine and $A_{2A}R$ upregulation (Fig. 5.1 a).

Furthermore the effect of CD73 inhibition promotes a response on LTP as the blockade of $A_{2A}R$, and taking into account that changes in this form of synaptic plasticity have been correlated with behavioural alterations (Kessels and Malinow, 2009), we found relevant to assess if the genetic deletion of CD73 altered hippocampal-related behaviour.

Indeed, hippocampal synaptosomes of β-amyloid ($A\beta_{1-42}$, 2 nmol) icv-treated mice modelling early AD Canas *et al.*, 2009) display: i) larger ATP release upon K^+-depolarization (n=12, 45.8±5.1% vs. 32.0±3.9% light /mg protein in control, t_{20}=2.150, *P*=0.044; Fig. 5.1 b); ii) increased CD73 density (n=3, 244.2±69.3% larger immunodensity, n=3, t5=3.525, P=0017; Fig. 5.1 c); iii) increased CD73 activity (AMP extracellular catabolism of 1.054±0.056 vs. 0.507±0.018 nmol/min/mg protein in control, n=5, t_{10}=9.361, *P*=0.008; Fig. 5.1 d); iv) increased $A_{2A}R$ density (specific ^{3}H-SCH58261 binding of 80.5±4.8 vs. 46.7±8.1 nmol/mg protein in control, n=3, t_4=3.591, *P*=0.023; Fig. 5.1).This prompts the hypothesis that the excessive formation of ATP-derived adenosine is the main responsible for early AD alterations. This entails that CD73 elimination should blunt the triad of modifications characteristic of early AD (Coleman *et al.*, 2004), namely impaired reference memory, synaptic dysfunction and loss of synaptic markers, all recapitulated after $A\beta_{1-42}$ administration (Canas *et al.*, 2009).

Remarkably, in CD73 KO mice no behavioural changes were observed in hippocampal-related tasks (Fig 5.2), with the performance in spatial and recognition memory tests being identical between WT and KO mice. This is a striking result since a similar pattern is perceived for the same tests upon $A_{2A}R$ blockade (Lopes *et al.*, 2015; Viana da Silva *et al.*, 2016), thus suggesting that the elimination of CD73 generates a phenotype similar to that of $A_{2A}R$ blockade. Moreover, $A_{2A}R$ KO mice only display a phenotypic alteration after being subjected to a noxious stimulus (Viana da Silva *et al.*, 2016). Therefore, to assess if the absence of phenotype of CD73 KO mice can also be altered in pathologic conditions, we performed an icv administration of oligomeric $A\beta_{1-42}$, a synaptotoxic peptide that is proposed as the

culprit of Alzheimer's disease (Ferreira and Klein, 2011) and probed for behavioural changes in a panel of tests with a strong focus on hippocampal-dependent functions.

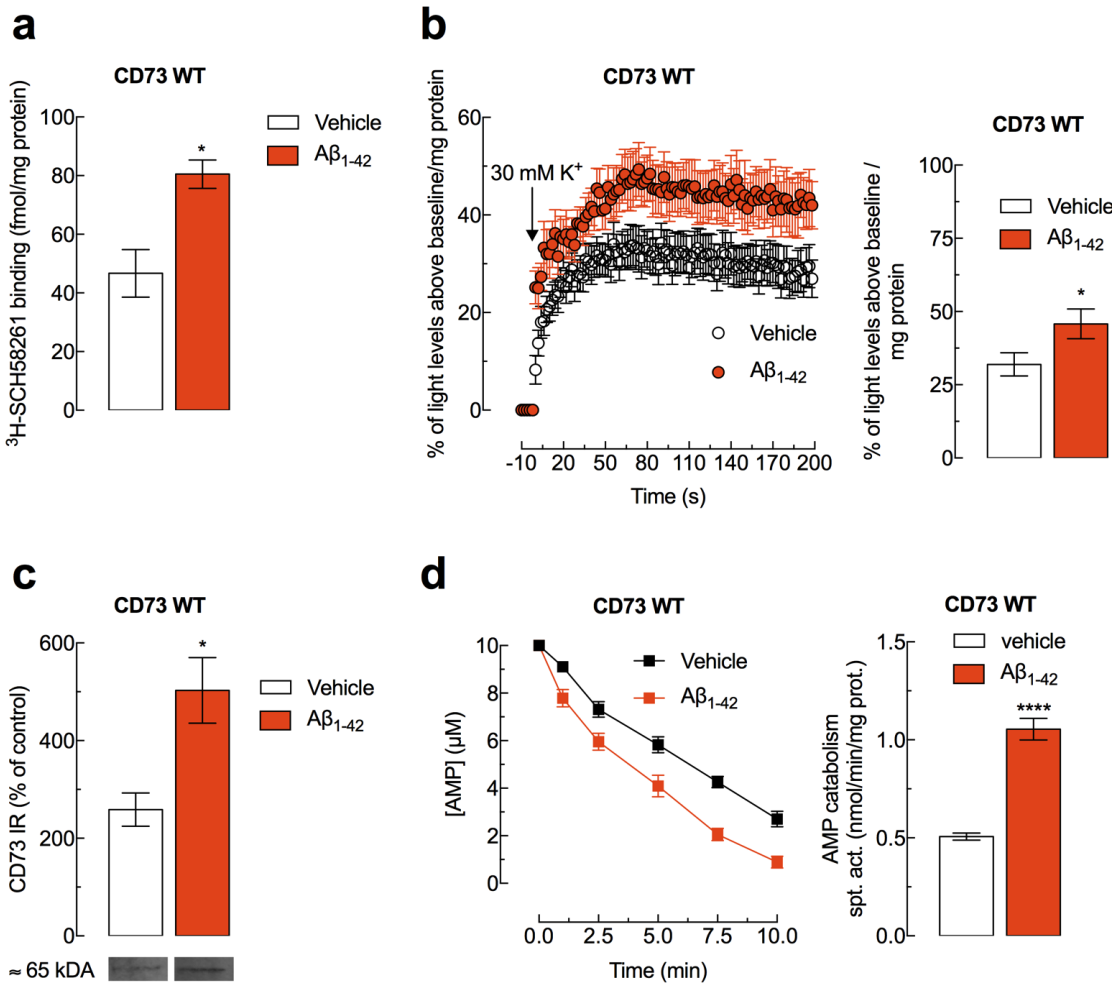

Figure 5.1: Increased formation of ATP-derived adenosine and $A_{2A}R$ upregulation in the hippocampus in an early AD model. In a mouse model of early AD based on exposure to $A\beta_{1-42}$ (2 nmol, icv, orange) compared to vehicle-treated (black), hippocampal synaptosomes display a higher $A_{2A}R$ density assessed by binding density of 2nM ^{3}H-SCH58261 (a); a higher luminometrically-detected release of ATP upon exposure to 30 mM K^+ (b), a higher Western blot immunoreactivity of CD73 (c), a higher CD73 activity assessed by formation of extracellular adenosine after addition of 10 μM AMP (d) *P<0.05, ****P<0.001, unpaired, two-tailed t-test.

Aβ-treated CD73 WT mice displayed a lower performance in the object displacement test (localization index: n=5-7, 30.5±5.8% *vs*. vehicle: 62.4±6.8%, $F_{1,19}$=13.1, *P*=0.002; Fig. 5.2 a), in the object recognition test (recognition index: n=5-7, 39.8±5.3% *vs*. vehicle: 84.2±3.8%, $F_{1,20}$=9.739, *P*=0.005; Fig. 5.2 b) and in the modified Y-maze test (time in the novel arm: n=5-7, 120.2±13.1s *vs*. vehicle: 166.3±8.0s, $F_{1,19}$=4.414, P=0.049; Fig. 5.2c) without locomotor alterations in an open field test (Fig. 5.2 d). contrast, Aβ-treated CD73 KO mice preserved their localization index (n=5-7, 53.0±3.8% *vs*. vehicle: 61.0±8.0%, $F_{1,19}$=5.304, *P*=0.033; Fig. 5.2 a), their recognition index (n=5-7, 74.4±7.6% *vs*. vehicle: 72.0±7.7%, $F_{1,20}$=12.13, P=0.002; Fig. 5.2b) and their time in the novel arm (n=5-7, 161.8±6.3s *vs*. vehicle: 157.1±14.8s, $F_{1,19}$=6.543, *P*=0.019; Fig. 5.2 c) without locomotor alterations (Fig. 5.2 d).

