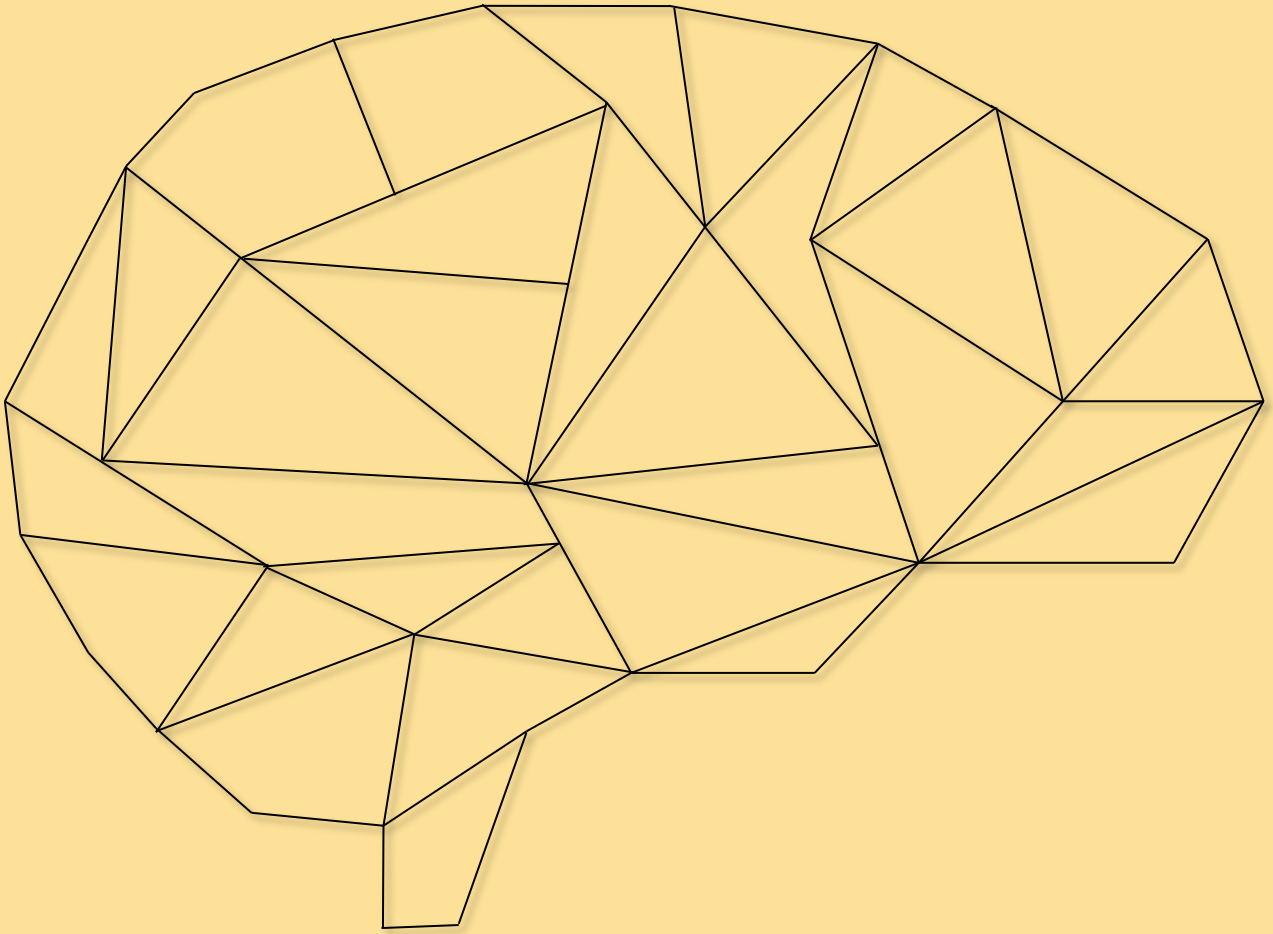

Elisabete de Oliveira Augusto

Characterization of ecto-5'-nucleotidase (CD73) in the brain – role in adenosine A_{2A} receptor activation

Tese de Doutoramento em Ciências da Saúde, ramo de Ciências Biomédicas, orientada pelo Senhor Professor Doutor Rodrigo Antunes da Cunha e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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Elisabete de Oliveira Augusto

Characterization of ecto-5'-nucleotidase (CD73) in the brain – role in adenosine A_{2A} receptor activation

Caracterização do enzima ecto-5'-nucleotidase (CD73) no cérebro – papel na activação dos receptores do subtipo A_{2A} para a adenosina

Coimbra
2014

Tese de Doutoramento apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Doutor em Ciências da Saúde, ramo Ciências Biomédicas, conduzida sob a orientação do Professor Doutor Rodrigo Antunes da Cunha.

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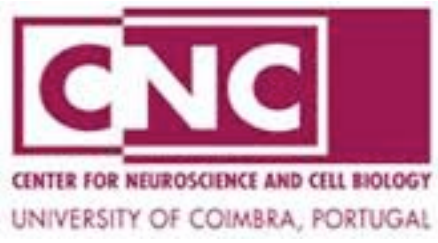

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List of publications

Part of the work presented in this thesis resulted in publications in international peer-reviewed scientific journals:

- **Augusto E**, Matos M, Sévigny J, El-Tayeb A, Bynoe MS, Müller CE, Cunha RA, Chen JF (2013). Ecto-5'-nucleotidase (CD73)-mediated formation of adenosine is critical for the striatal adenosine A2A receptor functions. J Neurosci., 33(28), 11390-11399.

- Wei CJ, **Augusto E**, Gomes CA, Singer P, Wang Y, Boison D, Cunha RA, Yee BK, Chen JF (2013). Regulation of Fear Responses by Striatal and Extrastriatal Adenosine A(2A) Receptors in Forebrain. Biol Psychiatry., S0006-3223 (13), 00411-3.

During the course of my doctoral work I actively participated in different projects that resulted in international peer-reviewed scientific journals:

- Matos M, **Augusto E**, Santos-Rodrigues A, Schwarzschild MA, Chen J-F, Cunha RA, Agostinho P (2012). Adenosine A2A receptors modulate glutamate uptake in cultured astrocytes and gliosomes. Glia, 60 (5), 702-716.

- Matos M, **Augusto E**, Machado NJ, Dos Santos-Rodrigues A, Cunha RA, Agostinho P (2012). Astrocytic Adenosine A2A Receptors Control the Amyloid-β Peptide-Induced Decrease of Glutamate Uptake. J Alzheimers Dis., 31(3), 555-567.

- Yao SQ, Li ZZ, Huang QY, Li F, Wang ZW, **Augusto E**, He JC, Wang XT, Chen JF, Zheng RY (2012). Genetic inactivation of the adenosine A(2A) receptor exacerbates brain damage in mice with experimental autoimmune encephalomyelitis. J Neurochem., 123(1), 100-112.

- Matos M, **Augusto E**, Agostinho P, Cunha RA, Chen JF (2013). Antagonistic interaction between adenosine A2A receptors and Na+/K+-ATPase-α2 controlling glutamate uptake in astrocytes. J Neurosci., 33 (47), 18492-18502.

“Duvido, portanto penso.”

Fernando Pessoa

List of abbreviations

A_1R - adenosine A_1 receptors
A_1R KO - *Adora1* global knockout
A_{2A}R - adenosine A_{2A} receptors
A_{2A}R KO - *Adora2A* global knockout
A_{2B}R - adenosine A_{2B} receptors
A_3R - adenosine A_3 receptors
AAV - adeno-associated virus
ADA - adenosine deaminase
ADK - adenosine kinase
ADP - adenosine 5'-diphosphate
a.m. - *ante meridiem;* before midday
AMP - adenosine 5'-monophosphate
AMPCP - adenosine 5'-[α, β-methylene]diphosphate
Amphetamine - (*RS*)-1-phenylpropan-2-amine (*RS*)-1-phenyl-2-aminopropane
ANOVA - analysis of variance
AP - anteroposterior
ATP - adenosine 5'-triphosphate
Avertin - 2,2,2-tribromoethyl alcohol
Bafilomycin - (3*Z*,5*E*,7*R*,8*S*,9*S*,11*E*,13*E*,15*S*,16*R*)-16- [(1*S*,2*R*,3*S*)-3-[(2*R*,4*R*,5*S*,6*R*)-2,4-dihydroxy-6- isopropyl-5-methyl-2-tetrahydropyranyl]-2- hydroxy-1-methylbutyl]-8-hydroxy-3,15- dimethoxy-5,7,9,11-tetramethyl-1- oxacyclohexadeca-3,5,11,13-tetraen-2-one
BCA - bicinchoninic acid
BDNF - brain-derived neurotrophic factor
BLA - basolateral amygdala
bp - base pairs
BSA - bovine serum albumin
CA1 - cornu ammonis 1
CA3 - cornu ammonis 3
CaMKII-α - alpha-Ca^{2+}/calmodulin-dependent protein kinase II
cAMP - 3'-5'-cyclic adenosine monophosphate
CB_1R - cannabinoid CB_1 receptors

CD39 - cluster of differentiation 39, also known as ENTPD1, ectonucleoside triphosphate diphosphohydrolase 1

CD73 - ecto-5'-nucleotidase

CD73 KO - global CD73 knockout

cDNA - complementary deoxyribonucleic acid

CeA - central nucleus of amygdala

CGS21680 - 3-[4-[2-[[6-amino-9-[(2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid

CMP - cytidine monophosphate

CMV - cytomegalovirus

CNS - central nervous system

Cocaine - methyl (1*R*,2*R*,3*S*,5*S*)-3- (benzoyloxy)-8-methyl-8-azabicyclo[3.2.1] octane-2-carboxylate

Co-IP - co-immunoprecipitation

CPu - caudate putamen

CREB - cAMP response element-binding protein

CS - conditional stimulus

CSF - cerebrospinal fluid

CTX - cortex

Cycloheximide - 4-[(2*R*)-2-[(1*S*,3*S*,5*S*)-3,5-Dimethyl-2-oxocyclohexyl]2hydroxyethyl] piperidine-2,6-dione

D_1R - dopamine D_1 receptors

D_2R - dopamine D_2 receptors

DAPI - 4',6-diamidino-2-phenylindole

DARPP-32 - dopamine and cAMP-regulated phosphoprotein, 32kDa

DARPP-32-p(Thr75) - dopamine and cAMP-regulated phosphoprotein, 32kDa phosphorylated at threonine 75

DG - dentate gyrus

Dlx5/6 - homeobox genes 5/6, mammalian homologs of the *Drosophila Distal-less* (*Dll*) gene

DNA - deoxyribonucleic acid

DPCPX - 8-cyclopentyl-1,3-dipropylxanthine

DPX - distrene plasticiser xylene

DTT - dithiothreitol

DV - dorsoventral

EAAT2 - excitatory amino acid transporters type 2

EDTA - ethylenediaminetetraacetic acid

Enk - enkephalin

E-NPP - ectonucleotide pyrophosphatase/phosphodiesterase

ENT - equilibrative nucleoside transporters

E-NTPDase - ectonucleoside triphosphate diphosphohydrolase

Fb - forebrain

GABA - gamma-aminobutyric acid

GAT-1 - gamma-aminobutyric acid transporters 1

Gentamicin - (3*R*,4*R*,5*R*)-2-{[(1*S*,2*S*,3*R*,4*S*,6*R*)-4,6-diamino-3-{[(2*R*,3*R*,6*S*)-3-amino-6-[(1*R*)-1-(methylamino)ethyl]oxan-2-yl]oxy}-2-hydroxycyclohexyl]oxy}-5-methyl-4-(methylamino)oxane-3,5-diol

Gfa2 A_{2A}R KO - GFAP driven A_{2A}R KO mice

GFAP - glial fibrillary acidic protein

GFAP-Cy3 - glial fibrillary acidic protein associated to cyanine dyes 3

GFP - green fluorescent protein

GLT-I - glutamate transporters 1

GMP - guanosine 5'-monophosphate

GP - globus pallidus

GPI - glycosyl-phosphatidyl-inositol

GS - glutamine synthetase

GTP - guanosine 5'-triphosphate

HBM - HEPES buffer medium

HIP - hippocampus

HEPES - 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

IgG - immunoglobulin G

IMP - inosine 5'-monophosphate

i.p. - intraperitoneal

IP - immunoprecipitation

IP_3 - inositol trisphosphate

IPB - immunoprecipitation buffer

IR - immunoreactivity

ITI - inter trial interval

KA - kainate; (2*S*,3*S*,4*S*)-3-(Carboxymethyl)-4-prop-1-en-2-ylpyrrolidine-2-carboxylic acid

KHR - Krebs-HEPES-Ringer

KO - knockout

L-DOPA - L-3,4-dihydroxyphenylalanine

LTD - long-term depression

LTP - long-term potentiation

MAPK - mitogen-activated protein kinases

ML - mediolateral

MTLE - mesial temporal lobe epilepsy

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MK-801 - dizocilpine; [5*R*,10*S*]-[+]-5-methyl-10,11- dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine

mGluR5 - metabotropic glutamate type 5 receptors

mGluR4 - metabotropic glutamate type 4 receptors

mRNA - messenger ribonucleic acid

MSNs - medium spiny neurons

NAc - nucleus accumbens

NeuN - hexaribonucleotide binding protein-3

NMDAR - N-methyl-D-aspartate receptors

NMG - N-methyl-D-glucamine

NTPDase - nucleotidases triphosphate diphosphohydrolases

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PD - Parkinson's disease

Phencyclidine - 1-(1-phenylcyclohexyl)piperidine

Pi - phosphate inorganic

PKA - protein kinase A

PKC - protein kinase C

PLA - proximity ligation assay

PlxC - plexus choroid

p.m. - *post meridiem*; after midday

PPi - inorganic pyrophosphate

PSD-95 - postsynaptic density-95

PSB-12404 - (2*R*,3*R*,4*S*,5*R*)-2-(6-amino-2-(2-cyclohexylethylthio)-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol

PSB-12405 - (2*R*,3*S*,4*R*,5*R*)-5-(6-amino-2-(2-cyclohexylethylthio)-9H-purin-9-yl)-3,4-dihydroxy-tetrahydro-furan-2-yl)methylphosphoric acid triethylammonium salt

qPCR - quantitative polymerase chain reaction

RAM - 8 radial arms maze

Raclopride - 3,5-dichloro-N-{[(2*S*)-1-ethylpyrrolidin-2-yl]methyl}-2-hydroxy-6-methoxybenzamide

RIPA - radioimmunoprecipitation assay

r.p.m. - rotations per minute

RNA - ribonucleic acid

RNase - ribonuclease

RT-PCR - reverse transcription polymerase chain reaction

SCH58261 - 7-(2-phenylethyl)-5-amino-2-(2-furyl)pyrazolo[4,3e][1,2,4]triazolo[1,5c]pyrimidine

SCH23390 - 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol

sec - seconds

SEM - standard error of the mean

SN - substantia nigra

SNc - substantia nigra pars compacta

STR - striatum

Sulpiride - (±)-5-(aminosulfonyl)-*N*-[(1-ethylpyrrolidin-2-yl)methyl]-2-methoxybenzamide

SSC - saline-sodium citrate

TBS-T - Tris-buffered saline Tween

TLE - temporal lobe epilepsy

TNAB-I - tissue-nonspecific alkaline phosphatase inhibitor

TH - tyrosine hydroxylase

TrkB - neurotrophin tyrosine kinase receptor type 2

UMP - uridine 5'-monophosphate

US - unconditional stimulus

VGAT - vesicular GABA transporters

VGLUT - vesicular glutamate transporters

VNUT - vesicular nucleotide transporters

WME - working memory error

WT - wild-type

XAC - 8-{4-[(2-aminoethyl)amino] carbonylmethyloxyphenyl}xanthine

ZM241385 - 4-(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-*a*} {1,3,5}triazin-5-yl-amino]ethyl)phenol

Resumo

A adenosina é um importante neuromodulador que participa em diferentes funções cerebrais e pode ser gerada extracelularmente por um mecanismo em cascata através de ectonucleotidases que desfosforilam ATP em adenosina. A ecto-5'-nucleotidase (CD73) é o enzima chave, participando no passo final de conversão do ATP extracelular em adenosina no cérebro. Neste estudo tirámos partido de um anticorpo selectivo para CD73 assim como de murganhos com delecção genética (*knockout*) de CD73 (CD73 KO), mostrando a sua elevada densidade no núcleo central da amígdala, no *globus pallidus* e no estriado; mostrámos ainda a sua presença no tubérculo olfactivo, nas meninges e no plexo coróide; e ainda, com níveis significativamente mais baixos, na substância nigra, no hipocampo e no córtex. Relativamente à sua distribuição celular mostrámos que a CD73 está presente nos astrócitos, mas está predominantemente localizada a nível pós-sináptico em todas as áreas cerebrais estudadas.

O fenótipo dos murganhos CD73 KO foi estudado em contextos fisiológicos, tendo-se observado défices de coordenação motora e hiperlocomoção num campo aberto, mas não na gaiola, sem modificações comportamentais quando testados no labirinto em cruz elevado. Estes resultados sugerem que a CD73 melhora a coordenação motora, sem influenciar a locomoção e a ansiedade. Este fenótipo foi acompanhado de uma diminuição no estriado quer da fosforilação da DARPP-32 na treonina 75 quer da captação de aspartato, sem modificações da densidade dos receptores para a adenosina ou para a dopamina ou na imunoreactividade da encefalina. Os murganhos CD73 KO apresentaram ainda uma melhoria das memórias de trabalho e reconhecimento e da aprendizagem do acto de evitar, assim como uma ausência da sensitização induzida por anfetamina, uma diminuição na actividade psicomotora induzida por MK-801 e na neurodegeneração induzida por 1-methyl-4-

phenyl-1,2,3,6- tetrahydropyridine. Deste modo este estudo aponta a CD73 como um possível alvo terapêutico para manipulação da coordenação motora e funções cognitivas, assim como em disfunções estriatais como as que ocorrem na viciação em drogas de abuso ou na esquizofrenia e na neurodegeneração que acontece na doença de Parkinson.

De modo a explorar a participação da CD73 como fonte específica da adenosina que activa os receptores A_{2A} para a adenosina ($A_{2A}R$) estriatais, começámos por mostrar que a CD73 se co-localiza e co-imunoprecipita com os $A_{2A}R$ no estriado, e é positivo no ensaio de ligação próxima com os $A_{2A}R$. De acordo com estes resultados, a formação de cAMP em sinaptosomas estriatais, assim como a hiperlocomoção induzida por uma nova prodroga que requer a metabolização por CD73 para activar os $A_{2A}R$, foi observada nos murganhos de estirpe selvagem, mas não nos murganhos CD73 KO ou $A_{2A}R$ KO. Estes resultados sugerem que a formação de adenosina mediada pela CD73 poderá ser responsável pela activação dos $A_{2A}R$ estriatais. Consequentemente, este estudo aponta a CD73 como um novo alvo que pode ajustar a actividade dos $A_{2A}R$ estriatais, sendo um possível alvo terapêutico para manipular as funções de controlo estriatal e a neurodegeneração mediados pelos $A_{2A}R$.

Sendo a adenosina um anticonvulsante endógeno, explorámos a participação da CD73 como fonte de adenosina activando os $A_{2A}R$ para controlar a progressão desta patologia. Os murganhos CD73 KO exibem um perfil de convulsão inalterado, mas apresentam uma diminuição da perda neuronal. Além disso, a deleção selectiva dos $A_{2A}R$ nos neurónios (Fb $A_{2A}R$ KO) resulta num perfil anticonvulsivo e neuroprotector, enquanto que a deleção selectiva dos $A_{2A}R$ nos astrócitos (Gfa2 $A_{2A}R$ KO) resulta num fenótipo oposto, proconvulsivo e neurotóxico. Os resultados sugerem que uma disfunção na captação de glutamato causada pela deleção dos $A_{2A}R$ está na base dos diferentes padrões fenotípicos, implicando uma acção coordenada dos $A_{2A}R$ nos

astrócitos e nos neurónios para controlar a transmissão glutamatérgica que estará perturbada em epilepsia.

Devido ao rápido aumento dos $A_{2A}R$ a nível pré-sináptico no hipocampo após diferentes tipos de estimulação, explorámos a sua possível síntese local; tendo-se mostrado a presença do mRNA para os $A_{2A}R$ e a sua síntese local nos terminais pré-sinápticos do hipocampo. Explorámos ainda a participação específica dos $A_{2A}R$ no hipocampo em comportamentos de memória e aprendizagem. Tirando partido de murganhos com redução selectiva dos $A_{2A}R$ no hipocampo, através da injecção local de um vector que expressa Cre em murganhos que têm um exão importante de *Adora2a* flanqueado por loxP, mostrámos que os $A_{2A}R$ do hipocampo não participam na aprendizagem motora ou na ansiedade (em contraste com os murganhos com deleção dos $A_{2A}R$ nos neurónios de todo o prosencéfalo (Fb $A_{2A}R$ KO)), mas apresentam uma melhoria da memória de trabalho (em semelhança com o fenótipo dos murganhos Fb $A_{2A}R$ KO), assim como um défice do medo condicionado pelo contexto (mas não pelo som).

Em conclusão, o trabalho aqui apresentado sugere a contribuição da adenosina derivada do enzima CD73 na activação dos $A_{2A}R$ estriatais, participando na coordenação motora, memória de trabalho, viciação em drogas de abuso, actividade psicomotora e neurodegeneração associada à doença de Parkinson. Para além disso, a adenosina derivada da CD73 parece participar na activação dos $A_{2A}R$ do hipocampo, cuja função se apresenta mais complexa do que anteriormente avaliado. Os nossos dados sugerem funções opostas dos $A_{2A}R$ do hipocampo nos neurónios *versus* nos astrócitos, no controlo da transmissão glutamatérgica com implicações para a epilepsia assim como a sua participação em processos cognitivos. Surpreendentemente, concluímos ainda que a síntese dos $A_{2A}R$ do hipocampo nos terminais pré-sinápticos

está impelido a uma rápida e local regulação de modo a permitir modificações rápidas do ambiente sináptico.

Abstract

Adenosine is an important neuromodulator that participates in different brain functions and can be extracellularly generated by an ectonucleotidase cascade that is able to catabolize ATP into adenosine. Ecto-5'-nucleotidase (CD73) catabolizes the final step of conversion of extracellular ATP into adenosine in the brain. Since the brain distribution of CD73 is still unclear, we here took advantage of a selective antibody against CD73 and of CD73 knockout (KO) mice to unveil the brain distribution of CD73, showing that it is highly expressed in the central nucleus of the amygdala, globus pallidus and striatum. CD73 is also present in the olfactory tubercle, meninges and choroid plexus and is also present at lower levels in the substantia nigra, hippocampus and cortex. Regarding the cellular and subcellular distribution of CD73 we here showed that, in addition to astrocytes, CD73 is prominently localized in postsynaptic sites in all the brain areas studied (cortex, hippocampus and striatum).

The phenotype of CD73 KO mice in physiological conditions suggest that CD73 is responsible for an improved motor coordination, without affecting locomotion or anxiety. This phenotype was accompanied by a lower phosphorylation of DARPP-32 at threonine 75 and a decreased D-aspartate uptake in the striatum, without modifications of adenosine or dopamine receptors levels or enkephalin immunoreactivity. Moreover, CD73 KO mice displayed improved working and recognition memories and avoidance learning. In addition we demonstrated the participation of CD73 in amphetamine-induced sensitization, MK-801-induced psychomotor activity and in MPTP-induced neurodegeneration. These findings suggest that CD73 can be a possible therapeutic target in brain dysfunctions characterized by altered motor coordination and cognitive functions and potential neurodegeneration, as well as neuropsychiatric disorders (e.g. drug addiction and schizophrenia).

Adenosine is a neuromodulator that acts mainly through inhibitory A_1 receptors

(A_1R) and facilitatory $A_{2A}R$, which have similar affinities for adenosine. In order to explore the participation of CD73 in the specific source of adenosine activating striatal $A_{2A}R$, we first showed that CD73 is closely associated with $A_{2A}R$ in the basal ganglia, as gauged by the co-localization, co-immunoprecipitation and positive proximity ligation assays between these two proteins. Accordingly, both the hypolocomotion and the cAMP formation in striatal synaptosomes induced by a novel $A_{2A}R$ prodrug that requires CD73 metabolization to activate $A_{2A}R$, were observed in wild type mice, but not in CD73 KO or $A_{2A}R$ KO mice. These results show that CD73-mediated formation of extracellular adenosine is responsible for the activation of striatal $A_{2A}R$ function. These findings suggest CD73 as a new target to fine-tune striatal $A_{2A}R$ activity, and therefore as a novel therapeutic target to manipulate $A_{2A}R$-mediated control of striatal function and neurodegeneration.

Since adenosine acting via A_1R is widely recognized as an endogenous anticonvulsant, we next explored the participation of CD73 as the molecular source of adenosine and the relevance of $A_{2A}R$ in the progression of epilepsy. CD73 KO mice exhibited an unaltered convulsive profile, albeit with a decreased hippocampal neuronal loss. In addition, mice with selective deletions on either neuronal or astrocytic $A_{2A}R$, displayed an opposite phenotype, with an anticonvulsive and neuroprotective profile observed for neuronal $A_{2A}R$ KO and a proconvulsive and neurotoxic profile for astrocytic $A_{2A}R$ KO. Notably, our data also suggest that a dysfunctional glutamate uptake caused by $A_{2A}R$ deletion might be at the basis of the different phenotypic patterns and imply a differential and delicate coordinated action between astrocytic $A_{2A}R$ and neuronal $A_{2A}R$ to fine-tune glutamatergic transmission that is disrupted in epilepsy.

Due to the participation of $A_{2A}R$ in noxious hippocampal conditions and in cognitive functions we investigated the role of this particular pool of $A_{2A}R$. Owing to the hippocampal increased of presynaptic $A_{2A}R$ after different types of stimulation, we

explored its possible local synthesis. We showed the presence of $A_{2A}R$ mRNA and its local synthesis in presynaptic nerve terminals of the hippocampus. Moreover, we explored the specific participation of hippocampal $A_{2A}R$ in learning and memory behaviors. Taking advantage of hippocampal $A_{2A}R$ knockdown mice, generated by injection into the dorsal hippocampus of a vector that expresses Cre in $A_{2A}R$-floxed mice, we further demonstrated that hippocampal $A_{2A}R$ do not participate in motor learning or anxiety (in contrast to forebrain (Fb) $A_{2A}R$ KO), but showed improved working memory (in similarity with Fb $A_{2A}R$ KO phenotype) and impaired context (but not tone) fear conditioning.

In conclusion, our work suggests that CD73-derived adenosine is involved in striatal $A_{2A}R$ activation, thus participating on the control of a vast number of functions such as motor coordination, working memory, drug addition, psychomotor activity and neurodegeneration associated with Parkinson's disease. In addition, CD73-derived adenosine seems to participate in hippocampal $A_{2A}R$ activation. Moreover, our data suggest opposite functions of neuronal and astrocytic hippocampal $A_{2A}R$ in the control of glutamatergic transmission with implications for epilepsy. Finally, we demonstrated that hippocampal $A_{2A}R$ synthesis at presynaptic nerve terminals is prompt to a rapid and local regulation in order to quickly change the synaptic environment and thus impacting on cognition. The complete understanding of CD73 physiology and mediation of $A_{2A}R$ activation may lead to the future development of strategies for modulating $A_{2A}R$ function in the treatment of different brain disorders.

CHAPTER 1

GENERAL INTRODUCTION

1. Adenosine overview

Adenosine is a widely distributed modulator of a broad spectrum of neurotransmitters, receptors and signaling pathways that contributes to the expression of a vast range of important brain functions. To date, there are no clear evidences that adenosine is stored in synaptic vesicles or released as a classical neurotransmitter in response to neuronal firing. Adenosine is a prototypic neuromodulator, which means it does not trigger direct neuronal responses but fine-tunes on-going synaptic transmission, controlling the flow of information through different neuronal circuits in the brain (Dunwiddie and Masino, 2001).

Moreover, adenosine can also affect brain metabolism (Newby et al., 1985; Magistretti et al., 1986; Håberg et al., 2000; Cunha, 2001a; Hammer et al., 2001), and it is still not clear if the neuromodulatory role of adenosine is related to the metabolic control in the brain or if the latter reflects a general homeostatic role of adenosine observed in different types of eukaryotic cells (Joo et al., 2007; Peart and Headrick, 2007; Wendler et al., 2007; Kim et al., 2008). Consequently, it should always be kept in mind that this ability of adenosine to control metabolic activity is expected to play a potentially relevant role in the control of both physiological and pathological brain adaptive changes, which are highly dependent on adequate metabolic support. Therefore, adenosine has been implicated in several key physiological processes in the central nervous system, ranging from neuromodulation, to neuroinflammation, blood brain barrier permeability and metabolic control.

The ability of adenosine to accomplish its functions depends on the adenosine receptors, which are cell-surface receptors belonging to the G-protein-coupled receptor family (Fredholm et al., 2000). To date, four adenosine receptors subtypes have been identified, purified and cloned (Fredholm et al., 2000; 2001) - A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. They were initially classified by their ability to inhibit (i.e., A_1R and A_3R) or stimulate (i.e.,

A_2R) adenylate cyclase (van Calker et al., 1979; Londos et al., 1980). The A_2R were further classified according to the presence of high-affinity ($A_{2A}R$) or low-affinity ($A_{2B}R$) binding sites for adenosine in brain (Daly et al., 1983). The higher density of A_1R and $A_{2A}R$ in the brain, and the modest impact of $A_{2B}R$ and A_3R manipulations on brain functions, led to the idea that the impact of adenosine on brain function might mostly depend on the actions of A_1R and $A_{2A}R$ (Fredholm et al., 2005b).

Importantly, under physiological conditions adenosine tonus is not very prominent, and an adopted strategy to study adenosine receptor functions on such basal conditions was through the study of adenosine receptors knockout (KO) mice. Over the past decade, the generation and characterization of genetic KO models for all four adenosine receptors confirmed and extended the neuromodulatory and integrated role of adenosine receptors in the control of a broad spectrum of normal and abnormal brain functions, in particular for A_1R and $A_{2A}R$ (Wei et al., 2010).

1.1. Adenosine A_1 receptors (A_1R)

The A_1R are the most conserved adenosine receptors subtype among species (Fredholm et al., 2000), and are expressed throughout the body with the highest levels observed in the brain, notably in the cortex, hippocampus, and cerebellum and moderately expressed in the striatum and elsewhere in the brain (Mahan et al., 1991; Dixon et al., 1996; Fredholm et al., 2000). Regarding the subcellular distribution, A_1R are found at both presynaptic and postsynaptic sites (Tetzlaff et al., 1987; ; Rebola et al., 2003a), but also non-synaptically (Greene and Haas, 1991) and in glial cells (Tsutsui et al., 2004; van Calker and Biber, 2005; Haselkorn et al., 2010). Moreover, within a specific tissue, adenosine receptors may show varying subregional and cellular expression patterns. For example, in the striatum, A_1R are localized in the postsynaptic striatonigral medium spiny neurons (MSNs) of the direct pathway, where interact with

dopamine D_1 receptors (D_1R) (Ferré et al., 1996; Fuxe et al., 1998), providing yet another mechanism from which A_1R can influence neuronal activity.

A_1R are coupled with $G_{i/o}$, inhibiting the adenylate cyclase and decreasing cyclic adenosine monophosphate (cAMP) (van Calker et al., 1979) that activates potassium channels, blocks calcium channels and increases inositol trisphosphate (IP_3) levels (Fredholm et al., 2001; Rogel et al., 2005; Tawfik et al., 2005). Consequently, A_1R activation modulates neuronal activity by inhibiting neurotransmitter release and reducing the firing rate. The most evident effect of A_1R activation in neuronal circuits of adult mammals is the selective depression of excitatory transmission (Dunwiddie and Haas, 1985).

Some of the roles of A_1R were unveiled through *Adora1*-KO (A_1R KO) mice. Two constitutive, global A_1R KO mice lines have been generated (Johansson et al., 2001; Sun et al., 2001; Fedele et al., 2006), as well as a brain-specific conditional A_1R KO mice (Bjorness et al., 2009) using the Cre/loxP strategy in which the Cre transgene expression was placed under the control of the alpha-Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII-α) promoter to provide both regional and temporal specificity of Cre expression and thus of A_1R gene (Tsien et al., 1996). Focal deletion of A_1R in hippocampal CA1 or CA3 neurons has been attained by local injection of adeno-associated virus (AAV) vectors containing the Cre transgene construct into those brain areas of mice with a critical exon of *Adora1* flanked by loxP sites (Scammell et al., 2003), which also allowed a temporal and regional specificity of A_1R deletion.

1.2. Adenosine A_{2A} receptors ($A_{2A}R$)

High levels of the $A_{2A}R$ are found in particular regions of the brain, namely in the dorsal and ventral striatum, as well as in the olfactory tubercle (Schiffmann et al., 1991b;

Fink et al., 1992; Dixon et al., 1996; Svenningsson et al., 1997a; 1997b; Rosin et al., 1998). Despite that, it is recognized that $A_{2A}R$ are also present throughout the brain, albeit with a considerably lower density, namely in the hippocampus and cortex (Dixon et al., 1996; Svenningsson et al., 1997a). In addition, $A_{2A}R$ show different subregional and cellular expression patterns within a specific tissue. For example, in the striatum, $A_{2A}R$ are mainly localized in the postsynaptic striatopallidal MSNs of the indirect pathway (Schiffmann et al., 1991a; Svenningsson et al., 1999b; Rebola et al., 2005a), where they interact with dopamine D_2 receptors (D_2R) (Ferré, 1997; Ferré et al., 1997; Hillion et al., 2002). $A_{2A}R$ are also detected at lower levels at presynaptic sites in cortico-striatal terminals (Popoli et al., 2002; Martire et al., 2010) and in the hippocampus (Rebola et al., 2005a), where they are also present postsynaptically (Rebola et al., 2008). In addition to the synaptic enrichment, $A_{2A}R$ are also located in astrocytes (Li et al., 2001; Nishizaki et al., 2002; Matos et al., 2012b), microglia (Fiebich et al., 1996; Küst et al., 1999) and in endothelial cells of brain capillaries, where they play an important role controlling brain vascular function (O'Regan, 2005; Mills et al., 2011).