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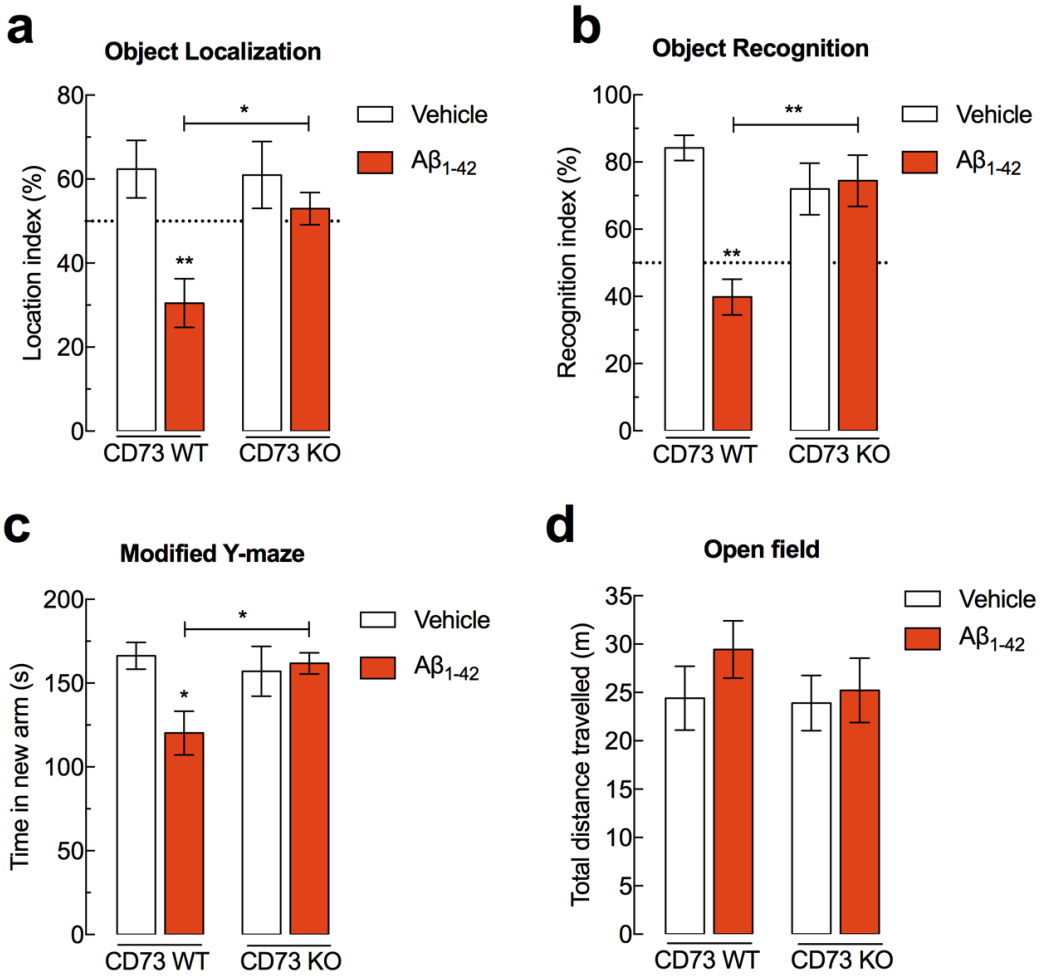

Figure 5.2: Genetic CD73 deletion prevents memory and synaptic impairments in a mouse model of early AD. Male CD73 KO mice and their wild type (WT) littermates (10 weeks old) were intracerebroventricularly injected with $A\beta_{1-42}$ (2nmol). After 14 days, $A\beta_{1-42}$ decreased memory performance in the object displacement test (**a**), object recognition test (**b**) and modified Y-maze (**c**) in WT but not CD73 KO mice, all without locomotor alterations (**d**).

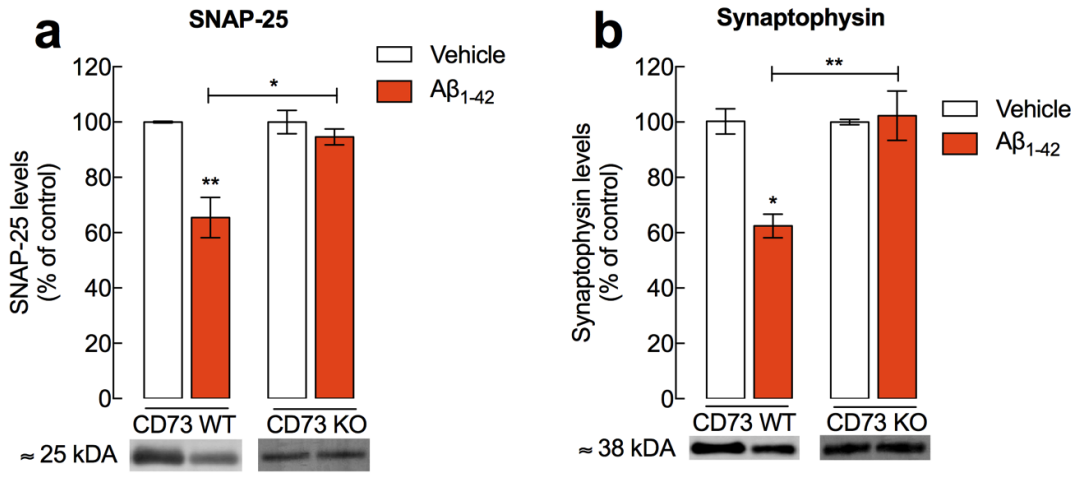

Figure 5.3: Genetic CD73 deletion prevents synaptic impairments in a mouse model of early AD. Male CD73 KO mice and their wild type (WT) littermates (10 weeks old) were intracerebroventricularly injected with $A\beta_{1-42}$ (2nmol). After 14 days, $A\beta_{1-42}$ decreased density of the synaptic proteins SNAP25 (**a**) and synaptophysin (**b**)*P<0.05, 2-way ANOVA.

5.4.2. Synaptic impairment triggered by $A\beta_{1-42}$ is abolished both by the pharmacologic and genetic elimination of CD73

$A_{2A}R$ have been deemed as critical and necessary for the deleterious effects of Aβ on behaviour and synaptic damage (Canas *et al.*, 2009; Viana da Silva *et al.*, 2016). Taking into account that the control of $A_{2A}R$ function by CD73 relies on the catabolism of ATP to adenosine, we assessed how Aβ exposure impacts on the triad ATP/CD73/$A_{2A}R$ overfunctioning the purinergic system as previously described. In the mice hippocampus, $A\beta_{1-42}$ triggers changes in adenosine control, not only boosting the release of ATP, but also concomitantly increasing the levels of the rate-limiting player in the conversion of ATP to adenosine, CD73, and the predicted target for this adenosine, neuronal $A_{2A}R$. These changes in the release of ATP, CD73 levels and $A_{2A}R$ densities are likely to translate into changes in synaptic plasticity. As Aβ

peptides have been shown to impair LTP (Wang *et al.*, 2009), we assessed how the elimination of CD73, both genetic and pharmacologic, affects the Aβ-induced alterations of this type of synaptic plasticity.

As shown on Fig 5.4, electrophysiological recordings in Schaffer fib-CA1 pyramid synapses of hippocampal slices from WT mice icv-injected with $A\beta_{1-42}$ display a significant impairment of long-term potentiation (LTP) when compared with mice injected with vehicle $A\beta_{1-42}$ -treated CD73 WT mice displayed lower LTP amplitude (n=5-7, 30.9±6.2% *vs*. vehicle: 63.5±5.1%, $F_{1,21}$=6.284, P=0.021; Fig. 5.4 a), whereas this did not occur for CD73 KO mice (LTP in Aβ-treated: n=5-7, 63.0±9.4% *vs*. vehicle: 63.7±7.7%, $F_{1,21}$=9.925, P=0.005; Fig. 5.4 a). Notably, pharmacological inhibition of either CD73 or $A_{2A}R$ acutely reverted $A\beta_{1-42}$-induced LTP deficits. Hippocampal slices from $A\beta_{1-42}$ -treated mice exposed to 100 μM AOPCP recovered their LTP (n=5-7, 24.8±6.7% without, 48.8±7.6% with AOPCP, $F_{1,15}$=17.69, P<0.001 (0.0008); Fig. 5.4 c) to values similar to vehicle-treated mice (n=5-7, 57.4±5.4%, $F_{1,15}$=0.153, P=0.702 *vs*. $A\beta_{1-42}$-treated mice without AOPCP; Fig. 5.4 c). As previously reported (Chen *et al.*, 2013), 50 nM SCH58261 also recovered LTP (n=5-7, 24.8±6.7% without, 51.9±7.3% with SCH58261, $F_{1,16}$=14.87, P<0.001; Fig. 5.4 b) to values similar to vehicle-treated mice (n=5-7, 57.4±5.4%, $F_{1,16}$=0.279, P=0.604 *vs*. $A\beta_{1-42}$-treated mice without SCH58261; Fig. 5.4 b).