In the brain $A_{2A}R$ are coupled with $G_{s/olf}$, stimulating the adenylate cyclase and increasing cAMP (Fredholm et al., 2000; Kull et al., 2000; Corvol et al., 2001; Hervé et al., 2001). $A_{2A}R$ signaling is classically described as occurring via a protein kinase A (PKA)-dependent pathway, though $A_{2A}R$ signaling through a protein kinase C (PKC)-dependent pathway in hippocampal synaptosomes has also been demonstrated (Gubitz et al., 1996; Nörenberg et al., 1998; Cunha and Ribeiro, 2000a; 2000b; Queiroz et al., 2003; Rebola et al., 2003b; Pinto-Duarte et al., 2005). Interestingly, $A_{2A}R$ seem to have limited impact on the control of 'basal' synaptic transmission but play a crucial role in controlling synaptic plasticity (Cunha, 2008a).

In addition, the scope of action and effects of $A_{2A}R$ manipulation, including the triggered signaling pathways, should be evaluated together with their ability to

heteromerize with different other G protein-coupled receptors, such as A_1R (O'Kane and Stone, 1998; Ribeiro, 1999; Ciruela et al., 2006a; 2006b), D_2R (Ferré, 1997; Ferré et al., 1997; Hillion et al., 2002), metabotropic glutamate type 5 receptors ($mGluR_5$) (Ferré et al., 2002; Tebano et al., 2005), N-methyl-D-aspartate receptors (NMDAR) (Nörenberg et al., 1998; Ribeiro, 1999), and cannabinoid CB_1 receptors (CB_1R) (Carriba et al., 2007; Ferré, 2007; Ferré et al., 2007; Tebano et al., 2009).

Some of the $A_{2A}R$ functions were also unveiled through *Adora2A*-KO ($A_{2A}R$ KO) mice. Four constitutive, global $A_{2A}R$ knockout mouse lines from different genetic backgrounds have been generated (Ledent et al., 1997; Chen et al., 1999; Day et al., 2003; Huang et al., 2006), as well as two different brain-regional deletion of $A_{2A}R$: in the forebrain (i.e., striatum, cortex, hippocampus) (Bastia et al., 2005; Xiao et al., 2006) or striatum (Shen et al., 2008; Yu et al., 2009), using the Cre/loxP strategy in which Cre transgene expression was placed under the control of the forebrain neuron-specific CaMKII-α or Dlx5/6 promoter, respectively.

1.2.1. Hippocampal $A_{2A}R$

In the hippocampus $A_{2A}R$ are mainly expressed in neurons (Rebola et al., 2003b; 2005a; 2005b), being enriched in the active zone of presynaptic terminals (Rebola et al., 2005a; 2005b), where they control the release of glutamate (Cunha and Ribeiro, 2000a), acetylcholine (Cunha et al., 1994b; 1995b; Jin and Fredholm, 1997; Rebola et al., 2002) and serotonin (Okada et al., 2001), among others (Sebastião and Ribeiro, 1996). However, hippocampal $A_{2A}R$ is particularly enriched in glutamatergic synapses (Rebola et al., 2005b), where these receptors play a tight control on the release of glutamate (Cunha and Ribeiro, 2000a; Lopes et al., 2002). The final target of $A_{2A}R$ modulation in nerve terminals seems to be calcium channels (Mogul et al., 1993; Umemiya and Berger, 1994; Gubitz et al., 1996; Gonçalves et al., 1997). Postsynaptic hippocampal

$A_{2A}R$ are able to modulate plasticity, being required for long-term potentiation (LTP) (Rebola et al., 2008; Fontinha et al., 2009; Diógenes et al., 2011; Dias et al., 2012), where blockade of these receptors impaired the response to conditioned behaviors (Fontinha et al., 2009). Hippocampal $A_{2A}R$ are also present in astrocytes (Nishizaki et al., 2002), where they control glutamate release and uptake through modulating the activity of glutamate transporters (Nishizaki et al., 2002; Matos et al., 2012b; Matos et al., 2013).

The overall control of extracellular glutamate levels (derived from neurons or astrocytes) and LTP by hippocampal $A_{2A}R$, may play crucial roles in physiological and noxious conditions affecting the hippocampal functions, like memory and epilepsy. Actually, several reports observed increased $A_{2A}R$ density in the hippocampus upon brain harmful conditions (Cunha et al., 1995a; Rebola et al., 2003b; Cunha, 2005; Cunha et al., 2006; Duarte et al., 2006; Canas et al., 2009a), namely in epilepsy (Doriat et al., 1999; Cognato et al., 2010), which seems to be responsible for an enhanced facilitation of glutamatergic synaptic transmission (Rebola et al., 2003b; Costenla et al., 2011) and acetylcholine release (Lopes et al., 1999a; 1999b).

In addition, it was shown that high frequency of neuronal firing in the hippocampus, leads to ATP release and a preferential activation of $A_{2A}R$ (Cunha et al., 1996a; 1996b; Cunha, 2005), which seems to be able to attenuate A_1R function (Cunha et al., 1994a; Lopes et al., 2002; Pinto-Duarte et al., 2005). In agreement, the percentage of nerve terminals with $A_{2A}R$ in the hippocampus that are A_1R-positive is around 80% (Rebola et al., 2005b). Consequently, $A_{2A}R$ have a major role in the control of A_1R functions, probably through intracellular transducing systems (Lopes et al., 1999a; 1999b) or maybe through receptors dimerization (Ciruela et al., 2006b).

Hippocampal $A_{2A}R$ are also able to transactivate TrkB receptors in the absence of the ligand (Lee and Chao, 2001), being required for normal BDNF levels and functions in the hippocampus (Diógenes et al., 2004; 2007; Tebano et al., 2008).

2. Adenosine and memory

The notion that adenosine potentially modulates cognition, namely memory functions, was strengthened from the general belief that consumption of caffeine (a non-selective adenosine receptors antagonist), the most widely used psychoactive compound, improves cognitive performance in humans (Nehlig, 2010). Several studies suggested that caffeine enhances memory performance in healthy volunteers consumed either acutely (Hogervorst et al., 1999; Rees et al., 1999; Smit and Rogers, 2000; Lieberman et al., 2002; Haskell et al., 2005; Heatherley et al., 2005; van Duinen et al., 2005), with continuous slow delivery (Patat et al., 2000; Beaumont et al., 2001) or with long-term intermittent consumption (Jarvis, 1993; Hameleers et al., 2000; Johnson-Kozlow et al., 2002; James and Rogers, 2005; James and Keane, 2007). In contrast, other studies found minor or no effects of caffeine on memory performance (Schmitt et al., 2003; Bonnet et al., 2005; Bichler et al., 2006; Childs and de Wit, 2006), which questions the classification of caffeine as a cognitive enhancer. The inconsistency can reflect the different doses tested, the schedule of administration (acute *versus* chronic) or the timing of administration (before training, affecting memory acquisition, or after training, affecting memory consolidation or retrieval). Nevertheless, the overall available evidences cautiously suggest that the continuous and moderate consumption of caffeine might afford beneficial effects on cognition (Jarvis, 1993; Cunha, 2008b; Cunha and Agostinho, 2010). In agreement, a recent and very well carried out study showed that caffeine administration enhances memory consolidation in humans (Borota et al., 2014). Animal studies also corroborate the idea that the consumption of moderate doses of

caffeine (or other drugs with pharmacological properties similar to caffeine) improves memory performance in rodents (Cestari and Castellano, 1996; Angelucci et al., 1999; Hauber and Bareiss, 2001; Angelucci et al., 2002; Prediger et al., 2005a; 2005b; Costa et al., 2008a; 2008b). In contrast, the effects of caffeine in memory retrieval are still unclear (Furusawa, 1991; Angelucci et al., 1999; Corodimas et al., 2000; Hauber and Bareiss, 2001; Angelucci et al., 2002). In addition, the pro-mnemonic effects of caffeine intake may reflect caffeine's impact on other processes such as arousal, attention, and mood, which can in turn influence performance on cognitive tasks (Takahashi et al., 2008; Chen et al., 2010), being difficult to pinpoint the specificity and direct targets of caffeine in the brain.

Nowadays it is known that endogenous extracellular adenosine, acting mainly through A_1R and $A_{2A}R$ in the CNS, controls and integrates a wide range of brain functions, namely the regulation of sleep, locomotion, anxiety, cognition and memory (Dunwiddie and Masino, 2001; Fredholm et al., 2005b). The involvement of the adenosinergic signaling in neuromodulation and neurodegeneration in the CNS is well established (Ribeiro and Sebastião, 2010; Gomes et al., 2011), including its participation in cognitive behavioral functions in pathological situations (Canas et al., 2009b; Cunha and Agostinho, 2010). However, the involvement of the adenosinergic signaling in cognitive behavioral processes in physiological conditions is just beginning to gain attention. Studies based on the use of antagonists and agonists, have suggested a role for adenosine and its receptors targets on learning and memory, but these findings have been inconsistent and the majority performed in pathological conditions (Gomes et al., 2011). This inconsistency possibly reflects the different contributions of the different adenosine receptors subtypes in distinct brain regions and differences in the timing of the pharmacologic manipulation across studies, but clearly emphasizes the role of these two receptors in the different pathological conditions.

Transgenic mice with low adenosine tone in the brain due to over-expression of adenosine kinase (ADK, that phosphorylate adenosine to AMP; see Figure 1) were characterized by impaired memory (Yee et al., 2007). This further suggests that adenosine regulates cognition under physiologic conditions. In agreement, adenosine acting through A_1R and $A_{2A}R$ is able to modulate neurotransmitter systems, neuronal excitability, and synaptic plasticity (e.g., LTP and long-term depression (LTD)) in brain regions relevant for learning and memory (Rebola et al., 2008; Fontinha et al., 2009; Wei et al., 2010). However, the contribution of A_1R and $A_{2A}R$ to adenosine's regulation of cognitive functions is still unclear. The potential modulatory effects of adenosine on cognition were traditionally attributed to A_1R, mainly due to its relative abundance in regions classically studied in learning and memory, like the hippocampus. However, the recent studies that explored the role of adenosine receptors in cognition under physiological conditions are starting to reveal the complexity and vastness of adenosine's functions in brain, as well as the importance of $A_{2A}R$ is gaining emphasis.

Surprisingly, the evidences gathered from studying A_1R KO mice suggest that A_1R may not play a critical role as once believed in mediating some of the mnemonic effects of adenosine. Global deletion of the A_1R in mice failed to produce any behavioral performance repercussion in the water maze test in three separate experiments from two different knockout mouse lines (Giménez-Llort et al., 2002; Lang et al., 2003). In fact, global A_1R KO mice showed normal acquisition and retention of a spatial reference memory, normal spatial working memory, and normal ability to learn the new position of a fixed platform during reversal learning (Giménez-Llort et al., 2002). These findings suggest that A_1R are maybe not critical for the expression of normal spatial reference memory or working memory under physiologic conditions. However, it should be taken into account that interfering with specific pools of A_1R in particular brain regions could have different outcomes. Despite this contention, global deletion of A_1R in mice seems to

afford a balanced and normal cognitive performance. However, old global A_1R KO mice showed spatial working memory deficits in the 6-arm radial tunnel maze (Giménez-Llort et al., 2005), thus corroborating an earlier pharmacologic study showing a role for hippocampal A_1R on working memory (Ohno and Watanabe, 1996). Though, this earlier finding, was instead attributed to reduced test environment habituation rather than to a mnemonic process (Giménez-Llort et al., 2005).

Nevertheless, transgenic and knockout studies with $A_{2A}R$ manipulation have recently provided direct evidence that $A_{2A}R$ are major players in adenosine's control of memory performance. Global $A_{2A}R$ KO mice showed improved spatial recognition memory in an elevated Y-maze (Wang et al., 2006), spatial water maze and also in the radial arm maze (Zhou et al., 2009; Wei et al., 2011). In agreement, transgenic rats over-expressing $A_{2A}R$ in the cortex exhibited impaired memory function in several behavioral paradigms including the water maze, 6-arm radial tunnel maze, and novel object recognition tasks (Giménez-Llort et al., 2007). Thus, findings from genetic $A_{2A}R$ studies generally support the notion that suppression of $A_{2A}R$ activity is pro-cognitive and raises the possibility that $A_{2A}R$ may represent a target for improving cognitive function under normal conditions.

3. Adenosine and motor function

Adenosine exerts inhibitory effects on spontaneous locomotor activity and thus the administration or intake of caffeine seems to result on the opposite effect, through the antagonism of adenosine receptors (Snyder et al., 1981; Barraco et al., 1983). In addition, $A_{2A}R$ activation on the nucleus accumbens was shown to mediate locomotor depression (Barraco et al., 1993). A predominant role for A_1R on the motor-activity effects in rats acutely administered with caffeine has been reported (Antoniou et al., 2005) and a combination of A_1R and $A_{2A}R$ blocking agents induces caffeine-like

enhancement of spontaneous locomotor activity in mice (Kuzmin et al., 2006). Nowadays, the striatal neuronal $A_{2A}R$ are believed to be the main effector of adenosine-based modulation of motor activity (Shen et al., 2008). Thus, the evident impact of adenosine on motor functions, is probably mainly due to the high density of $A_{2A}R$ in the striatum, a brain region critically involved on motor control.

$A_{2A}R$ are highly expressed in the striatum, being predominantly expressed at postsynaptic striatopallidal MSNs of the indirect pathway (Schiffmann et al., 1991b; Svenningsson et al., 1997a; 1997b; 1999b; Rebola et al., 2005a), where they antagonistically interact with D_2R (Ferré, 1997; Ferré et al., 1997; Schwarzschild et al., 2006). This anatomic segregation is functionally significant and is relevant for understanding the role of these receptors in motor control, psychomotor activity and motor disorders, like Parkinson's disease (PD) (Ferré et al., 1997; Svenningsson et al., 1999a; Schwarzschild et al., 2006). Even so, A_1R have a low expression in the striatum, being predominantly expressed at postsynaptic striatonigral MSNs of the direct pathway (Ferré et al., 1996).

3.1. Adenosine and spontaneous locomotion

$A_{2A}R$ antagonism induces motor stimulation and in agreement, $A_{2A}R$ activation induces motor depression (Chen et al., 2001a). In fact, adenosine receptor KO mice studies confirmed that $A_{2A}R$ are the main effectors of adenosine-based modulation of motor activity and of caffeine's motor stimulant effects, as well as the identification of D_2R-independent mechanism for motor outcomes of $A_{2A}R$ manipulation (Chen et al., 2001a; Wei et al., 2010; Lazarus et al., 2011). In addition, it was shown that $A_{2A}R$ antagonist-induced motor stimulation was absent in striatal $A_{2A}R$ KO mice, strengthening the evidence that $A_{2A}R$ in the striatopallidal MSNs are the main target of the motor stimulating effects of $A_{2A}R$ antagonists (Shen et al., 2008).

Oddly, while $A_{2A}R$ antagonists are well known to induce motor stimulation, genetic $A_{2A}R$ deletion has failed to produce a similar effect on basal motor activity. Instead, adult global $A_{2A}R$ KO mice (from different genetic backgrounds) consistently exhibited reduced spontaneous motor activity compared to their wild-type (WT) controls (Ledent et al., 1997; Chen et al., 1999; 2000; Yang et al., 2009; Sturgess et al., 2010). On the other hand, forebrain $A_{2A}R$ KO or striatal $A_{2A}R$ KO mice do not show differences in basal activity levels (Bastia et al., 2005; Shen et al., 2008; Yu et al., 2008). This difference between global $A_{2A}R$ KO mice and conditional $A_{2A}R$ KO mice suggests that the phenotype observed in the global $A_{2A}R$ KO mice might be non-specific or a result of adaptive effects of constitutive global gene deletion, or may also reflect the activity of $A_{2A}R$ at non-neuronal (i.e., astrocytic or microglial) sites.

Besides, global A_1R KO mice have a minimal impact on spontaneous motor activity (Johansson et al., 2001; Giménez-Llort et al., 2002; Halldner et al., 2004; Yang et al., 2009) and failed to affect motor coordination (Giménez-Llort et al., 2002).

3.2. Adenosine and psychomotor activity

It is suggested that the psychomotor effects are mainly generated by both, the dopaminergic as well as the glutamatergic tonus in the striatum (Svensson et al., 1995). In addition, adenosine and its receptors are known to modulate these neurotransmitters systems in that brain area. In agreement, $A_{2A}R$ have been shown to modulate the psychomotor effects produced by various drugs such as cocaine, amphetamine (i.e., dopamine enhancers) and phencyclidine (a NMDAR antagonist) (Turgeon et al., 1996; Hauber and Münkle 1997; Ferré 1997; Chen et al., 2003; Bastia et al., 2005; Shen et al., 2008; Hobson et al., 2012).

Overall, $A_{2A}R$ KO mice have been shown to have a reduced psychomotor response to dopaminergic compounds, like cocaine and amphetamine and to the

NMDAR antagonist, phencyclidine (Chen et al., 2003; Bastia et al., 2005; Shen et al., 2008). Accordingly, global $A_{2A}R$ KO mice were shown to exhibit a selective attenuation in the motor response to cocaine or amphetamine, without changes on D_1R or D_2R direct agonist-induced motor stimulation or suppression, respectively (Chen et al., 2001a; Chen et al., 2000; 2003; Fredholm et al., 2005b). Forebrain $A_{2A}R$ KO mice also displayed a reduction in their motor responses to cocaine (Shen et al., 2008), amphetamine (Bastia et al., 2005) and phencyclidine (Shen et al., 2008). In contrast, $A_{2A}R$ deletion restricted to postsynaptic striatal neurons in striatal $A_{2A}R$ KO mice, enhanced rather than attenuated the hyperlocomotor response to a single injection of cocaine or phencyclidine (Shen et al., 2008). These results indicate that striatopallidal $A_{2A}R$ predominantly inhibit psychomotor activity, which is consistent with the $A_{2A}R$-D_2R antagonistic interaction at striatopallidal MSNs (Ferré et al., 1997). Notably, the comparative behavior analysis of the psychomotor response profile to dopamine enhancers or NMDAR blockade in global, forebrain or striatal $A_{2A}R$ KO mice revealed a critical and preponderant role of extra-striatal $A_{2A}R$ in the modulation of psychomotor activity (Shen et al., 2008). Altogether, these results suggested that the excitatory effect of extra-striatal $A_{2A}R$ predominates and counters the inhibitory effect of striatopallidal $A_{2A}R$ on psychomotor activity. Thus, stimulation of $A_{2A}R$ on forebrain neurons appears to be important for the full expression of hyperlocomotor responses to psychoactive drugs. This effect of extra-striatal $A_{2A}R$ was speculated to result from pre-synaptic $A_{2A}R$ modulation of glutamate release at cortico-striatal nerve terminals. This aspect is important for the treatment of addiction, since the results from global and forebrain $A_{2A}R$ KO mice also reflect the lack of locomotor sensitization to cocaine and amphetamine (Chen et al., 2000; 2001a; 2003; Bastia et al., 2005). It is also significant to psychiatric disorders like schizophrenia, since NMDAR antagonists (namely phencyclidine and dizocilpine (MK-801)) elicit behavioral abnormalities related to symptoms of

schizophrenia, such as enhanced spontaneous locomotor activity.

3.3. Adenosine and Parkinson's disease (PD)

PD results primarily from the death of dopaminergic neurons in the substantia nigra pars compacta (SNc) that lead to a profound loss of dopamine in the striatum (Hornykiewicz and Kish, 1987), which in turn attenuate the control of striatal circuits, particularly reducing the inhibitory tonus of the indirect pathway (that selectively expresses $A_{2A}R$) that becomes overactive (Gerfen, 2006).

The involvement of $A_{2A}R$ in PD was first suggested by the epidemiologic inverse relation between the consumption of caffeine (an antagonist of adenosine receptors) and the risk of developing PD (Ross et al., 2000a; 2000b; Ascherio et al., 2001; Ross and Petrovitch, 2001; Chen et al., 2001b). Currently there are strong evidences that highlight a critical role for $A_{2A}R$ in the pathophysiology of PD (Kanda et al., 1998; Grondin et al., 1999; Richardson et al., 1999; Shiozaki et al., 1999; Aoyama et al., 2000; Chen et al., 2001b; Casetta et al., 2013). $A_{2A}R$ participates in the control of motor functions (see section 2.1.) and is able to regulate glutamatergic transmission in the cortico-striatal pathway (Popoli et al., 1995; 2002) that suffers compensatory plastic adaptations in PD (Pisani et al., 2005; Day et al., 2006; Deutch, 2006). In addition, $A_{2A}R$ blockade leads to increased motor activity in different rodent and primate models of PD, alone or co-administered with dopaminergic drugs (Fenu et al., 1997; Kanda et al., 1998; Shiozaki et al., 1999; Koga et al., 2000; Hauber et al., 2001) and confers neuroprotection (Richardson et al., 1997; 1999; Chen, 2003; Schwarzschild et al., 2003; 2006; Jenner et al., 2009). Moreover, when administered after the onset of the most severe side effect of levodopa (L-3,4-dihydroxyphenylalanine, also known as L-DOPA and a precursor of dopamine and first-line pharmacotherapeutical strategy in PD), dyskinesia, $A_{2A}R$ antagonists have an additional beneficial effect upon motor disability and do not worsen

dyskinesia (Kanda et al., 1998; Grondin et al., 1999; Kanda et al., 2000; Jenner, 2003; Lundblad et al., 2003). Furthermore, the current therapeutic strategy is unable to stop the ongoing degenerative process. In this regard, $A_{2A}R$ blockade or genetic deletion, reduce dopaminergic cell loss and counteract undergoing striatal dopamine depletion, underpinning an effective neuroprotective role, whose mechanism has not yet been determined, but which seems to be different from that mediating motor effects of these ligands (Chen et al., 2001b; Ikeda et al., 2002; Pierri et al., 2005; Yu et al., 2008). Accordingly, $A_{2A}R$ antagonists have emerged as prominent non-dopaminergic drugs for the treatment of PD and 25 clinical trials have been conducted (Chen et al., 2013). Different clinical Phase IIb and Phase III trials involving patients with advanced PD have been reported (Hauser et al., 2011). Most of them showed a modest but significant reduction in the 'off-time' compared to the optimal levodopa dose regimen (Jenner et al., 2009). Currently, several Phase IIb and Phase III trials for $A_{2A}R$ antagonists are still underway, persisting as one of the leading non-dopaminergic treatment candidates for PD (Meissner et al., 2011; Chen et al., 2013).

4. Sources of extracellular adenosine

Adenosine causes different and most often opposite actions by activating different receptors that, in addition can be co-localized, at least in nerve terminals (Ciruela et al., 2006a, 2006b). Thus, it becomes of upmost importance to understand how the differential activation of the different adenosine receptors can be effectively controlled to meet the needs of the system. One possibility would be that the different adenosine receptors might have different affinities for adenosine. However, most results suggest that the affinity of adenosine for A_1R and $A_{2A}R$ is similar, in the low nanomolar range like the ones observed in the brain in basal conditions, which was estimated to be circa 20 nM (Fredholm et al., 2005b). Thus, it has been suggested that there might be different

ways of generating adenosine to activate either A_1R or $A_{2A}R$ (Cunha, 2001a). In fact, the source of endogenous extracellular adenosine during physiological conditions of neuronal firing has been one of the less studied aspects of adenosine neuromodulation.

Two main mechanisms have been identified in nerve terminals for the generation of extracellular adenosine (Figure 1): one is based on the release of adenosine as such through bi-directional non-concentrative (or equilibrative) nucleoside transporters (ENT); the other is its formation from released ATP, followed by extracellular catabolism through the action of ecto-nucleotidases (Cunha, 2001a). In addition, recent studies suggested that adenosine could be released as such (Frenguelli et al., 2007; Klyuch et al., 2012) through mechanisms still controversial, which may involve carrier systems independent of nucleoside transporters (Sperlágh et al., 2003), but blocked by bafilomycin and modulated by metabotropic glutamate type 4 receptors (mGluR4) activation (Klyuch et al., 2012).

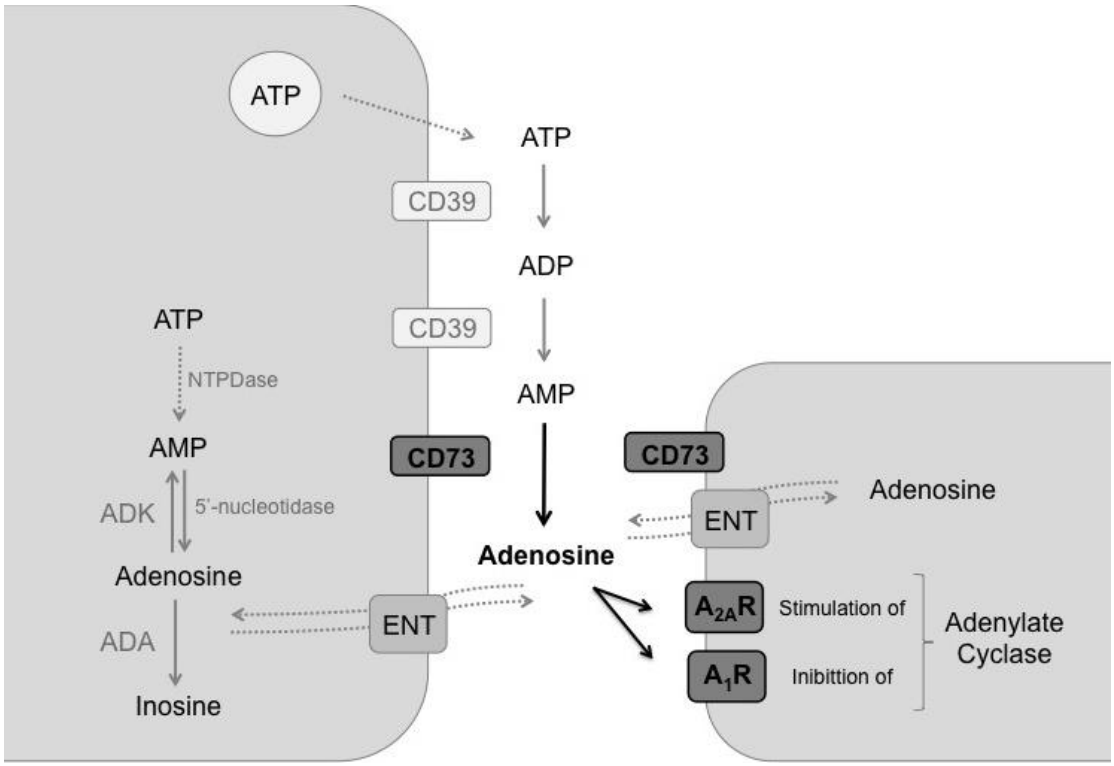

Figure 1. Summary of the principal components of the adenosinergic system, showing the principal sources of adenosine and its metabolism. Released ATP is extracellularly degradated to adenosine by CD39 and CD73, followed by its reuptake by equilibrative nucleoside transporters (ENT) and further phosphorylated by adenosine kinase (ADK) back into ATP. Adenosine can also be metabolized into inosine through the action of adenosine deaminase (ADA). Extracellular adenosine can activate different adenosine receptors, namely A_1R or $A_{2A}R$. NTPDase; nucleotidases triphosphate diphosphohydrolases; CD39, ecto-nucleotidases triphosphate diphosphohydrolases; CD73, ecto-5'-nucleotidase; ENT, equilibrative nucleoside transportes; ADK, adenosine kinase; ADA, adenosine deaminase.

The mechanism of controlling A_1R versus $A_{2A}R$ activation according to the levels of released ATP seems to be valid for hippocampal excitatory nerve terminals (Cunha, 2001a; 2005) and phrenic nerve endings (Correia-de-Sá et al., 1996). However, different types of nerve terminals have different organizations of extracellular adenosine metabolism, transporters and adenosine receptors, and in more integrated brain preparations, the different sources of extracellular adenosine and A_1R or $A_{2A}R$ activation are not clear. Extracellular ATP is the strongest candidate to act as a primary source of adenosine since it is released in a controlled manner from neurons (Zimmermann,

1994), astrocytes (Wang et al., 2000; Arcuino et al., 2002; Bal-Price et al., 2002; Ballerini et al., 2002; Stout et al., 2002; Coco et al., 2003; Koizumi et al., 2003; Newman, 2003; Zhang et al., 2003; Anderson et al., 2004; Parkinson and Xiong, 2004), as well as from activated microglia (Seo et al., 2004). Actually, most cell types in the brain can release ATP (Fields and Burnstock, 2006) and are endowed with ecto-nucleotidases that are able to convert extracellular ATP into adenosine (Zimmermann, 2000; Cunha, 2001b; Cunha et al., 2001). In addition, there is now compelling evidence supporting an important role for this ATP-source of adenosine, at least under physiological conditions (Correia-de-Sá et al., 1996; Cunha et al., 1996a; 1996b; Dale, 2002; Koizumi et al., 2003; Newman, 2003; Zhang et al., 2003; Pascual et al., 2005).

One key aspect of the mechanistic explanation coupling the extracellular metabolism of released ATP with the preferential activation of $A_{2A}R$, is the proximal localization between ecto-5'-nucleotidase (CD73, responsible for last step of the formation of ATP-derived adenosine) and $A_{2A}R$. This has, so far, not been directly demonstrated, however, it is striking to note that several physiological (Cunha et al., 1996b; 2001) and pathological situations (Agostinho et al., 2000) cause a parallel increase on the activity of CD73 and density of $A_{2A}R$, in contrast to A_1R. In agreement, it has been shown that different noxious stimuli cause a parallel increase on the expression of CD73 and of $A_{2A}R$ (Kobayashi and Millhorn, 1999; Kobayashi et al., 2000; Napieralski et al., 2003; Deaglio et al., 2007), strongly supporting the view that these two molecules are tightly interconnected. Yet, it is possible that different sources of ATP could facilitate the activation of different adenosine receptors. While synaptic ATP release has been associated with $A_{2A}R$ activation (Cunha et al., 1996a; Cunha, 2005; 2008a), astrocytic ATP release was associated with tonic A_1R-mediated inhibition on synaptic transmission in hippocampal slices (Pascual et al., 2005). This implies that the endogenous extracellular adenosine responsible for the tonic A_1R-mediated inhibition of

excitatory synaptic transmission is largely derived from astrocytes. However, this was not supported by a recent study performed by Lovatt et al. (2012). In addition, it has also been proposed that cAMP could be released from neurons and hydrolyzed into adenosine (Rosenberg and Li, 1995), though this contribution is at best limited (Brundege et al., 1997).

Adenosine nucleotide : adenosine ratios are around 10 000 : 1 (Fredholm et al., 2005a), the maintenance of which requires powerful and very orchestrated mechanisms. Different ectonucleotidases are involved in the conversion of adenosine nucleotides (e.g. ATP, ADP, AMP) into adenosine, and the intracellular adenosine levels are regulated by enzymes such as adenosine kinase (ADK) and adenosine deaminase (ADA; Figure 1). Clearly, further work is required to elucidate the pathways of generation of extracellular adenosine in the brain, which is a pre-requisite to understand the dynamics of activation of adenosine receptors in different physio-pathological conditions.

4.1. Equilibrative nucleoside transporters (ENT)

Equilibrative (i.e., non-concentrative and bidirectional) nucleoside transporters are assumed to be present (still to be experimentally documented) in all cell types (Kong et al., 2004). Although the release of adenosine as such through these transporters was classical and widely accepted hypothesis for the build-up of extracellular adenosine in the brain, it has only received episodic experimental confirmation (MacDonald and White, 1985; Cunha et al., 2000a). In less intact preparations, like nerve terminals or *in vitro* preparations, inhibition of ENT indeed decrease the extracellular levels of adenosine (MacDonald and White, 1985; Cunha et al., 2000a); which is in accordance with a build-up of extracellular adenosine through its release from ENT, maybe in particular compartment of the brain, like the hippocampal nerve terminals (Cunha et al., 2000a). Oddly, the effect of pharmacologically manipulation of ENT *in vivo* or in more

intact brain preparations, is an increase of the extracellular levels of adenosine implying that their role is to capture rather than release adenosine (Latini and Pedata, 2001; Fredholm et al., 2005a; Melani et al., 2012). Thus, in integrated brain preparations under physiological conditions, there are no studies directly supporting the idea that adenosine is released directly through ENT.

4.2. ATP release

ATP is present in high concentrations within the brain, varying from approximately 2mM/kg in the cortex to 4mM/kg in the putamen and hippocampus (Kogure and Alonso, 1978). It is now well accepted that all cell types in the brain release ATP, namely, neurons, astrocytes or microglia cells, by mechanisms that remain controversial (Bodin and Burnstock, 2001). ATP can be released from various cell types by multiple mechanisms: from uncontrolled leakage from necrotic cells or other forms of cell death (Elliott et al., 2009), to controlled release through pannexin hemichannels (Chekeni et al., 2010), such as P2X purinergic receptor 7 (Kanneganti et al., 2007; Anselmi et al., 2008; Faigle et al., 2008). Furthermore, ATP can also be co-released from storage vesicles through exocyotosis (Zhang et al., 2007) together with other neurotransmitters (Burnstock, 2013), a mechanism that is more relevant for synaptic plasticity.

In neurons, it is known that ATP is stored in synaptic vesicles and that nerve terminals release ATP after stimulation (Zimmermann, 1994). ATP is present in synaptic and/or secretory vesicle, and can be co-stored and co-released with other neurotransmitters (e.g. gamma-aminobutyric acid (GABA), noradrenaline or glutamate), and some neuronal terminals might even contain vesicles only enriched in ATP (Pankratov et al., 2006; 2007).

As revealed recently, the accumulation of ATP into vesicles can be mediated by a vesicular nucleotide transporters (VNUT) (Sawada et al., 2008) that preferentially

recognize ATP, GTP and ADP (Sawada et al., 2008). These transporters are highly expressed in the brain and are preferentially localized in subpopulations of astrocytes (Larsson et al., 2012). In addition, it was shown that VNUT are also associated with synaptic vesicles and co-localize with other vesicular neurotransmitter transporters, namely vesicular glutamate transporters (VGLUT) and vesicular GABA transporters (VGAT) (Larsson et al., 2012). Although controversial, there are already some evidences suggesting that ATP can be released separately from other neurotransmitters (Pankratov et al., 2006).