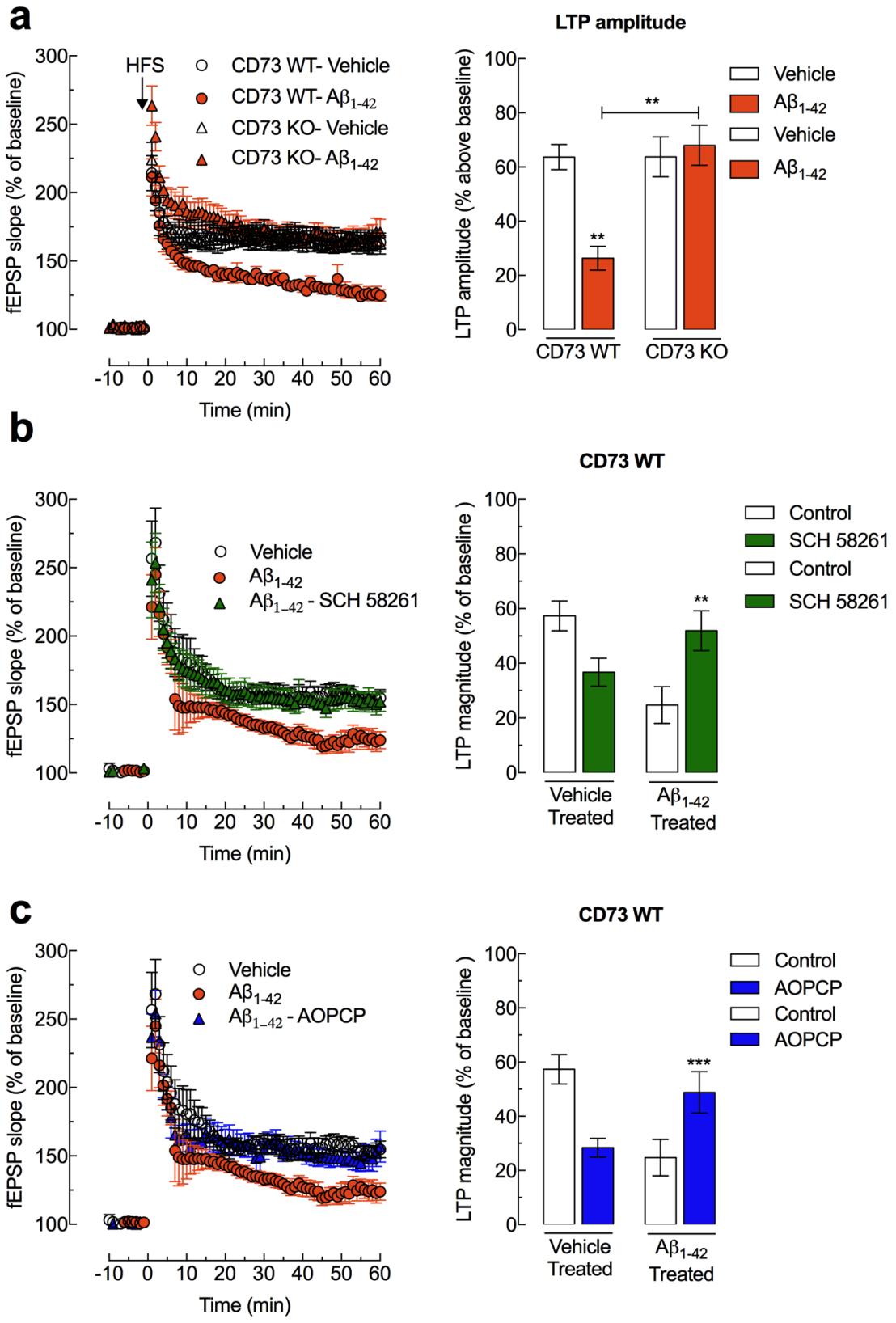

Figure 5.4: Genetic CD73 deletion prevents plasticity impairments in a mouse model of early AD. Male CD73 KO mice and their wild type (WT) littermates (10 weeks old) were intracerebroventricularly injected with $A\beta_{1\text{-}42}$ (2nmol). $A\beta_{1\text{-}42}$ decreased LTP amplitude in WT

but not CD73 KO mice. This lower LTP in $A\beta_{1-42}$-treated mice was reverted to control values by acutely applying () or (**c**) to slices. *P<0.05, 2-way ANOVA

These observations indicate that the elimination of CD73, either genetic or pharmacologic, is able to counteract the synaptic plasticity deficits induced by $A\beta_{1-42}$ in a manner identical to the blockade of $A_{2A}R$, thus further reinforcing the idea that the abrogation of CD73 activity generates a phenocopy of $A_{2A}R$ elimination.

5.5. Discussion

Besides the control of neuronal $A_{2A}R$ by CD73 in the hippocampus, the data from electrophysiological recordings (Fig 5.4) in conjugation with the results of hippocampal-dependent behavioural tests (Fig 5.2) allowed us to uncover another aspect of the tight link between CD73 activity and $A_{2A}R$ function: similarly to what has been observed in $A_{2A}R$ KO mice, in CD73 KO mice there is an absence of phenotypical alterations. Indeed, although previous studies point out to improvements of striatal-dependent behaviour output upon genetic deletion of CD73 (Augusto *et al.*, 2013; Kulesskaya *et al.*, 2013) as occurs upon striatal $A_{2A}R$ blockade (Wei *et al.*, 2011), we did not observe any changes in spatial and recognition memory or in locomotor behaviour (Fig 5.2). However, the absence of phenotype in $A_{2A}R$ KO is not maintained in pathological conditions. Indeed, it has been reported a robust neuroprotection by $A_{2A}R$ blockade in animal models of brain diseases such as Alzheimer's or Parkinson's diseases, epilepsy or ischaemia (Cunha *et al.*, 2008; Pedata *et al.*, 2005; Canas *et al.*, 2009). Therefore, we verified if the manipulation of CD73 afforded a neuroprotective benefit against brain insults identical to that observed for $A_{2A}R$ blockade (Cunha, 2005; Chen *et al.*, 2007). $A\beta_{1-42}$ is a neurotoxic

peptide deemed as culprit of Alzheimer's disease (AD) (Ferreira and Klein, 2011) and whose effect on synaptotoxicity and behavioural impairment was shown to be prevented by the elimination of $A_{2A}R$ (Canas *et al.*, 2009; Viana da Silva *et al.*, 2016). We observed that the genetic elimination of CD73 was also able to prevent the behavioural deficits and synaptotoxicity triggered by the icv administration of Aβ (Fig 5.2). This similarity between the two knockout models points out to an exacerbation of the mechanism observed in physiological conditions, where CD73 control $A_{2A}R$ via the modulation of catabolism to . Moreover, under brain pathological conditions, the enhancement in extracellular levels of ATP (Di Virgilio, 2000) has been proposed as a danger signal and occurs along with a parallel increase of the activity of CD73 and the density of $A_{2A}Rs$ (Cunha, 2005). Indeed, we observed that the prolonged exposure to Aβ increased the release of ATP and bolstered CD73 levels and $A_{2A}R$ density (Fig 5.1), unbalancing synaptic plasticity processes in CA1 region (Fig 5.4).

Changes in behaviour are known to reflect alterations of synaptic plasticity (Kessels and Malinow, 2009), which in turn is affected both by the synaptotoxicity (Fig 5.4) and loss of adenosine control triggered by $Aβ_{1-42}$. Modifications of LTP induced by Aβ were prevented by the genetic deletion of CD73 (Fig 5.4), much alike what we observed for behavioural changes and impairment of synaptic markers (Fig 5.3). However, besides the preventive action of CD73 deletion it was also crucial to assess the potential of CD73 inhibition in reverting a noxious stimulus, as a possible therapeutic option in brain disorders. In fact, exposure of hippocampal slices of mice injected icv with Aβ was able to significantly revert the impairment of LTP (Fig 5.4 c) and, strikingly, this effect is identical in magnitude to that achieved with the $A_{2A}R$

antagonist SCH58261 (Fig 5.4 b). This further reinforces our contention of a crucial link between CD73 activity and $A_{2A}R$ control of hippocampal LTP.

In summary, our results provide the first demonstration that a purported ancillary neuromodulation system operated by purines actually has a main impact on synaptic plasticity and a key role in synaptic and memory dysfunction in a model of early AD. In fact, our results show that irrespective of changes in the morphology or in the glutamate receptor setup of excitatory synapses, it is the overfunctioning of the purinergic system, namely of the increased formation of ATP-derived adenosine funnelled into $A_{2A}R$ activation that critically disrupts synaptic and memory dysfunction in a mouse model of early AD. The next challenge is to understand how $A_{2A}R$ shift their unknown transducing system(s) from bolstering synaptic plasticity in naïve mice into disrupting LTP in injured synapses, a phenocopy of NMDA receptors, which are critical to enable physiological LTP, but are paramount to trigger neurodegeneration.

CHAPTER 6

ATP-derived adenosine controls fear conditioned-related memories

6.1. Abstract

$A_{2A}R$ control synaptic plasticity and memoryrelated processes in different brain areas. Recently it was described that $A_{2A}R$ are closely linked to the control fear-related responses. Genetic deletion of $A_{2A}R$ in different brain areas has different impacts on fear conditioning protocols. During this dissertation we described CD73 as the main responsible the formation of ATP-derived adenosine that selectively activates $A_{2A}R$. Thus, we decided to look for the impact of CD73 in controlling fear-related responses and if this impact is similar to $A_{2A}R$ as described in the literature. For that, we tested the pharmacological inhibition and the genetic deletion of CD73 on fear-related responses. Selective inhibition of CD73 through a infusion of AOPCP in the lateral ventricle (icv administration of 1 nmol of AOPCP), decrease fear conditioning responses. This emerges as the first observation that CD73, being the main source of ATP-derived adenosine in the brain, control fear-related processes.