4.3. Ectonucleotidases

Following release, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotidases, which is functionally important because ATP and associated metabolites act as physiological ligands for various purinergic receptors. The ectonucleotidases not only control the lifetime of nucleotide ligands but, by degrading or interconverting the originally released ligands, they are also able to produce ligands for additional adenosine receptors (Zimmermann, 2006a).

Ectonucleotidases are considered ubiquitous enzymes. All ectonucleotidase families identified so far are expressed in the brain, forming an efficient enzymatic pathway to convert ATP into adenosine (Zimmermann, 2000) and consequently capable of controlling the extracellular concentrations of ATP and adenosine present in the brain.

Extracellular nucleotide hydrolysis is surprisingly complex and ectonucleotidases belong to several enzyme families. They differ in functional and molecular properties, differing in substrate specificity and product formation, but reveal a partially overlapping expression pattern. The family of ectonucleotidases include ectonucleoside triphosphate diphosphohydrolase (E-NTPDase), ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP), alkaline phosphatases and ecto-5′-nucleotidase (CD73) (Zimmermann, 2006a;

Yegutkin, 2008). E-NTPDases and E-NPPs hydrolyze ATP and ADP into AMP, which is further hydrolyzed to adenosine by CD73. Alkaline phosphatases are also able to hydrolyze nucleoside tri, di and monophosphates (Table 1) (Zimmermann et al., 2012).

Enzyme	Hydrolysis pathway
E-NTPDases	ATP → ADP + Pi
	ADP → AMP + Pi
	ATP → ADP + Pi → AMP + Pi
E-NPPs	ATP → AMP + PPi
	ADP → AMP + Pi
Alkaline Phosphatases	ATP → ADP + Pi
	ADP → AMP + Pi
	AMP → Adenosine + Pi
CD73	AMP → Adenosine + Pi

Table 1. Ectonucleotidases and their hydrolysis of nucleotides in the brain.

The members of the E-NTPDase family hydrolyze nucleoside tri- and diphosphates, namely ATP and ADP, but not nucleoside monophosphates. At least three known cell-surface located NTPDases (NTPDase1–3) are present in rat brain (Kegel et al., 1997; Belcher et al., 2006). Despite some structural similarities, these enzymes differ distinctly in their substrate specificity. Thus, NTPDase1 (also known as CD39, ecto-apyrase, ecto-ATP diphosphohydrolase) is able to convert ATP and ADP to AMP with similar efficiency, while NTPDase2 (ecto-ATPase) hydrolyzes triphosphonucleosides to the respective diphosphonucleosides (Heine et al., 1999; Kukulski and Komoszyński, 2003). NTPDase3 (also known as CD39L3) is a functional intermediate that dephosphorylates ATP into AMP with a transient accumulation of ADP (Lavoie et al., 2004). NTPDase8 has not yet identified in the mammalian brain (Bigonnesse et al., 2004).

Members of the E-NPP family (NPP1, NPP2 (autotaxin) and NPP3) hydrolyze 5′-monodiester bonds in nucleotides and their derivatives, resulting in the release of, for example, AMP and inorganic pyrophosphate (PPi) from extracellular ATP (Goding et al.,

2003; Stefan et al., 2005). They also hydrolyze dinucleoside polyphosphates (Vollmayer et al., 2003).

Alkaline phosphatases are nonspecific phosphomonoesterases, i.e. they release inorganic phosphate from a large variety of organic compounds and equally degrade nucleoside 5′-tri-, -di-, and –monophosphates (Langer et al., 2008).

CD73 only hydrolyzes nucleoside monophosphates and plays an important role in the formation of adenosine from extracellular AMP and the subsequent activation of adenosine receptors (Cunha, 2001a).

The coordination of purinergic regulatory systems in the CNS relies on the control of a local network regulated by the balance between the effects of ATP, adenosine and ectonucleotidases on synaptic transmission (Kato et al., 2004; Matsuoka and Ohkubo, 2004). Importantly, in neither case appear the ecto-forms to be identical with cytosolic enzymes exhibiting similar catalytic activity (Zimmermann, 1996).

4.3.1. Ecto-5'-nucleotidase (CD73)

CD73 is highly conserved between vertebrate species (Zimmermann, 1996) and recently the crystal structure of human CD73 was published (Heuts et al., 2012). CD73 belongs to the family of 5′-nucleotidases, but while most 5′-nucleotidases are located intracellularly, CD73 is a cell-surface enzyme that faces the extracellular medium, being linking to the outer part of the plasma membrane through a glycosyl-phosphatidyl-inositol (GPI) anchor in its carboxylic terminal being thus hydrophobic in nature (Low and Finean, 1978; Ogata et al., 1990; Misumi et al., 1990a; 1990b); however a soluble form can also exist if the GPI anchor is cleaved (Braun et al., 1997). Nevertheless, attempts to solubilize CD73 from intact membranes by addition of phosphatidylinositol specific phospholipase C have generally resulted in the release of only a fraction of the enzyme (Zimmermann, 1992).

The different 5'-nucleotidases are filogenetically distant and there are no significant similarities between the primary structures of these proteins (Bianchi and Spychala, 2003), so CD73 can be distinguished from all the other enzymes. Actually, the soluble cytoplasmic enzymes represent proteins different from CD73 (Zimmermann, 1992) and the biochemical properties of the intracellular soluble forms differ from each other and in particular from the cell-surface-anchored CD73 (Zimmermann, 1992). CD73 is codified by a single gene in mammalians although it has been reported the appearance of different glycosylated forms (Cunha et al., 2000b; Zimmermann et al., 2012). CD73 was found to have an apparent molecular mass of 62 to 74 kDa and occurs as a dimer with inter-chain disulfide bridges (Zimmermann, 1996).

CD73 hydrolyze non-cyclic nucleoside monophosphates or deoxynucleoside monophosphates (such as AMP, CMP, UMP, IMP and GMP), to (deoxy)nucleosides and inorganic phosphate (Borowiec et al., 2006). Although it has a broad spectrum of substrates it seems to hydrolyze with the highest efficiency AMP into adenosine (Zimmermann, 1992; Bianchi and Spychala, 2003), being the key enzyme that catalyzes the production of extracellular adenosine from AMP (Zimmermann, 1996; Zimmermann et al., 2012). ATP and ADP are competitive inhibitors, as well as the nucleotide analogue adenosine 5'-[α,β-methylene]diphosphate (AMPCP) (Cunha, 2001b).

CD73 catalytic activity has been detected in the rodent CNS (Langer et al., 2008), and was classically predominantly assigned to the surface of glial cells, however, neuronal localization of CD73 has also been reported (Langer et al., 2008). Nevertheless, the relative expression of CD73 in glial (Schoen et al., 1992) versus neuronal cells (Heiman et al., 2008) is still unclear.

Additionally, there are evidences suggesting that CD73 can interact with other components of the extracellular matrix, particularly to laminin and fibronectin, and therefore involved in cell/cell or cell/matrix interactions, particularly during development

(Zimmermann and Braun, 1996; Zimmermann, 2006b). These matrix proteins are involved in several crucial biological processes, such as cell adhesion, growth, spreading and also migration, which might give us an insight about the other potential functions of CD73 (Langer et al., 2008).

Recently, CD73 knockout (CD73 KO) mice have been successfully generated, showing around 90% reduction of ATP to adenosine metabolism in the brain (Klyuch et al., 2012), and thus reduced endogenous adenosine as well as reduced adenosine receptor sensitivity (Koszalka et al., 2004; Thompson et al., 2004; Colgan et al., 2006). Given the pivotal role of CD73 as a key regulator of purinergic signaling controlling the extracellular provision of adenosine, CD73 KO mice provide a unique opportunity to examine the role of CD73 on different behavioral processes.

4.4. Challenges and new perspectives

Different sources of adenosine, as well as different sources of ATP, which in turn can be metabolized into adenosine (see section 2; Figure 1), can participate in the activation of different adenosine receptors, as well as in different pathways and functions in the brain. Therefore, determining the adenosine source and receptors subtype, which are singularly associated with specific physiological process or disease status, in different definable extracellular domains within the brain parenchyma (that is, neuronal and/or synaptic, astrocytic, microglial or vascular domains), is crucial in order to develop new therapeutic strategies for brain disorders (Chen et al., 2013).

The difficulty in distinguishing the several sources of extracellular adenosine under physiological and pathological conditions is a major challenge and caveat in the adenosinergic field. The difficulties start with the different handling of preparations, which generally produce a massive extracellular accumulation of adenosine that occurs after different types of insults (Latini and Pedata, 2001). The challenges continue with the lack

of tools to provide a reliable quantification of adenosine in a nanomolar range, like the ones observed in physiological conditions, and the possibility to perform the quantifications in specific cellular and subcellular domains, like the synaptic cleft.

A further major issue contributing to the inability to pinpoint the contribution of the different sources of endogenous extracellular adenosine is the inability to determine the location and role of the different ectonucleotidases. This is probably related to the general lack of pharmacological tools to specifically manipulate particular enzymes in this large family of enzymes (Zimmermann, 2000), as well as to the lack of tools to accurate recognize them. In fact, we know considerably more about the molecular biology of ectonucleotidases than of their localization and kinetic properties in native tissues that ultimately define their physiological role (Cunha, 2005).

Despite the fact that adenosine receptor ligands are metabolically stable, they are able to reach all receptors (and they are found on many cells in the body) contributing to a substantial risk of inducing side effects. In addition, because of their generally high affinity, would provide prolonged stimulation. Alternatively, instead of directly targeting adenosine receptors, an optimal therapeutic approach would be to manipulate a specific source of endogenous adenosine, which could provide some degree of specificity (Chen et al., 2013). An elegant use of network pharmacology is the development of a novel type of prodrug that needs to be metabolized in order to become available as an adenosine receptor agonist (El-Tayeb et al., 2009). Accordingly, a 5´-phosphate prodrug of adenosine receptor agonist was generated in order to be hydrolized at sites where CD73 is highly expressed, in order to release the active adenosine receptor agonist (Flögel et al., 2012). This prodrug approach not only allows the site-specific action within the tissues where CD73 is enriched but may also avoid some of the side effects.

The adenosinergic field has greatly improved and gained prominence due to the awareness of the gaps and the technical limitations that were still hampering the field.

This led to the enhancement and optimization of experimental tools and techniques to perform a multiplicity of different studies and thus on resultant expansion of the available data. Hopefully, in a near future, this will be reflected by an increased consequential knowledge and on new therapeutic strategies.

GOALS

Adenosine is a prototypic neuromodulator that fine-tunes on-going synaptic transmission, controlling the flow of information through different neuronal circuits in the brain (Dunwiddie and Masino, 2001). Some of adenosine receptors' roles in the brain are well known, however, the source of adenosine is a caveat in the adenosinergic field. Importantly, under physiological conditions adenosine tonus is not very prominent, and the adopted strategy to study the source of extracellular adenosine was trough CD73 knockout (KO) mice. CD73 is the key enzyme that catabolizes the last step of the catabolism of ATP to adenosine and we proposed **to refine the characterization and role of CD73 in the central nervous system (CNS).**

On **chapter 2** we started **to investigate the presence of CD73 in the different brain areas**, as well as, its cellular and subcellular localization in physiological settings.

On **chapter 3** we aim **to explore the role of CD73 in locomotion, memory and learning** paradigms, taking advantage of CD73 KO mice.

Due to the results obtained on chapters 2 and 3, with a segregation of CD73 on basal ganglia, on **chapter 4** we decided **to explore the role of CD73 in different pathological conditions that are patent in the striatum**, namely drug addiction, psychomotor activity and Parkinson's disease, by taking advantage of CD73 KO mice.

Thanks to the results obtained on the previous chapters that pointed CD73 with a particular role on the activation of $A_{2A}R$, on **chapter 5** we proposed **to explore if CD73 provides the particular pool of extracellular adenosine selectively responsible for activating striatal $A_{2A}R$.**

Adenosine has a huge impact in the hippocampus and causes different and most often opposite actions by activating different receptors, namely A_1R and $A_{2A}R$ that, in addition can be co-localized, at least in nerve terminals (Cunha, 2008). Thus, it becomes of upmost importance to understand how the differential activation of the different adenosine receptors can be effectively controlled to meet the needs of the system.

In deleterious conditions like in epilepsy, adenosine has a huge impact, being known to act as a powerful endogenous anticonvulsive, mainly through A_1R activation (Boison, 2012). In order **to define and characterize the involvement of the adenosine catabolic mediator CD73 and $A_{2A}R$ in epilepsy**, on **chapter 6** we compared mice models with selective cellular deletions of $A_{2A}R$ (neuronal or astrocytic) and mice deficient in CD73 on a mice model of mesial temporal lobe epilepsy.

Since hippocampal $A_{2A}R$ are known to be involved in important functions like long-term potentiation (Rebola et al., 2008; Fontinha et al., 2009) and different types of memories (Wei et al., 2010), on **chapter 7** we proposed **to explore the role of hippocampal $A_{2A}R$ in different behavior paradigms** and **to investigate a possible local (synaptic) synthesis of $A_{2A}R$ in the hippocampus**.

CHAPTER 2

DISTRIBUTION OF CD73 IN THE BRAIN

1. Abstract

Ecto-5'-nucleotidase (CD73) is an important enzyme in the brain but its distribution and characterization are ill defined due to the lack of tools to perform the studies. The production of a selective antibody and the creation of a rodent CD73 knockout triggered the opportunity to characterize CD73, namely to unveil its brain localization. We now show that CD73, the major enzyme able to convert extracellular AMP into adenosine in the brain, is highly expressed in central nucleus of amygdala, globus pallidus and the striatum, being differentially expressed in the different nuclei of basal ganglia. Moreover, CD73 is also present in the olfactory tubercle, meninges and plexus choroid. In addition, this enzyme is also present, but less abundantly, in the substantia nigra, hippocampus and cortex. Regarding the cellular and subcellular distribution we here show that in addition to astrocytes, CD73 is prominently localized in the postsynaptic sites in all the brain areas studied.

2. Introduction

Signaling via extracellular nucleotides is an important mechanism in the brain (Burnstock et al., 2011) and ecto-5'-nucleotidase (CD73) is a key enzyme in the purinergic system, dephosphorylating adenosine monophosphate (AMP) to adenosine (Zimmermann and Braun, 1996). Therefore, CD73 is the enzyme that forms most of the adenosine originating from hydrolysis of released adenine nucleotides. Adenosine through activation of adenosine receptors, is an important neuromodulator in the central nervous system (CNS), participating in a multitude of processes, from synaptic plasticity to different behaviors and progression of distinctive neurological diseases (Fredholm et al., 2005b). The physiological impact of different ecto-nucleotidases, namely CD73 in the control of different neuronal, glial, and vascular functions was demonstrated in distinct studies (Zimmermann, 2006a).

Until recently it was difficult to identify individual enzymes from the ecto-nucleotidase family and its specific location. The majority of studies exploring the regional and cellular localization of ecto-nucleotidases, namely CD73, rely on biochemical analysis or on enzyme histochemical techniques that do not allow differentiating between individual enzymes, since until recently specific inhibitors of each member of ecto-nucleotidase family were lacking and many enzymes hydrolyze the same substrate and can be present in the same cell type (Kegel et al., 1997; Nedeljkovic et al., 2003). In the case of CD73, it is particularly difficult because the potential contribution of the intracellular enzymes, different from CD73, and other surface-located phosphatases should be carefully excluded. Therefore, many of the earlier histochemical studies do not provide secure information regarding the type of enzyme investigated (Zimmermann, 1996; 2006a; Zimmermann and Braun, 1999). More recently, antibodies have become available but problems related with their selectivity produced discrepancies between immunohistochemical and enzyme histochemical data (Schoen et al., 1988; Braun et al., 1994; Zimmermann, 1996). In addition, a number of caveats are overlooked in a considerable number of studies. The general discussion of the regional distribution of CD73 is hampered by the incongruent results obtained by enzyme cytochemistry and immunocytochemistry and even the significant interspecies differences. Thus, reliable histological information regarding CD73 cellular distribution is a considerable gap in the adenosine field, and the information on the cellular and subcellular localization of CD73 is thus still incomplete.

CD73 was classically known as a marker of myelin (Cammer and Tansey, 1986; Kreutzberg et al., 1978) and of astrocytes, as well as activated microglial cells in the mature nervous system (Kreutzberg and Barron, 1978; Kreutzberg et al., 1978; Kreutzberg and Hussain, 1982; Gehrmann et al., 1991). However, the recent studies where the distribution of CD73 activity in WT and CD73 KO mice were analyzed (Langer

et al., 2008) do not corroborate this classical view, but showed that the previous enzymatic histochemical analyses had correctly identified the distribution of CD73 in mouse brain (Braun and Zimmermann, 1998; Schoen et al., 1999).

Since the understanding of the pattern of distribution of CD73 and its cellular location are essential for elucidating the control of purinergic signaling in the brain, in the present study we proposed to refine the distribution of CD73 in the central nervous system (CNS), exploring its presence and density in the different areas, as well as, its cellular and subcellular localization in physiological settings, using the CD73 knockout (KO) mice as a key control.