6.2. Introduction

Although the information collected in the last two chapters (chapter 4 and 5) allowed to claim that ATP-derived adenosine, influences physiological and pathological processes that are deeply related with memory performance, the truth is that, when it was compared LTP or memory performance between CD73 WT and CD73 KO, we observed no differences between groups (Fig. 4.2 and Fig. 5.2). However, we found that ATP-derived adenosine is crucial to sustain plasticity processes on mice hippocampus through the selective activation of neuronal $A_{2A}R$. In other hand, we also found that memory and synaptic deficits present in an early AD model (that showed higher neuronal density of $A_{2A}R$) were prevented by CD73

blockade *i.e.* blocking ATP-derived adenosine formation. In fact, the only modulation effect on memory performance upon genetic deletion of CD73 was observed in animals displaying a pathological condition (icv-injected with Aβ, Fig. 5.2). As we argued before, CD73 KO mice, as an $A_{2A}R$ KO mice's phenocopy, may have undergone adaptive mechanisms that make it impossible to understand the impact of CD73 in the control of mechanisms in CD73 KO.

Assuming that all of our observations are correct until now, we now propose to look for the impact of the pharmacological *in vivo* manipulation of CD73 on memory performance. For that, we decided to look for fear-related memory, performing fear condition analysis in animals injected with the CD73 inhibitor AOPCP.

Fear conditioning is a behavioural paradigm in which organisms learn to predict aversive events (Maren,). It is a form of learning in which an aversive stimulus (in this case a controlled electrical shock that is known as an unconditioned stimulus (US)) is associated with a particular neutral context or a neutral stimulus (cue toneconditioned stimulus (CS)), resulting in the expression of fear responses to the originally neutral stimulus or context. Fear conditioning to either a cue or a context represents a form of associative learning that has been used in many species (Kim and Jung, 2006).

The encoding of fear-related memory is well established and it is generally, associated to the presence of abnormal plastic changes of information processing mainly in amygdala circuits (Johansen *et al.*, 2011; Mahan and Ressler, 2012). However, context fear conditioning requires learning and memorization of the context and, because of that, it is intimately connected with hippocampal function, especially from dorsal hippocampus and CA3 region. It is important to say that this hippocampal

input is not necessary specific for learning of cue associations (Clark and Squire, 1998).

Regarding the influence of purinergic signalling in fear-related memory, it is already know that adenosine through the activation of striatal and extrastriatal $A_{2A}R$ exertopposite control over fear conditioning.nactivation of striatal $A_{2A}R$ facilitates pavlovian fear conditioning, while inactivation of extrastriatal $A_{2A}R$ in the forebrain inhibits fear conditioning and also affects anxiety-related behaviour (Wei *et al.*, 2014). This last observation (regarding the forebrain $A_{2A}R$) identifies the previously under-appreciated $A_{2A}R$ in cortex and hippocampus as contributors to regulat conditioned fear, demonstrat that selective deletion of $A_{2A}R$ in the forebrain yields alterations in all three of the defining features of PTSD (Wei *et al.*, 2014). Recently, glutamatergic $A_{2A}R$ in the amygdala were found to control synaptic plasticity and contextual fear memory,the assumption that $A_{2A}R$ blockade decreases fear memory (Simões *et al.*, 2016). This paves the way to consider $A_{2A}R$ antagonists as novel candidate drugs to manage psychiatric conditions associated with excessive expression of aversive memories such as post-traumatic stress disorders.

As we described in the chapters, CD73 is the main supplier of adenosine that selective activates $A_{2A}Rs$ to control synaptic plasticity and memory formation. Consequently, this chapter describes a group of experiments exploring the impact of CD73 genetic deletion and pharmacological inhibition in fear conditionrelated memory formation. For that we take advantage of the CD73 KO mice and the selective inhibitor of CD73, AOPCP.

6.3. Material and Methods

6.3.1. Animals

We used male C57\6 mice with 8-12 weeks of age, obtained from Charles River (Barcelona, Spain), as well as male mice with the same age range from our in-house colonies of CD73 KO mice together with their respective control littermates. The animals were housed in groups of up to five mice per cage in a 12-h light/dark cycle (lights on at 7:00 AM), under controlled temperature (23±2°C), with free access to food and water. Animal procedures were in accordance with the European Community guidelines (Directive 2010/63/EU) and the Portuguese law on animal care (1005/92), and approved by the Animal's Ethics Committee of the Center for Neuroscience and Cell Biology (Orbea 78-201) and the Portuguese Veterinarian Office. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort, following ARRIVE guidelines (Kilkenny *et al.*, 2010). Thus, the animals were anaesthetized in halothane atmosphere before decapitation and, whereas the hippocampus was used in this study, other tissues from these animals were collected for use in different projects at our research centre.

6.3.2. Drugs

AOPCP Sigma, Sintra, Portugal) was to a 10 mM stock solution and used at a supra-maximal and selective dose of 1 nmol/µl (Saute *et al.*, 2006).

6.3.3. Intracerebroventricular Cannulation

The animals were anaesthetized with avertin (1.3% tribromoethanol, 0.8% amylalcohol) and placed in a stereotactic apparatus (Stoelting, Wood Dale, IL, USA). Then, mice were subjected to icv cannulation (x= -0.22 mm; y= -1 mm; z= -2.25

mm). This procedure consisted in the of a cannula Plastics One which allowed us to administrate/infuse 1 nmol AOPCP (at the rate of 1 µl/min) to the lateral ventricle in different time points of the behavioural protocol. AOPCP was administrat three different times (24 h interval) before (the third time was 1 h before starting the acquisition in fear condition protocol) or after (the first time was administrated immediately after the acquisition).

6.3.4. Behavioural analysis

All behavioural tests were conducted as previously described (Kaster *et al.*, 2015), between 9:00 AM and 1:00 PM (light phase) in a sound attenuated room with 15 lux illumination, where the had been habituated for 1 h before beginning the tests. The apparatus and objects were cleaned with a 70% alcohol solution and rinsed with water after each session. The behaviour was video-monitored with ANY-maze^{TM} (Stoelting) and ranked by two observers blind to the treatments.

Locomotion was evaluated in an open-field arena, measuring the distance travelled during a 10 min period, as previously described (Kaster *et al.*, 2015).

Fear conditioning was performed in context A with four presentations of an auditory conditioned stimulus (CS; 80 dB for 20s at 4kHz) paired with a foot-shock unconditioned stimulus (US; 0.5 mA for 2s, delivered 20s after the beginning of CS) with a 120s inter-trial interval total time of 12 min (this consist on the acquisition. At day 2, mice were returned to context A to test their contextual freezing behaviour for 12 min. At day 3, mice were placed in a completely different chambre (different environment and lux conditionscontext B), and the CS was presented in the same way

it was in context A, and the freezing behaviour was measured for 12 min. This protocol was adapted from Simões *et al.*, 2016.

After the acquisition of the behavioural performance of different groups we evaluated two types of fear conditioning; contextual fear conditioning and cued fear conditioning. Contextual fear conditioning is the most basic of the conditioning procedures. It involves taking an animal and placing it in a novel environment, providing an aversive stimulus (US after CS). When the animal is returned to the same environment a second time, it generally will demonstrate a freezing response if it remembers and associates that environment with the aversive stimulus. Freezing is a species-specific response to fear, which has been defined as "absence of movement except for respiration". This may last for seconds to minutes depending on the strength of the aversive stimulus, the number of presentations and the degree of learning achieved by the subject. Cued fear conditioning is similar to contextual conditioning, with one notable exception: a CS is added to the context. The animals learn to associate the CS to the US and when they are exposed to a completely different environment and they are subjected to the CS they will show a freezing response.

6.3.5. Statistics

Results are given as mean ± SEM of n animals, and significance was considered at $P<0.05$ using Student's *t* test for comparison between two groups and one-way ANOVA (followed by a Bonferroni's post hoc test) or two-way ANOVA (followed by a Newman-Keuls post hoc test) for comparison of multiple groups. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, CA, USA).

6.4. Results

6.4.1. CD73 pharmacological inhibition decreased context fear conditioning

A_{2A}Rs are described to control fear conditioned related processes (Simões *et al.*, 2016; Wei *et al.*, 2014) and we now report that in some brain areas such as hippocampus, CD73 is crucial for the activation of A_{2A}Rs to control synaptic plasticity and memory formation related processes. Thus, we tested if the selective blockade of CD73 through the icv administration of AOPCP before or after the conditioning session (training session) was sufficient to affect fear conditioned (Fig 6.1).

First, we confirmed that mice injected in the lateral ventricle had a similar spontaneous locomotion in the open field (Fig 6.1 a).

In the first day of conditioning we observed that the administration of AOPCP before the condition session did not change the conditioning curve in CD73 WT animals as we applied a tone-CS paired with a shock-US (Fig 6.1 b).

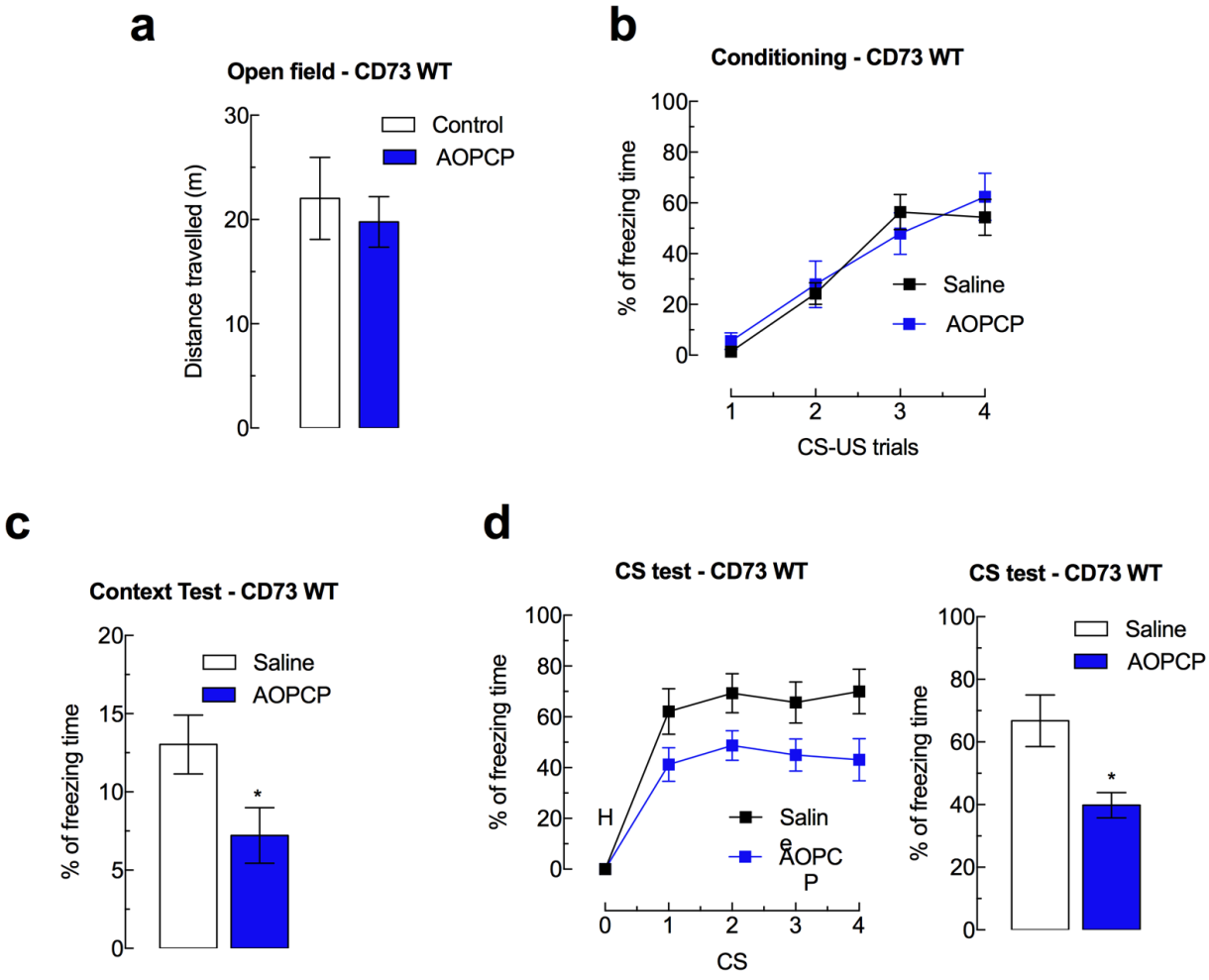

Figure 6.1: Pharmacological blockade of CD73 before the conditioning session decreased conditioned in CD73 WT . (a) Locomotor activity of administ with AOPCP and saline. CD73 WT infused with 1 nmol of AOPCP did not change locomotor profile compared to control group; **(b)** Conditioning: Freezing responses to four repeated presentations of a 20 s tone (CS) paired with an unconditioned stimulus (US) (2 s foot-shock) are illustrated. All mice showed a comparable increase in freezing with each successive CS-US paired trial; **(c)** Context Testing: Freezing responses to the conditioning context in the absence of the CS 1 day later are illustrated. CD73 WT mice administ with AOPCP exhibited a pronounced decrease freezing throughout the entire test relative to saline treated mice; **(d)** CS Testing: Freezing responses during four consecutive CS tones after a 2 min habituation (H), in a new context are illustrated; as during the conditioning experiment (a) each tone had the duration of

20 s; AOPCP treated animals showed a decreased fear to the CS compared to the saline treated animals. The values are mean±SEM *P<0.05 unpaired t test

Re-exposure to the same context, 1 day after conditioning, in the absence of CS and US, induced a freezing behaviour in all mice. context freezing was decreased by the pharmacological blockade of CD73 (Fig 6.1 c)the % of freezing the animals (7.218 ± 1.767 % *vs* vehicle 13.03 ± 1.881 %, t_{11}=2.227, P=0.0478) during the session that had exactly the same time compar the conditioning session.

The next day, mice were placed in a novel context (context b) and presented with the tone CS. While this elicited freezing in all groups of mice, the freezing response was significantly reduced in the group administ with 1 nmol of AOPCP (39.82 ± 4.059 % *vs* vehicle 66.79 ± 8.196 %, t_{12}=2.948, P=0.01) (Fig 6.1 d).

After observing the effect of the icv administration of 1 nmol of AOPCP before the conditioning session, we looked for the impact of the pharmacological blockade of CD73 after the conditioning session. For that, immediately after the conditioning of the CD73 WT animals, one single administration of 1 nmol of AOPCP was performed.

to what was in Fig 6.1, the administration of AOPCP after the conditioning session did significantly modify neither the context fear conditioning nor the CS fear (n=7-10) (Fig 6.2).