3. Materials and methods

Animals

Approval from the Institutional Animal Care and Use Committee at Boston University School of Medicine and the Portuguese Veterinarian Office was granted for all experiments conducted in Boston and Coimbra, respectively. They adhered to the NIH Guide for the Care and Use of Laboratory Animals, the Portuguese Law and Ordinance on Animal Protection, and European Council Directive 86/609/EEC. The global CD73 knockout (CD73 KO) mice used, with a C57Bl/6 genetic background, were previously characterized (Thompson et al., 2004). In all experiments males and females adult (2-3 months old) mice were used.

Separation of total membranes

Mice were euthanized by decapitation after deep anesthesia with isoflurane, and the brain tissues were homogenized in sucrose (0.32 M) solution containing 1 mM EDTA, 10 mM HEPES, 1 mg/mL bovine serum albumin (BSA; Sigma), pH 7.4 at 4 ºC. The homogenates were centrifuged at 3,000 *g* for 10 min at 4 °C and the supernatants

then centrifuged at 14,000 *g* for 10 min at 4 °C. The pellets were washed in Krebs-HEPES-Ringer (KHR) solution containing 140 mM NaCl, 1 mM EDTA, 10 mM HEPES, 5 mM KCl, 5 mM glucose, pH 7.4 at 4 ºC and further centrifuged at 14,000 *g* for 10 min at 4 ºC. The pellets were either resuspended in the radioimmunoprecipitation assay (RIPA) buffer for Western blot analysis.

Purification of synaptosomes and gliosomes

After the homogenization of the brain tissue, synaptosomes and gliosomes were obtained using a discontinuous Percoll gradient (2, 6, 15, and 23% v/v of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4), as previously described (Matos et al., 2012b). The mixture was centrifuged at 31,000 *g* for 5 min at 4 ºC with braking speed set down to 0 after reaching 1,500 *g* (Dunkley et al., 2008). The layers between 2 and 6% of Percoll (gliosomal fraction) and between 15 and 23% of Percoll (presynaptosomal fraction) were collected, washed in 10 mL of HEPES buffered medium containing 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4, and further centrifuged at 22,000 *g* for 15 min at 4 ºC to remove myelin components and postsynaptic material from the gliosomal and synaptosomal fractions, respectively. Both fractions were resuspended in RIPA buffer for Western blot analysis.

Separation of pre-, post- and extrasynaptic fractions

The separation of the presynaptic active zone, postsynaptic density and non-synaptic fractions from nerve terminals was carried out by combining solubilization steps and changes in pH, as previously described (Rebola et al., 2005a). Briefly, a solution with sucrose (1.25 M) and $CaCl_2$ (0.1 mM) was gently added to the tissue homogenates under agitation. Another sucrose solution (1 M) containing 0.1 mM $CaCl_2$ was gently

stratified over the homogenate, followed by centrifugation (100,000 *g* for 3 h at 4 ºC) to separate nuclei and debris (pellet), myelin (top layer) and the synaptosomes (interface between 1.25 and 1 M of sucrose), which were diluted 1:10 in sucrose solution (0.32 M) containing 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF and centrifuged (15,000 *g* for 30 min at 4 ºC). The pellet (synaptosomes) was diluted 1:10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2x solubilization buffer (2 % Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The membranes were incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junctions) pelleted (40,000 *g* for 30 min at 4 °C). The supernatant (extrasynaptic fraction) was decanted and proteins precipitated with six volumes acetone at -20 °C and recovered by centrifugation (18,000 *g* for 30 min at -15 °C). The synaptic junctions pellet was washed in the solubilization buffer (pH 6.0) and resuspended in 10 volumes of a second solubilization buffer (1 % Triton X-100, 20 mM Tris but at pH 8.0). This increase of pH allows the dissociation of the extracellular matrix that maintains the presynaptic active zone tightly bound to the postsynaptic density (Phillips et al., 2001). Hence, the active zone is solubilized whereas the postsynaptic density is essentially preserved because the amount of detergent is not enough for its solubilization (Phillips et al., 2001). After incubation for 30 min on ice with mild agitation, the mixture was centrifuged and the supernatant (presynaptic fraction) processed as described for the extrasynaptic fraction, whereas the final insoluble pellet corresponds to the postsynaptic fraction. The samples were resuspended in RIPA buffer for Western blot analysis.

Western blot

Western blotting was performed as previously described (Rebola et al., 2005a), using non-reducing conditions for rabbit anti-murine CD73. Incubation with the primary antibodies, namely, mouse anti-synaptophysin (1:50,000; Sigma), mouse anti-syntaxin

(1:50,000; Sigma), mouse anti-postsynaptic density-95 (PSD-95; 1:100,000; Sigma), mouse anti-β-actin (1:20,000; Sigma), and rabbit anti-murine CD73 (1:1,000; Fausther et al., 2012), all diluted in Tris-buffered saline (137 mM NaCl and 20 mM Tris–HCl, pH 7.6) with 0.1 % Tween (TBS-T) and 5 % BSA (fatty acid free), was carried out overnight at 4 ºC. After washing twice with TBS-T, the membranes were incubated with appropriate IgG secondary antibodies conjugated with alkaline phosphatase (Amersham) for 2 h at room temperature. After washing, the membranes were revealed using an ECF kit (Amersham) and visualized with an imaging system (VersaDoc 3000, Bio-Rad) and the densiometric analysis of protein bands was performed using the Quantity One software (Bio-Rad).

Immunohistochemistry

Mice were anesthetized with avertin and brain fixation was performed through transcardiac perfusion with 4 % paraformaldehyde in PBS, postfixation overnight in PBS with 4 % paraformaldehyde and cryopreservation in PBS containing 25 % sucrose. Frozen brains were sectioned (30 μm coronal slices) with a Leica CM3050S cryostat (Leica Microsystems). The sections were first rinsed for 5 min with PBS at room temperature and then permeabilized and blocked with PBS containing 0.2 % Triton X-100 and 5 % donkey serum during 1 h, incubated in the presence of the rabbit anti-murine CD73 antibody (1:500; Fausther et al., 2012) overnight at room temperature, rinsed three times for 10 min in PBS, and then incubated with donkey anti-rabbit secondary antibodies conjugated with a fluorophore (Alexa Fluor 488 or Alexa Fluor 555, 1:200, Invitrogen) for 2 h at room temperature. After rinsing three times for 10 min in PBS, the sections were mounted on slides and allowed to dry. Vectashield mounting medium with DAPI (Vector Laboratories) was applied as well as the cover glass. All sections were examined under a fluorescence Nikon eclipse E600 microscope, with

SPOT software 4.7 (Diagnostic instruments, Inc.).

Statistical analysis

Results are presented as mean ± SEM. Data with one condition and one variable (e.g. genotype) were analyzed with Student's *t* test. Data from more than one condition (e.g. different brain's preparations) were analyzed with one-way ANOVA followed by a Tukey's or Newman-Keuls multiple comparison post-hoc test. Unless otherwise indicate the significance level was 95 %.

4. Results

4.1. CD73 has a high density in the striatum

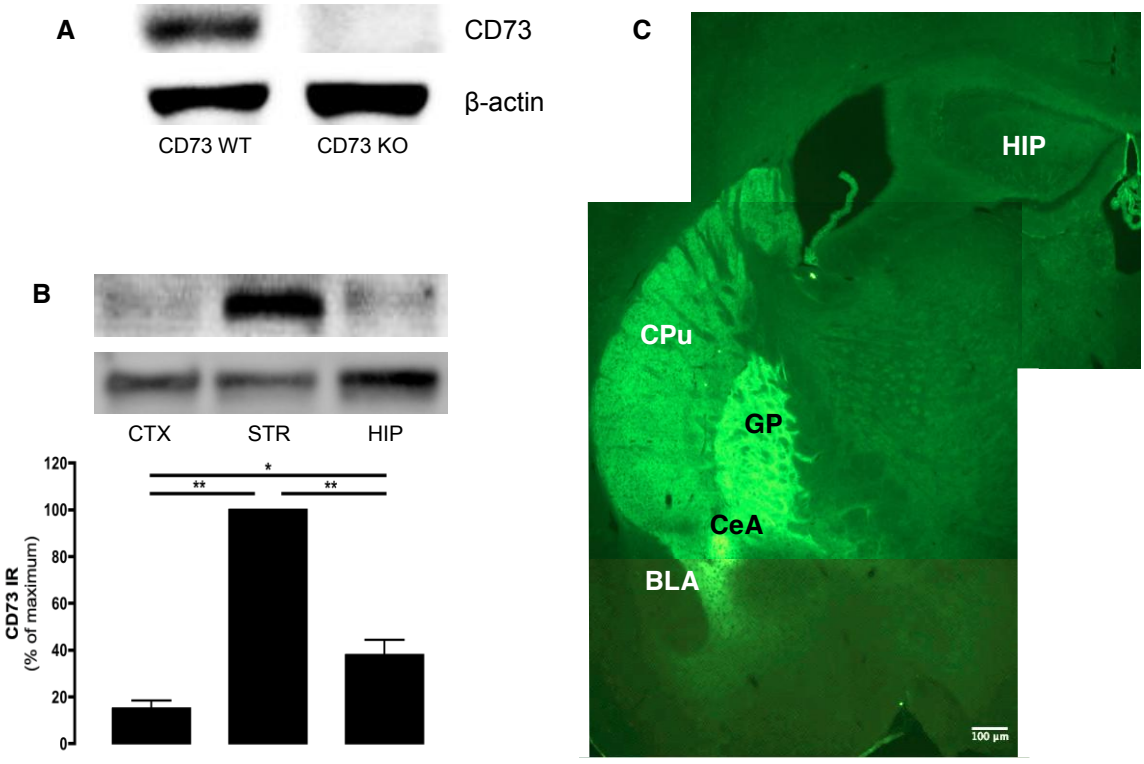

Figure 2.1. CD73 have a high density in the striatum. Using an antibody that selectively recognizes CD73 in the striatum of WT but not of CD73 KO mice, as shown in panel A (representative of n = 4), a Western blot analysis (B) revealed that CD73

immunoreactivity was more densely located in the striatum (STR) than in the hippocampus (HIP) or cortex (CTX) (n = 3). Immunohistochemistry (C) showed that CD73 displayed greater abundance in the globus pallidus (GP) and central nucleus of amygdala (CeA), than in the caudate putamen (CPu), than in the hippocampus (HIP) and the basolateral amygdala (BLA) (image representative of n = 5). Data are mean ± SEM and a one-way ANOVA test was used, followed by a Tukey's multiple comparison test; *$p < 0.01$; **$p < 0.001$.

To determine the brain distribution of CD73, we first certified the selectivity of our anti-CD73 antibody through Western blot analysis, which we found to recognize a band (≈ 65 kDa) in striatal membranes of WT mice, without detectable signal in CD73 KO mice (Fig. 2.1A). We then compared the density of CD73 in different brain regions; Western blot analysis of total membranes showed that CD73 is more abundant in the striatum ($p < 0.001$) than in the hippocampus or prefrontal cortex (Fig. 2.1B). This was confirmed by immunohistochemical analysis (Fig. 2.1C) that showed a higher CD73 immunoreactivity in different basal ganglia areas, as well as in central nucleus of amygdala, when compared with the hippocampus or cerebral cortex.

4.2. CD73 is differently expressed in basal ganglia and central nucleus of amygdala

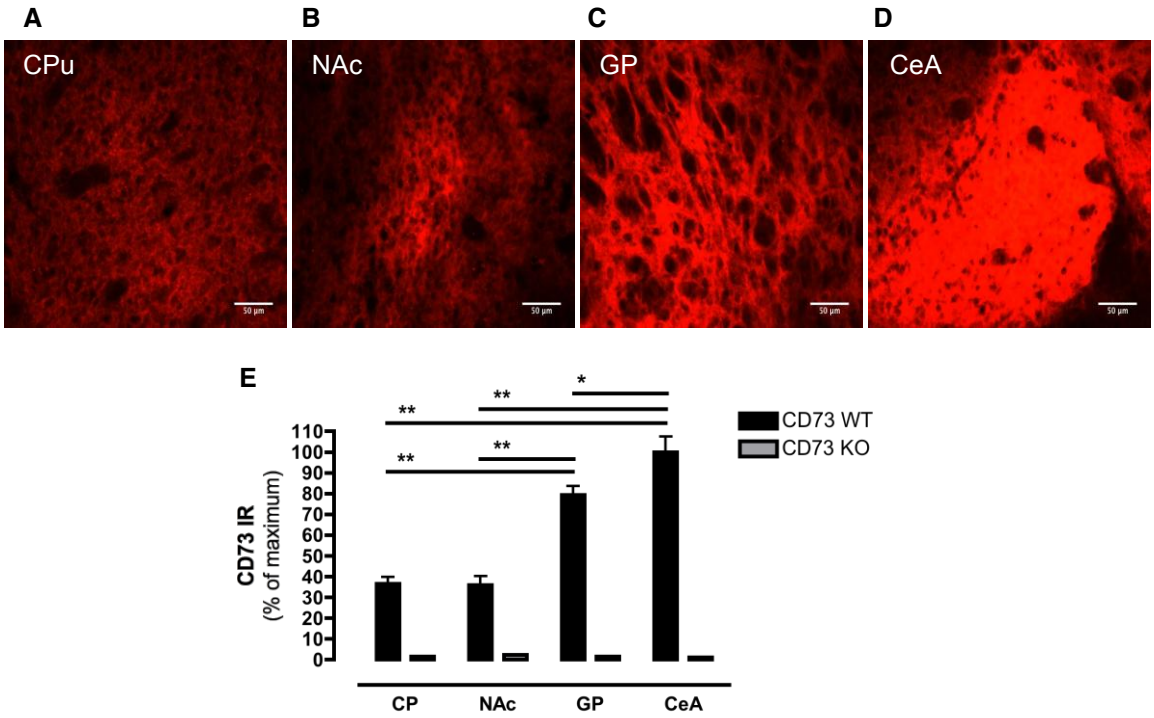

Figure 2.2. CD73 density in basal ganglia and central nucleus of amygdala. The density of CD73 was explored in the brain areas where it is highly expressed, using sections of the striatum from WT and CD73 KO mice. Panels A to D are representative CD73 immunohistochemistry images of caudate putamen (CPu; A) nucleus accumbens (NAc; B), globus pallidus (GP; C) and central nucleus of amygdala (CeA; D) sections from WT mice (representative from n = 2). Panel E displays the quantification of the CD73 immunoreactivity (IR), showing that CD73 displayed greater abundance in the caudate putamen (CPu) and nucleus accumbens (NAc), being higher in globus pallidus (GP) and even higher in central nucleus of amygdala (CeA), without staining in sections from CD73 KO mice in any of those brain areas (data not shown). The data are mean ± SEM; *p < 0.05; **p < 0.01 using a One-way ANOVA followed by Newman-Keuls multiple comparison test).

Basal ganglia are comprised by several subcortical nuclei, with different nucleus controlling different physiological process and participating in different pathological conditions. We here show the differences of CD73 immunoreactivity (IR; Fig. 2.2) in the brain areas where CD73 has a higher density (Fig. 2.1). CD73 immunoreactivity is significantly higher in central nucleus of amygdala (CeA), when compared with the globus pallidus (GP; + 20.7 ± 7.9 %, $p < 0.05$, One-way ANOVA followed by Newman-Keuls multiple comparison test; CeA, 100.0 ± 7.5 %, n = 2; GP, 79.3 ± 4.2 %, n = 2). CeA has also higher CD73 IR when compared with the nucleus accumbens (NAc; + 67.9 ± 8.6 %, $p < 0.01$, One-way ANOVA followed by Newman-Keuls multiple comparison test; NAc, 32.4 ± 4.7 %, n = 2). A similar result is observed comparing CD73 IR in CeA and caudate putamen (CP; + 63.4 ± 7.5 %, $p < 0.01$, One-way ANOVA followed by Newman-Keuls multiple comparison test; CP, 36.6 ± 3.1 %, n = 2). Within the basal ganglia CD73 immunoreactivity was higher in the globus pallidus than in the caudate putamen (+ 46.9 ± 6.5 %, $p < 0.01$, One-way ANOVA followed by Newman-Keuls multiple comparison test) and nucleus accumbens (+ 42.7 ± 5.5 %, $p < 0.01$, One-way ANOVA followed by Newman-Keuls multiple comparison test), without significant differences between caudate putamen and nucleus accumbens.