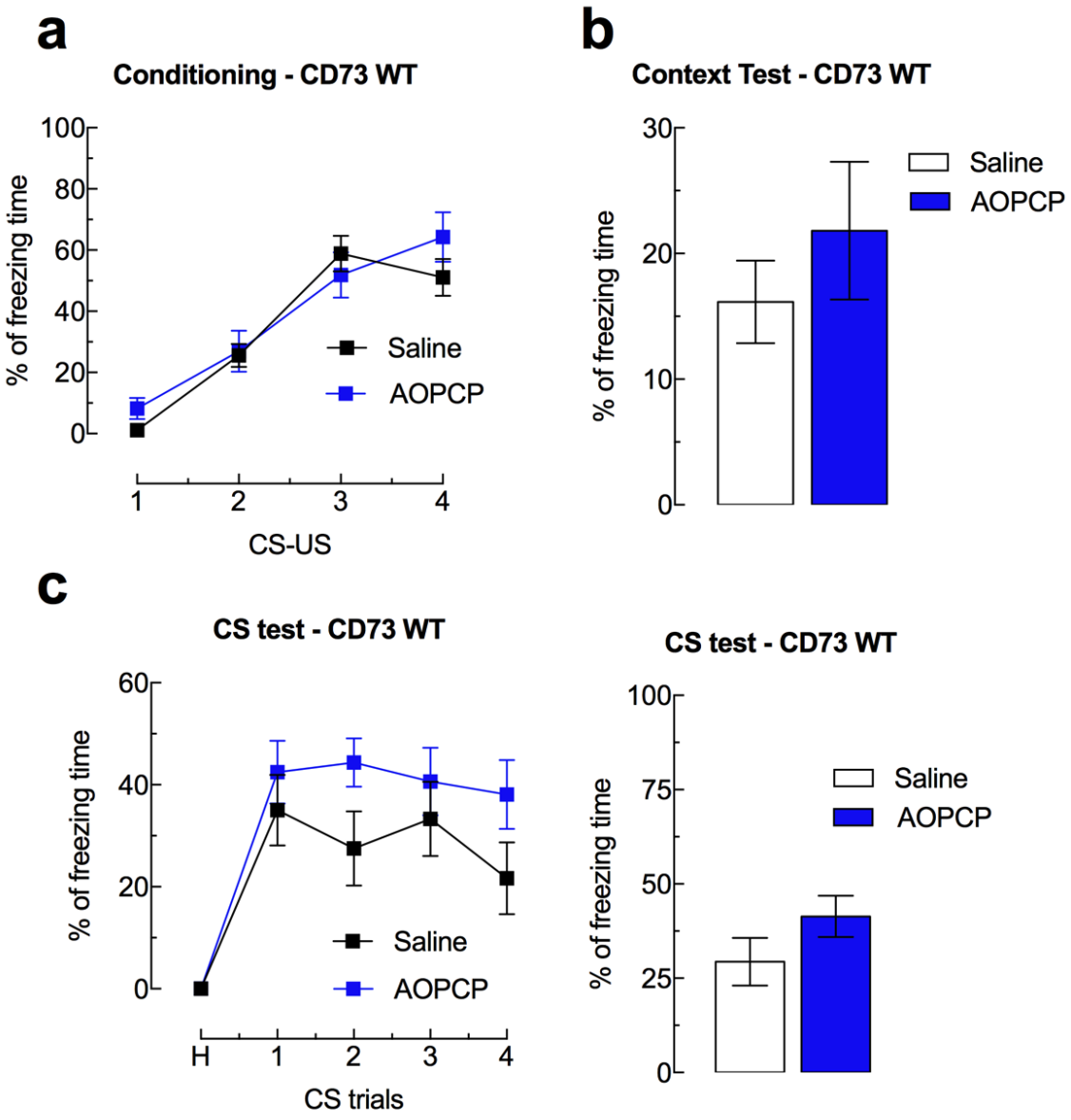

Figure 6.2: Pharmacological blockade of CD73 after the conditioning session did not modify fear. (a) Conditioning: Freezing responses to four repeated presentations of a 20 s tone (CS) paired with an unconditioned stimulus (US) (2-sec foot-shock US) are illustrated. All mice showed a comparable increase in freezing with each successive CS-US paired trial; **(b)** Context Test: Freezing responses to the conditioning context in the absence of the CS 1 day later are illustrated. CD73 WT mice administrated with AOPCP exhibited a similar freezing throughout the entire test relative to saline treated mice; **()** CS Test: Freezing responses during four consecutive CS tones after a 2 min habituation (H), in a new context are illustrated; as during the conditioning experiment (a) each tone had the duration of 20 s;

AOPCP treated animals showed a similar fear to the CS compared to the saline treated animals. The values are mean±SE

6.4.2. AOPCP administration before the conditioning session decrease fear conditioning through the selective inhibition of CD73 To ensure the selective effect of AOPCP in decreasing fear conditioning we took advantage of the CD73 KO animals. First, we observed that the genetic deletion of CD73, oppositely of what happened with the pharmacological blockade, does not change freezing profile in CD73 KO mice compared with the WT littermates (n=11) (Fig 6.3).

Finally, CD73 KO animals ensure us the selectivity of AOPCP in inhibiting CD73, since the icv administration of 1 nmol of AOPCP in CD73 KO animals did not modify fear conditioning responses (n=8-11) (Fig 6.4) as it did in CD73 WT as described before (Fig 6.1).

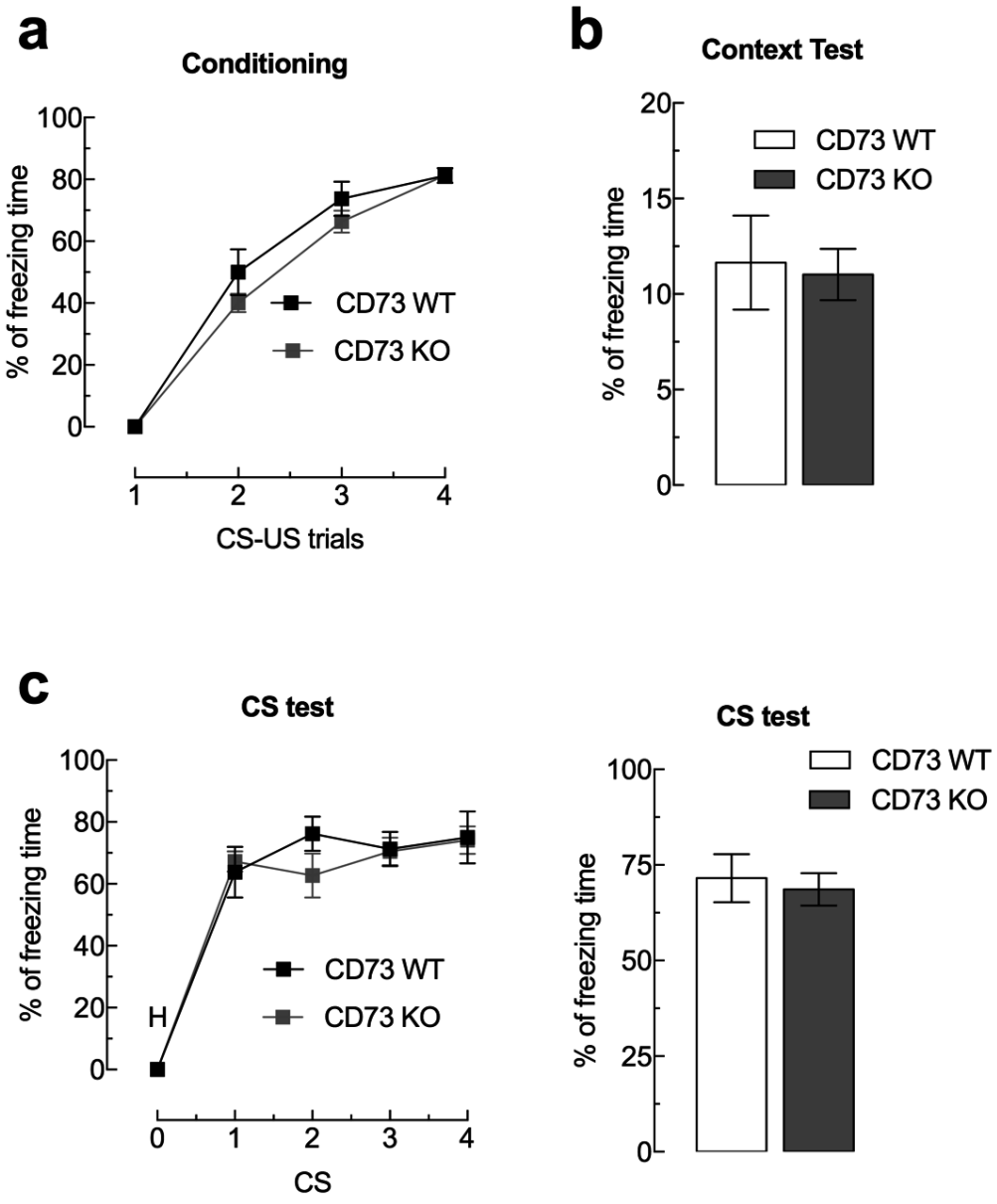

Figure 6.3: The genetic deletion of CD73 did not modify fear response. (a) Conditioning: Freezing responses to four repeated presentations of a 20 s tone (CS) paired with an unconditioned stimulus (US) (2-sec foot-shock US) are illustrated. All mice showed a comparable increase in freezing with each successive CS-US paired trial; **(b)** Context Testing: Freezing responses to the conditioning context in the absence of the CS 1 day later are illustrated. CD73 KO mice exhibited a similar freezing throughout the entire test relative to WT littermates; **()** CS Testing: Freezing responses during four consecutive CS tones after a 2 min habituation (H), in a new context are illustrated; as during the conditioning experiment

(a) each tone had the duration of 20 s; CD73 KO animals showed a similar fear conditioned to the CS compared to the CD73 WT. The values are mean±SEM

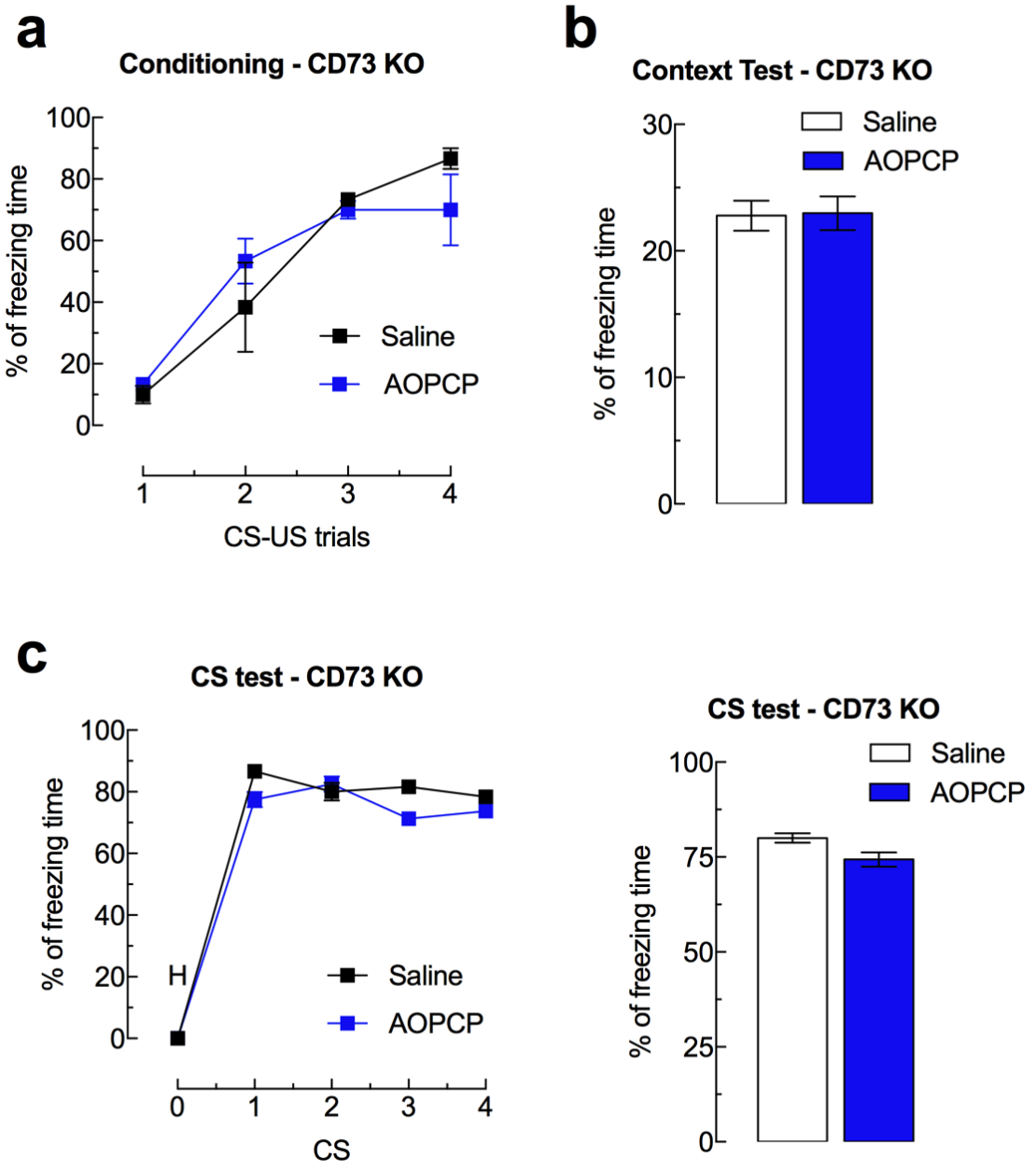

Figure 6.4: AOPCP is selective inhibiting CD73 to control fear conditioning it did not have its typical effect in CD73 KO mice. (a) Conditioning: Freezing responses to four repeated presentations of a 20 s tone (CS) paired with an unconditioned stimulus (US) (2-sec foot-shock US) are illustrated. All mice showed a comparable increase in freezing with each successive CS-US paired trial; **(b)** Context Testing: Freezing responses to the conditioning

context in the absence of the CS 1 day later are illustrated. CD73 KO mice administ with AOPCP exhibited a similar freezing throughout the entire test relative to saline treated mice; **()** CS Testing: Freezing responses during four consecutive CS tones after a 2 min habituation (H), in a new context are illustrated; as during the conditioning experiment (a) each tone had the duration of 20 s; AOPCP treated animals showed a similar fear conditioned to the CS compared to the saline treated animals. The values are mean±SEM

6.5. Discussion

In some brain regions, such as hippocampus and amygdala (Simões *et al.*, 2016), synaptic plasticity is controlled by the adenosine neuromodulation system (Fredholm *et al.*, 2005), which involves a coordinated action of inhibitory A_1 and facilitatory $A_{2A}R$ to fine-tune brain neurotransmission (Cunha, 2008). In hippocampal circuits, $A_{2A}R$ are found in synapses (Rebola *et al.*, 2005a), namely in glutamatergic synapses (Rebola *et al.*, 2005a), and are selectively engaged to control synaptic plasticity under high levels of stimulation (Rebola *et al.*, 2008; Costenla *et al.*, 2011). The importance of this modulation system is best heralded by the observation that the overactivation of hippocampal $A_{2A}R$ is necessary and sufficient to trigger spatial memory dysfunction (Li *et al.*, 2015; Pagnussat *et al.*, 2015). During this dissertation we added new information to this pool of premises. In mouse hippocampus, ATP-derived adenosine, formed through the action of CD73 is selectively activating neuronal $A_{2A}R$ to facilitate synaptic plasticity.

During these years of research in purinergic signalling, it was not possible to disentangle a clear function of $A_{2A}R$ in animal behaviour in a physiological state. Though $A_{2A}R$ are key regulators of synaptic plasticity, $A_{2A}R$ KO mice for instance do not show a clear phenotype in terms of behaviour. By our experiment, and by what is

described in the literature (Kulesskaya *et al.*, 2013), the same happens with CD73 KO mice.

Recently, it was described that mice with downregulat $A_{2A}R$, through the action of a lentivirus silencsmall hairpin RNA targeting $A_{2A}R$ ($shA_{2A}R$)), showed a decreased fear phenotype (Simões *et al.*, 2016). Other studies showed that $FBA_{2A}R$ KO and $STA_{2A}R$ KO ($A_{2A}R$ genetically removed in the striatum) promoted different impacts in fear . Selectively deleting $A_{2A}R$ in the striatum increased pavlovian fear conditioning (both context and tone) in $STA_{2A}R$ KO mice, but extending the deletion to the rest of the forebrain apparently spared context fear conditioning and attenuated tone fear conditioning in $FBA_{2A}R$ KO mice (Wei *et al.*, 2014). Moreover, focal deletion of hippocampal $A_{2A}R$ by AAV5-Cre injection selectively attenuated context (but not tone) fear conditioning (Wei *et al.*, 2014).

Based on these assumptions it was decided to advance to test the impact of CD73 in controlling fear conditioning related processes. It was observed a clear impact of CD73 on controlling context fear conditioning and CS fear conditioning (Fig 6.1). In fact, when CD73 was pharmacologically blocked before the conditioning session (Fig 6.1 b) it was observed a decreased context freezing time and a decreased CS freezing time. The impact was clear and without interfering with the acquisition. Briefly, the animals injected with AOPCP and saline showed a similar performance in acquiring the relationship between the tone (CS) and the shock (US). However, when the same animals where tested 24 h in the same context the animals administ with AOPCP were more comfortable and consequently showed a decreased freezing (Fig 6.1 c). Fig 6.1 d allowed us to observe that the pharmacological blockade of CD73 also interferes with the CS freezing. When animals were tested in a completely different environment that they did not recognize as an aversive context (as it was

observed in Fig 6.1 d, animals did not show freezing before the first application of tone (CS). After that, and trying to target a memory phase more related with the consolidation CD73 WT animals were icv administ with a single of 1 nmol of AOPCP immediately after the conditioning session (training session, acquisition). It was not observed a significant difference between groups (Fig 6.2). In fact, the analysis of the results us to admit that AOPCP is promoting an effect in the freezing profile opposite to what was promoted before. Although it was not statistically significant, the pharmacological blockade of CD73 seemed to increase the context and the CS freezing responses. The cause of this fact may be on the different administrations performed. Animals infused before the conditioning session were injected three times in three different days, and animals infused after the conditioning session were injected with a single infusion due to the limitation of the protocol. Context fear conditioning test had to be done 24h after the conditioning session (see material and methods).

Taking advantage of CD73 KO mice, it was ensured the selectivity of AOPCP in inhibiting CD73 to have an impact on the protocol. CD73 KO mice treated with AOPCP did not show a different freezing profile compared with CD73 KO mice treated with saline (Fig 6.4). It important to say that the genetic deletion of CD73 did not modify fear conditioning between CD73 KO and CD73 WT littermates (Fig 6.3).

In conclusion, this group of results appear as the first preliminary evidence that ATP-derived adenosine control fear conditioning related processes or memories. Furthermore, it is possible to attribute a new role for CD73 that showed to be crucial for implementation/early consolidation phases of fear-related memories and it did not show to control late consolidation phases of fear-related memories.

CHAPTER 7

Conclusion

The purine modulation system is actually constituted by two parallel systems functioning simultaneously (operated by ATP and by adenosine). Their different hierarchical importance and their crosstalk are currently unknown, but it is believed that they are probably adjusted in a dynamic manner according to the relative engagement of the different cell types and compartments in the brain. These intertwined ATP and adenosine purinergic modulation systems, with multiple signalling roles, emphasize the critical function of the dynamic control of the extracellular levels of ATP, the activity of ecto-nucleotidases, of adenosine transporters and of the adenosine-metabolizing enzymes namely adenosine deaminase and principally adenosine kinase (Boison, 2011), to detail which of the purinergic modulation systems is engaged and where. This massive caveat in our current understanding of the dynamics of extracellular purines has been forcing evaluat the role of purines based on the impact of the manipulation of their receptors. Considerably more is known about adenosine than about ATP (Rodrigues *et al.*, 2015), since better pharmacological tools are available for the two main adenosine receptors than for the 15 different ATP (P2) receptors.

We started by improving the characterization of the pattern of ATP release from nerve terminals (Raiteri and Raiteri, 2000). It was important to find out how the main source of adenosine (ATP) is released and under which conditions. That enabled us to better understand why ATP is preferentially release under plasticity and brain damage associated processes. At this point it was found that: 1) ATP release displays a sensitivity to the intensity of stimulation different from classical neurotransmitters (glutamate, GABA or); 2) ATP release mostly depends on the recruitment of L-type voltage-sensitive calcium channels in contrast to classical neurotransmitters

(glutamate, GABA or) and 3) ATP is more abundant in large dense core vesicles rather than in small synaptic vesicles.