4.3. CD73 distribution throughout the brain

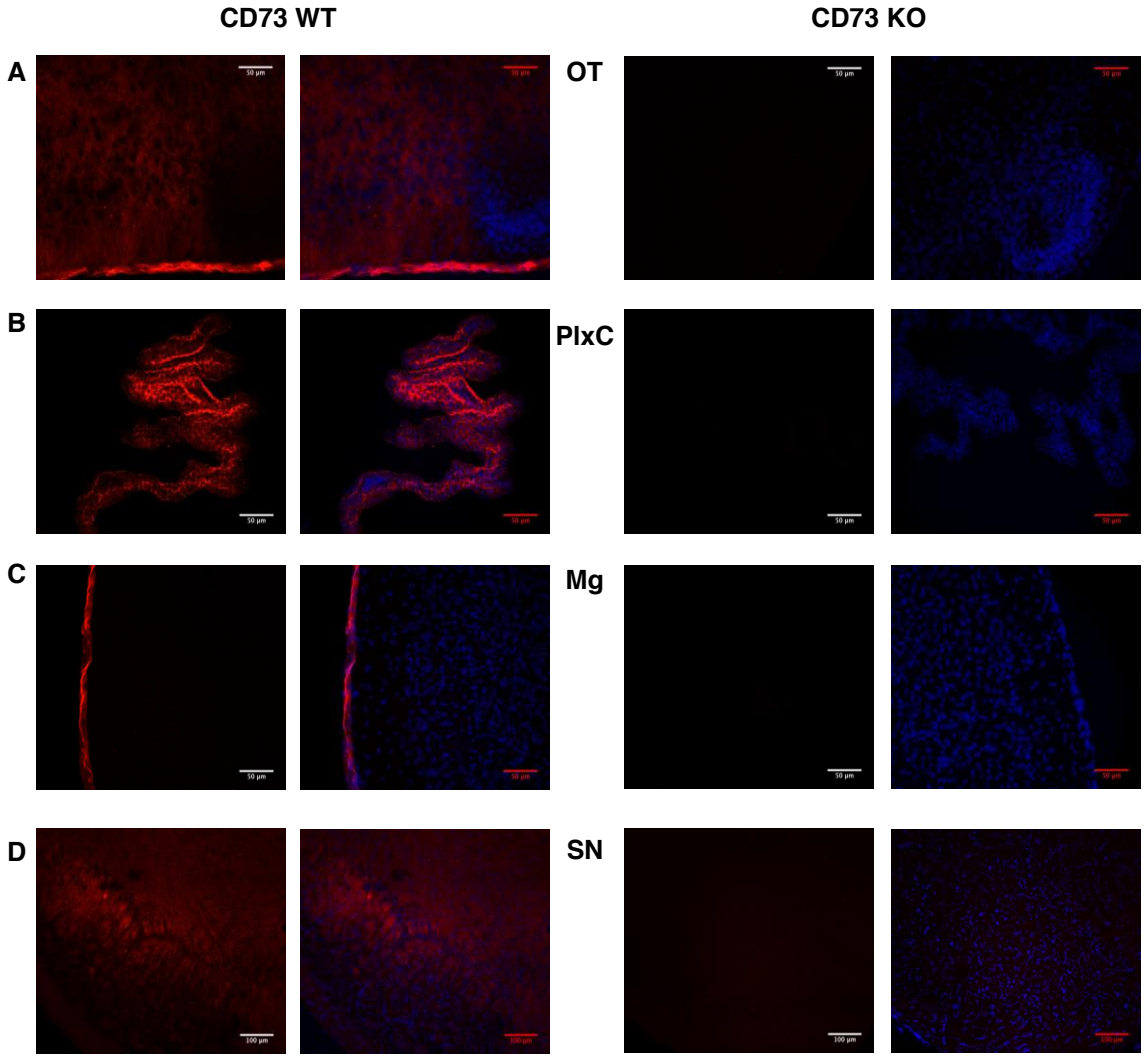

Figure 2.3. CD73 distribution throughout the brain. The distribution of CD73 was explored in different areas using brain sections from WT and CD73 KO mice. Panel A shows representative images of CD73 immunohistochemistry from olfactory tubercle (OT) sections, showing CD73 immunoreactivity (red) in sections from WT without staining in CD73 KO, but with similar DAPI staining (blue) in both. Panel B are representative images of CD73 immunohistochemistry from plexus choroid (PlxC) sections, showing CD73 immunoreactivity (red) in sections from WT without staining in CD73 KO, but with similar DAPI staining (blue) in both. Panel C are representative images of CD73 immunohistochemistry from meninges (Mg) sections, showing CD73 immunoreactivity (red) in sections from WT without staining in CD73 KO, but with similar DAPI staining (blue) in both. Panel D are representative images of CD73 immunohistochemistry from substantia nigra (SN) sections, showing CD73 immunoreactivity (red) in sections from WT without staining in CD73 KO, but with similar DAPI staining (blue) in both.

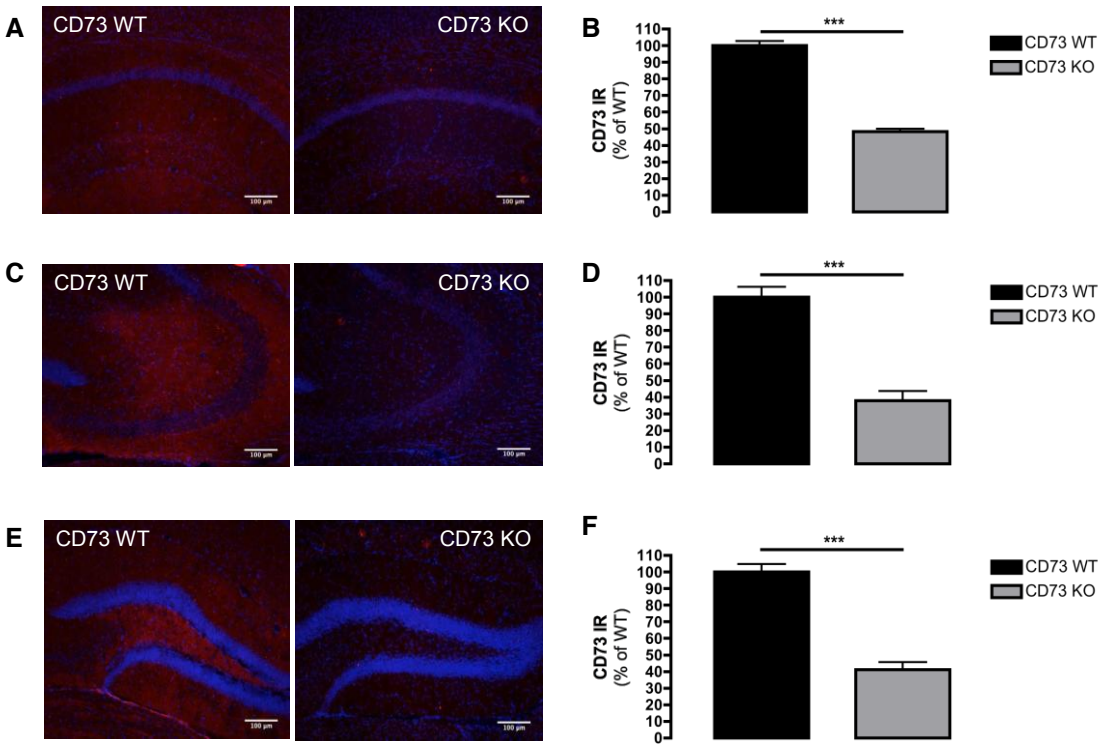

Figure 2.4. CD73 is present in the hippocampus. Using an antibody selectively recognizing CD73 in WT but not in CD73 KO mice, immunohistochemical analysis (A, C, E) showed that CD73 its present in different areas of hippocampus: CA1 (A, B), CA3 (C, D) and DG (E, F). A are representative images showing CD73 immunoreactivity in CA1 in CD73 WT but not CD73 KO mice and B is the quantification of CD73 immunoreactivity (IR) in CA1. C are representative images showing CD73 immunoreactivity in CA3 in CD73 WT but not in CD73 KO mice and D is the quantification of CD73 IR in CA3. E are representative images showing CD73 immunoreactivity in DG in CD73 WT but not CD73 KO mice and F is the quantification of CD73 IR in DG. Data are mean ± SEM; a Student's *t* test was used; ***$p < 0.001$.

It was previously showed that CD73 activity is present in brain areas other than the striatum (Langer et al., 2008) . We here show by immunohistochemistry the presence of CD73 in olfactory tubercle (OT; Fig. 2.3A), but also in the plexus choroid (PlxC; Fig. 2.3B), meninges (Mg; Fig. 2.3C) and substantia nigra (SN; Fig. 2.3D), without staining in CD73 KO mice sections. These findings open new avenues to explore the role of CD73 in different physiopathological conditions that involve the participation of these brain areas.

In addition, it was previously demonstrated the presence of CD73 activity in the hippocampus (Langer et al., 2008) in naïve conditions. In agreement, we here showed the presence of CD73 in hippocampal total membranes (Fig. 2.1B). This was corroborated by immunohistochemical analysis (Fig. 2.4), showing the presence of CD73 in CA1 (Fig. 2.4A-B), CA3 (Fig. 2.4C-D) and DG (Fig. 2.4E-F) of CD73 WT and not from CD73 KO mice sections (Fig. 2.4A-F).

4.4. CD73 has a predominant postsynaptic localization

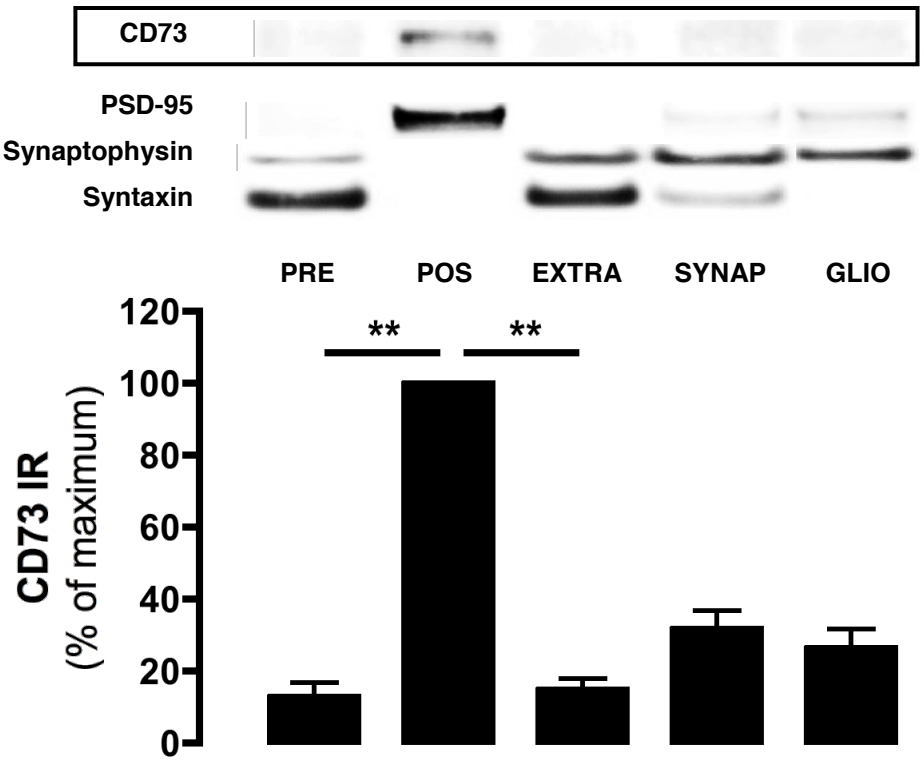

Figure 2.5. CD73 has a predominant postsynaptic localization in the prefrontal cortex. In the prefrontal cortex, subcellular fractionation (n = 2) showed that CD73 had a higher density in postsynapyic density (POS), when compared with pre- (PRE) and extra-synaptic (EXTRA) fractions; the density was similar between synaptosomes (SYNAP) and gliosomes (GLIO). Data are mean ± SEM; a one-way ANOVA test was used, followed by a Tukey's multiple comparison test; $^{**}p < 0.001$.

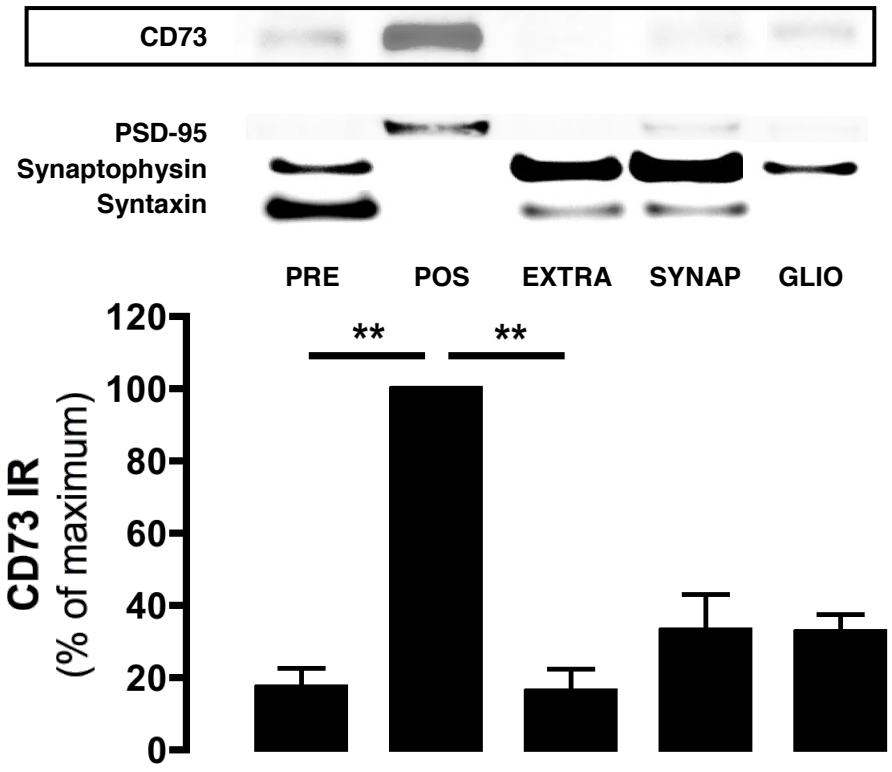

Figure 2.6. CD73 has a predominant postsynaptic localization in the hippocampus. In the hippocampus, subcellular fractionation (n = 2) showed that CD73 had a higher density in postsynapyic density fraction (POS), when compared with pre- (PRE) and extra-synaptic (EXTRA) fractions; the density was similar between synaptosomes (SYNAP) and gliosomes (GLIO). Data are mean ± SEM; a one-way ANOVA test was used, followed by a Tukey's multiple comparison test; $^{**}p < 0.001$.

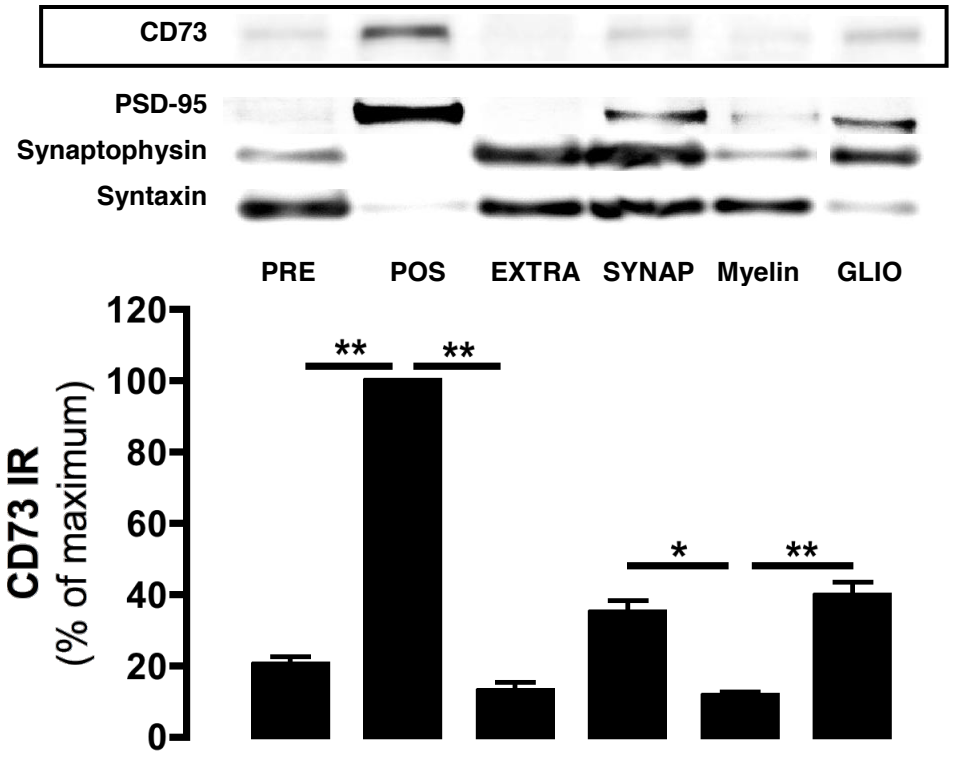

Figure 2.7. CD73 has a predominant postsynaptic localization in the striatum. In the striatum, subcellular fractionation (n = 2) showed that CD73 had a higher density in postsynapyic density (POS), when compared with pre- (PRE) and extra-synaptic (EXTRA) fractions and was more densely located in synaptosomes (SYNAP) and gliosomes (GLIO) than in myelin preparations. Data are mean ± SEM and a one-way ANOVA test was used, followed by a Tukey's multiple comparison test; *$p < 0.01$; **$p < 0.001$.

We next attempted to define the cellular and subsynaptic localization of CD73 in different brain areas. In accordance with the previously described localization of CD73 in astrocytes and neurons (Schoen and Kreutzberg, 1997), we found that CD73 was present and with a similar density in gliosomes (astrocytic plasmalemal vesicles) and in synaptosomes, in prefrontal cortex (Fig. 2.5), hippocampus (Fig. 2.6) and striatum (Fig. 2.7). In the prefrontal cortex (Fig. 2.5), within synapses, CD73 was more abundantly located in the postsynaptic density than in the presynaptic active zone (- 86.97 ± 3.65 %; $p < 0.001$) and perisynaptic regions (extra-synaptic fraction; - 84.99 ± 2.89 %; $p <$

0.001). In the hippocampus (Fig. 2.6) a similar result was obtained, with CD73 being more abundantly located in the postsynaptic density than in the presynaptic active zone (- 82.57 ± 5.20 %; $p < 0.001$) and perisynaptic regions (extra-synaptic fraction; - 83.58 ± 5.83 %; $p < 0.001$). Furthermore, in the striatum (Fig. 2.7), CD73 was more abundantly located in the postsynaptic density than in the presynaptic active zone (- 79.46 ± 1.99 %; $p < 0.001$) and was scarcely located in perisynaptic regions (extra-synaptic fraction; - 86.96 ± 3.35 %; $p < 0.001$).