The obtained results indicate that the evoked release of ATP from nerve terminals occurs in a vesicular-manner, but follows a pattern different from classical neurotransmitters, requiring higher intensities of stimulation and mainly depending on calcium entry through L-type calcium channels. Accordingly, higher amounts of ATP were present in LDCV than in SSV. Overall, this prompts the new conclusion that ATP release from nerve terminals mostly occurs in a peptidergic-like manner. Another interesting observation that we collected refers to the fact that the increase of extracellular availability of ATP or ADP, but not AMP, depresses the evoked release of more ATP in a negative feedback-like pattern, through a process that we still do not fully understand. This result appears as a golden opportunity to understand better how ATP can control the release of ATP by itself. The use of pharmacological tools for the selective or generic inhibition of P2 receptors can be assumed as the key piece to finish this puzzle. All together, these results show that ATP release from nerve terminals occurs in a vesicular manner, but follows a pattern different from the release of classical neurotransmitters, occurring mainly with more intense depolarizations, in a peptidergiclikemanner.

Once understood how and why under certain conditions the main source of adenosine is preferentially released, we looked for the physio-pathological role of CD73 as a way to modulate $A_{2A}R$. Conditions associated with memory deterioration trigger an upregulation of $A_{2A}Rs$ in the hippocampus leading to abnormal synaptic plasticity (Costenla *et al.*, 2011; Kaster *et al.*, 2015), and $A_{2A}R$ blockade prevents memory impairment in conditions such as stress, ageing, or Alzheimer's disease (Batalha *et al.*, 2013; Laurent *et al.*, 2016; Orr *et al.*, 2015; Prediger *et al.*, 2005), an

effect mimicked by caffeine (a nonselective adenosine receptor antagonist) both in animal models and in humans (reviewed in Cunha and Agostinho, 2010; Chen *et al.*, 2013). This has led to the initiation of different drug development programs fostering effective $A_{2A}R$ antagonists to manage abnormal plasticity associated with the motor system (Parkinson's disease), memory system (Alzheimer's disease) and mood system (depression) (Gomes *et al.*, 2011) In all these conditions, it seems that the symptomatic benefits of chronic caffeine consumption result from its action on $A_{2A}R$ controlling the plasticity of glutamatergic synapses, whereas the neuroprotection afforded might result from combined neuronal and glial effects (Cunha and Agostinho, 2010).

Albeit exciting, the long term use of $A_{2A}R$ antagonists will not be an option for some individuals intolerant to caffeine, since they show a particular polymorphism of $A_{2A}R$ (Rogers *et al.*, 2010). Hence, it has become urgent to begin exploring the neuroprotective potential of novel targets that can fine-tune $A_{2A}R$-mediated control of neurodegeneration. One such target might be CD73, the enzyme proposed to form extracellularly the adenosine responsible for the activation of $A_{2A}R$ upon catabolism of released adenine nucleotides.

First of all, we showed that CD73, through the regulation of ATP catabolism to adenosine, controls the activation of neuronal $A_{2A}R$, thus impacting on hippocampal LTP. This observation provided the first evidence showing a clear physiological role of CD73 in mouse hippocampus, proving that adenosine generated from ATP catabolism mediated by CD73 is strictly directed towards the activation of neuronal $A_{2A}R$ and consequently controlling synaptic plasticity. Besides the control of neuronal $A_{2A}R$ by CD73 in the hippocampus, the data from electrophysiological recordings in conjugation with the results of hippocampal-dependent behavioural tests

allowed us to uncover another aspect of the tight link between CD73 activity and $A_{2A}R$ function: similarly to what has been observed in $A_{2A}R$ KO mice, in CD73 KO mice there is an absence of phenotypical alterations. Indeed, although previous studies point out to behavioural improvements upon genetic deletion of CD73 (Augusto *et al.*, 2013; Kulesskaya *et al.*, 2013) and ST $A_{2A}R$ (Wei *et al.*, 2011), we did not observe a characteristic phenotype in these animals. Although CD73 seems to have a clear physiological role in controlling plasticity phenomena through the modulation of neuronal activity, the data gathered on Chapter 4 clearly lacked the synaptic and the sub-cellular localization of CD73. Therefore, we proceeded with the gathering of such information, which integrated Chapter 5, having found that, much like $A_{2A}R$, in mouse hippocampus CD73 was enriched on synapses

Trying to better understand the physio-pathological role of CD73 we decided to use an animal model of a neurodegenerative disease that expresses in the hippocampus a higher density of $A_{2A}R$, hypothetically leading to an engagement by CD73, as previously showed. For that, we administ an oligomeric $A\beta_{1-42}$ (Canas *et al.*, 2009) in CD73 WT and KO animals and probed for changes in hippocampal-dependent memory performance and plasticity patterns. We observed changes in the release of ATP, CD73 levels and $A_{2A}R$ density triggered by "chronic" exposure to $A\beta_{1-42}$ which were likely to translate into changes in synaptic plasticity, thus implying a unbalance of the purinergic system. We demonstrated that, whereas the genetic elimination of CD73 has a protective effect against the impairments induced by Aβ, pharmacologic CD73 inhibition significantly reverts Aβ-triggered LTP deficits. This provided the first demonstration that a purported ancillary neuromodulation system operated by purines actually has a main impact on synaptic plasticity and a key role in synaptic and memory dysfunction in a model of early AD. In fact, our results show

that, irrespective of changes in the morphology or in the glutamate receptor setup of excitatory synapses, it is the overfunctioning of the purinergic system, namely of the increased formation of ATP-derived adenosine funnelled into $A_{2A}R$ activation that critically disrupts synaptic and memory dysfunction in a mouse model of early AD. Consequently, we propose CD73 as new target for the regulation of $A_{2A}R$ function, with a potential therapeutic application in the prevention/reversion of brain disorders. The next challenge is to understand how $A_{2A}R$ shift their unknown transducing system(s) from bolstering synaptic plasticity in naïve mice into disrupting LTP in injured synapses, a phenocopy of NMDA receptors, which are critical to enable physiological LTP, but are paramount to trigger neurodegeneration.

Finally, in Chapter 6, we were able to demonstrate with preliminary results that CD73, as an ATP catabolism controller, can *per se* modulate behavioural characteristics in the animals. The selective inhibition of CD73 proved to be efficient in controlling fear memories. Indeed, CD73 inhibition tends to decrease fear-related responses. However, since the mechanism for such an effect remains poorly understood, it is still to be confirmed if this effect of CD73 blockade results from an indirect control of $A_{2A}R$. To try to disentangle these questions it will be required a future set of experiments using animals with the genetic deletion of $A_{2A}R$. Although we have obtained some controversial data, this group of results appear as the first preliminary evidence that ATP-derived adenosine controls fear conditioning-related processes or memories.

In conclusion, this thesis highlights the potential of manipulating the ATP catabolism neuromodulation system as a novel strategy to manage brain disorders and control physiological functions. However, in spite of this potential interest, it is also clear that a long road lies ahead to attempt confirming the effectiveness of

manipulating this system to obtain some clinical benefit in the management of brain disorders. There are obvious and immediate questions related to the how and when to manipulate ATP catabolism. As a way of conclusion there seems to be a parallel benefit of manipulating $A_{2A}R$ and CD73. In fact, it seems that manipulating CD73 is the easiest way to manipulate $A_{2A}R$. The last years have witnessed a substantial increase in the number of reports documenting the ability of the pharmacological or genetic blockade of $A_{2A}R$ to attenuate brain damage caused by different insults. We now add to the pool the CD73 factor. However, the underlying mechanisms modulated (intracellular pathways) are still to be unravelled, and there seem to be nearly as many possible mechanisms as researchers dedicated to the subject (Tomé *et al.*, 2010). The role of $A_{2A}R$ in the control of brain damage is further complicated by the plasticity of its expression and its promiscuity in terms of recruitment of intracellular signalling pathways, which leads to the hypothesis that the newly expressed $A_{2A}R$ upon brain insults (Fredholm *et al.*, 2005) may fulfil roles different from constitutively expressed $A_{2A}R$. According to previous studies, and reinforced now in this thesis, CD73 seems to follow the density variances of $A_{2A}R$ under physiological and pathological conditions. However, we still don't know in which kind of cells or in which part of the cells CD73 is more expressed and why. And certainly without this fundamental supportive information, we currently lack a rationale to logically sustain any proposal on when and how to manipulate CD73 as a way to manipulate $A_{2A}R$ function to attempt controlling the demise(s) of brain disorders.

There is another question of particular importance when considering the translational interest of CD73: we still know surprisingly little about $A_{2A}R$ or CD73 in the human brain, either in terms of its pharmacological properties, localization,

function(s) and modifications upon brain disorders. And this is crucial information to evaluate the extent to which animal models are representative of the role of this receptor in the human brain.

Albeit there are several open questions that demand careful attention, the general impression is that there is a great potential to further explore this system to develop novel strategies to control both neurological and psychiatric brain disorders.

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