5. Discussion

In this study we provide a characterization of the distribution of CD73 in the CNS, taking advantage of a selective antibody against CD73 and validated in the CD73 KO mice. We here show that CD73 is highly expressed in the central nucleus of amygdala, the globus pallidus and the striatum, being differentially expressed in the different nuclei of basal ganglia. Moreover, CD73 is also present in the olfactory tubercle, meninges and plexus choroid, being less abundant in the substantia nigra, hippocampus and cortex. Regarding the cellular and subcellular distribution, in addition to astrocytes, CD73 is prominently localized in postsynaptic sites in all the brain areas studied.

In previous studies using enzyme histochemistry, the levels of 5'-nucleotidase were detected in the entire brain, although with variations between regions and species. In a previous work it was shown that in mice, 5'-nucleotidase have a very high concentrations in the olfactory tubercle, the nucleus accumbens, the caudate putamen and the globus pallidus; intermediate levels were observed in the ventral nucleus of the thalamus and the substantia nigra (Fastbom et al., 1987). However, in this study the surface location of the reaction product could not be verified, thus it was not clear whether the results solely refer to membrane-bound 5'-nucleotidase, i.e. CD73. Recently, the distribution of CD73 activity in WT and CD73 KO mice has been analyzed

(Langer et al., 2008). When comparing the catalytic activity for AMP hydrolysis in sections from several brain regions of WT and CD73 KO mice, Langer et al. (2008) observed a similar distribution pattern to that previously observed (Fastbom et al., 1987) and an almost complete elimination of staining in the CD73 KO mice sections. This finding indicates that CD73 is the major enzyme for extracellular AMP hydrolysis in the brain regions investigated and that the previous enzymatic histochemical analyses had correctly identified the distribution of CD73 in mouse brain. We here corroborated the previous reports (Fastbom et al., 1987; Langer et al., 2008) showing the presence of CD73 with high density in the striatum (Fig. 2.1B), and other nuclei of basal ganglia, specifically in the caudate putamen, the nucleus accumbens and globus pallidus (Figs. 2.1, 2.2), without immunoreactivity in the CD73 KO mice in any of these areas (Fig. 2.1A, 2.2). The presence of CD73 activity in the CNS outside the striatum observed before (Langer et al., 2008), for example in the olfactory tubercule, was also corroborated by our data (Fig. 2.3A). In addition, our study was able to show the presence of CD73 in other areas that were not clearly identified before, namely central nucleus of amygdala, plexus choroid, meninges, substantia nigra and hippocampus. Actually, previous performed enzyme cytochemical and immunocytochemical analyses showed that the plexus choroid, the main source of cerebrospinal fluid, was immunostained for 5'-nucleotidase (Braun et al., 1994). However, this was not corroborated by Langer et al. (2008) that also showed staining in the CD73 KO mice. Nevertheless, our results clearly show the specific distribution of CD73, and not of other enzymes able to dephosphorylate AMP, since we here used a selective antibody validated in the CD73 KO mice and not a tool that is activity-based.

Despite a considerable amount of enzyme histochemical evidences, previous antibodies directed selectively against CD73 failed to recognize the majority of sites detected by enzyme histochemistry in the brain (Schoen et al., 1988; Braun et al., 1994;

Zimmermann, 1996). As a result of this, controversy existed regarding the expression of individual subtypes of ectonucleotidases by neurons, astrocytes and microglia (Zimmermann, 2006a). Consequently, the information on the cellular and subcellular localization of CD73 was still incomplete (Suran, 1974a; Suran, 1974b; Hess and Hess, 1986). Ultrastructural studies have demonstrated 5'-nucleotidase activity in neurons (Marani, 1977) and synapses (Bernstein et al., 1978), as well as in myelin (Kreutzberg et al., 1978) and different types of glial cell membranes (Kreutzberg et al., 1978). Biochemical analysis revealed the activity of CD73 in neuronal (Meghji et al., 1989), glial cultures including astrocytes (Renau-Piqueras et al., 1992), oligodendrocytes (Snyder et al., 1983), as well as in isolated synaptosomes (Nagy et al., 1983; Nagy et al., 1986; Centelles et al., 1986; Cunha et al., 1992; Dowdall, 1978; Zimmermann and Bokor, 1979). At the synaptic level, it was suggested that CD73 reaction product was solely confined to the clefts of certain populations of asymmetrical terminals (Schoen and Kreutzberg, 1997). Together, these data suggest that CD73 could be present on both glial and neural cells, where it can have a specialized location. Regarding the cellular distribution, our study shows that CD73 is present both in gliosomes (i.e. in astrocytes from mature brain and in naïve conditions) as well as neurons, where it is mainly located at the postsynaptic density, in all brain areas analyzed.

In conclusion, we here outline the macroscopic localization of CD73 in the brain, as well as its cellular and subcellular distribution. Since an understanding of the pattern of distribution of ectonucleotidases is essential for elucidating the control of nucleotide signaling in the brain, our data open up new avenues for the study of purinergic signaling in pathophysiological conditions in the CNS. This study is especially crucial since alterations in the adenosine concentrations through CD73 could have dramatic effects on functions and behavior of the whole CNS.

6. Acknowledgements

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CHAPTER 3

THE ROLE OF CD73 IN THE BRAIN IN PHYSIOLOGICAL CONDITIONS

1. Abstract

Adenosine is an important neuromodulator that participates in different brain functions. Adenosine can be extracellularly generated by an ectonucleotidase cascade that is able to convert ATP into adenosine. We now show that ecto-5'-nucleotidase (CD73), the major enzyme able to convert extracellular AMP into adenosine in the brain, participates in different brain functions. The phenotypical characterization of CD73 KO mice revealed an impaired motor coordination and hyperlocomotion in the open field, but not in home cages, without further modifications in the elevated plus maze. These results show that CD73 is involved in improving motor coordination, without affecting general locomotion or the anxiety profile. This phenotype was accompanied by a lower phosphorylation of DARPP-32 at Thr 75 and reduced glutamate uptake in the striatum, without modifications in adenosine and dopamine receptors levels or alterations on enkephalin immunoreactivity. Moreover, CD73 KO mice displayed an increased performance of working and recognition memories and an improved avoidance learning capacity. This study points CD73 as a possible therapeutic target to manipulate motor functions, specifically motor coordination, but also cognitive processes.

2. Introduction

Adenosine is an important neuromodulator in the central nervous system (CNS), participating in different physiological conditions, including memory, learning and locomotion (Fredholm et al., 2005a). Adenosine can be generated intracellularlly and theoretically be released directly via equilibrative nucleoside transporters (ENT) (Brundege and Dunwiddie, 1998) or by exocytosis (Klyuch et al., 2012). In addition it can be indirectly delivered as ATP, followed by the extracellular enzymatic catabolism by an ectonucleotidase cascade, where ecto-5'-nucleotidase (CD73) plays a key role by dephosphorylating adenosine monophosphate (AMP) to adenosine (Zimmermann,

1996).

Adenosine executes its actions through different receptors, from which adenosine A_1 receptor (A_1R) and adenosine A_{2A} receptor ($A_{2A}R$) have a higher abundance and play crucial roles in the CNS (Cunha, 2008a). While A_1R are abundantly expressed throughout the brain (Dunwiddie and Masino, 2001), $A_{2A}R$ are highly expressed in the striatum specifically in striatopallidal neurons (Schiffmann et al., 1991a), where they control the indirect pathway's outputs (Azdad et al., 2009). However, $A_{2A}R$ are also present in presynaptic glutamatergic terminals from cortico- and thalamo-striatal projections (Rosin et al., 2003), where they modulate glutamate levels in the striatum (Corsi et al., 2000; Popoli et al., 2003; Pintor et al., 2004; Quarta et al., 2004). The role of $A_{2A}R$ outside the striatum is also established (Rebola et al., 2005a; 2005b), as well as its participation in memory and learning processes (Wei et al., 2011).

Interestingly, we previously showed that CD73 has a distribution pattern in the central nervous system (CNS) very similar to $A_{2A}R$, with a higher density in the striatum (Langer et al., 2008; see Chapter 2), and a predominant postsynaptic subcellular location (see Chapter 2). However, the basal ganglia are composed of several subcortical nuclei, including the caudate putamen, the nucleus accumbens, the globus pallidus, the substantia nigra and others, and the different nuclei play different roles in different behavioral tasks. In addition, we revealed the presence of CD73 outside the basal ganglia (see Chapter 2), where it can affect crucial processes. Therefore, we proposed to refine the characterization of CD73 in the central nervous system, exploring its role in locomotion, memory and learning paradigms, taking advantage of CD73 knockout (KO) mice.

3. Materials and methods

Animals

Approval from the Institutional Animal Care and Use Committee at Boston University School of Medicine and the Portuguese Veterinarian Office was granted for all experiments conducted in Boston and Coimbra, respectively. They adhered to the NIH Guide for the Care and Use of Laboratory Animals, the Portuguese Law and Ordinance on Animal Protection, and European Council Directive 86/609/EEC. The global CD73 knockout (CD73 KO) mice used, with a C57Bl/6 genetic background, were previously characterized (Thompson et al., 2004). In all experiments males and females adult (2-3 months old) mice were used.

Separation of total membranes

Mice were euthanized by decapitation after deep anesthesia with isoflurane, and the brain tissues were homogenized in sucrose (0.32 M) solution containing 1 mM EDTA, 10 mM HEPES, 1 mg/mL bovine serum albumin (BSA; Sigma), pH 7.4 at 4 ºC. The homogenates were centrifuged at 3,000 *g* for 10 min at 4 °C and the supernatants then centrifuged at 14,000 *g* for 10 min at 4 °C. The pellets were washed in Krebs-HEPES-Ringer (KHR) solution containing 140 mM NaCl, 1 mM EDTA, 10 mM HEPES, 5 mM KCl, 5 mM glucose, pH 7.4 at 4 ºC and further centrifuged at 14,000 *g* for 10 min at 4 ºC. The pellets were either resuspended in the incubation buffer for binding studies or in radioimmunoprecipitation assay (RIPA) buffer for Western blot analysis.

Purification of synaptosomes and gliosomes

After the homogenization of the brain tissue, synaptosomes and gliosomes were obtained using a discontinuous Percoll gradient (2, 6, 15, and 23% v/v of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4), as previously described

(Matos et al., 2012b). The mixture was centrifuged at 31,000 *g* for 5 min at 4 ºC with braking speed set down to 0 after reaching 1,500 *g* (Dunkley et al., 2008). The layers between 2 and 6% of Percoll (gliosomal fraction) and between 15 and 23 % of Percoll (synaptosomal fraction) were collected, washed in 10 mL of HEPES buffered medium containing 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4, and further centrifuged at 22,000 *g* for 15 min at 4 ºC to remove myelin components and postsynaptic material from the gliosomal and synaptosomal fractions, respectively. Both fractions were resuspended in incubation buffer for D-[^{3}H]aspartate uptake.

Binding assay

The binding assays were performed as previously described (Wei et al., 2011). Briefly, the total membranes (see total membranes preparation) were resuspended in a preincubation solution (containing 50 mM Tris, 1 mM EDTA, 2 mM EGTA, pH7.4) and a sample was collected to determine the protein concentration using the BCA assay (Thermo Scientific). For the binding of adenosine receptors adenosine deaminase (ADA, 2 U/mL, Roche) 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 $MgCl_2$, for $A_{2A}R$ binding, 50 mM Tris and 2 mM $MgCl_2$, for A_1R binding, 50 mM Tris, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM KCl, 120 mM NaCl, 0.1 % ascorbic acid for D_2R binding, or 50 mM Tris and 4 mM $MgCl_2$ for D_1R binding, pH 7.4) with 4 U/mL of ADA for adenosine receptors binding. Binding with 3 nM of the selective $A_{2A}R$ antagonist, [^{3}H]ZM241385 (Perkin Elmer) was performed for 1 h and binding with 2 nM of the selective A_1R antagonist, [^{3}H]DPCPX (Perkin Elmer) was performed for 2 h, both at room temperature with 0.1-0.2 mg of protein, with constant swirling. Binding with

3 nM of the selective D_1R-like antagonist, [^{3}H]SCH23390 (Perkin Elmer) was performed for 1 h at 30 ºC, with 0.1-0.2 mg of protein and 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 microfiber filters (GE Healthcare) in a filtration system (Millipore). The radioactivity was measured after adding 5 mL of scintillation liquid (Perkin Elmer). 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), a mixed A_1R/A_{2A}R antagonist, for adenosine receptors binding, and 5 mM fluphenazine dihydrochloride (Sigma) for D_1R binding. All binding assays were performed in duplicate.

D-[^{3}H]aspartate uptake

The uptake of the non-metabolizable glutamate analogue D-[^{3}H]aspartate is a validated readout of the activity of glutamate transporters (Anderson and Swanson, 2000) and was carried out as previously described (Matos et al., 2012a; 2012b). Briefly, the gliosomal or synaptosomal fractions were diluted in N-Methyl-D-glucamine (NMG) buffer and equilibrated at 37 °C for 10 min. Triplicates (150 μL) of each fractions were added to 150 μL of Krebs or NMG medium containing a final concentration of 50 nM D-[^{3}H] aspartate (11.3 ci/mmol; PerkinElmer, USA). The mixtures were incubated for 10 min at 37 °C and the reaction terminated by rapid vacuum filtration over glass microfibre filters Whatman GF/C (GE Healthcare) and further washed 3 times with ice cold NMG buffer. The filters were dried overnight and the radioactivity was measured after adding 5 ml of scintillation liquid (Perkin Elmer). The specific uptake of D-[^{3}H]aspartate was calculated by subtraction from the total uptake of the non-specific uptake measured in a Na^+-free medium (NMG).

Immunohistochemistry

Mice were anesthetized with avertin and brain fixation was performed through transcardiac perfusion with 4 % paraformaldehyde in PBS, postfixation overnight in PBS with 4 % paraformaldehyde and cryopreservation in PBS containing 25 % sucrose. Frozen brains were sectioned (30 μm coronal slices) with a Leica CM3050S cryostat (Leica Microsystems). Four of every sixth hippocampal sections (i.e., four sections separated by 150 μm from each other) were selected for independent stainings for quantification. The sections were first rinsed for 5 min with PBS at room temperature and then permeabilized and blocked with PBS containing 0.2 % Triton X-100 and 5 % donkey serum during 1 h, incubated in the presence of the rabbit anti-D_2R antibody (1:300; Millipore), rabbit anti-D_1R antibody (1:300; Abcam), mouse anti-Enk (1:200; Abcam) or rabbit anti-DARPP-32-p(Thr75) (1:250; Cell Signalling) overnight at room temperature, rinsed three times for 10 min in PBS, and then incubated with donkey anti-mouse and/or donkey anti-rabbit secondary antibodies conjugated with a fluorophore (Alexa Fluor 488 or Alexa Fluor 555, 1:200, Invitrogen) for 2 h at room temperature. After rinsing three times for 10 min in PBS, the sections were mounted on slides and allowed to dry. Vectashield mounting medium with DAPI (Vector Laboratories) was applied as well as the cover glass. All sections were examined under a fluorescence Nikon eclipse E600 microscope, with SPOT software 4.7 (Diagnostic instruments, Inc.).

Locomotor activity in home cage

The horizontal locomotor activity of mice was assessed in standard polypropylene cages (15 x 25 cm, i.e. clean home cages) and recorded with infrared photobeams (San Diego Instrument). Ambulation was quantified as the number of sequential breaks in adjacent beams. All mice were habituated to the test cage (except in the habituation

experiment) for at least 120 min before recording basal locomotion in 5 min bins.

Open-field test

The open-field test was used to evaluate the locomotion behavior. The open-field arena consists of a white plastic box (41 cm × 41 cm × 25 cm) and was placed 50 cm above the floor. The arena was divided into a central field (center, 15 cm × 15 cm) and an outer field (periphery). Thirty minutes prior to the test the animals were acclimatized to the room. Individual mice were placed in the center of the open-field and the activity was recorded with a video camera during 8 min period. The ANY-maze software assessed the total distance traveled, as well as the number of entries, distance traveled and time spent in the center area.

Elevated plus maze test of anxiety

Unconditioned fear was assessed as previously described (Hagenbuch et al., 2006) using the elevated plus maze to evaluate anxiety behavior. Thirty minutes prior to the test the animals were acclimatized to the room. Briefly, a mouse began the test in the central platform facing an open arm and was allotted 5 min to freely explore the maze under video recording. The ANY-maze software assessed the total distance traveled on the maze that provided a measure of general locomotors activity, the reluctance to venture into the open arms comprised the main measures of anxiety: (i) time spent in open arms and (ii) number of entries into open arms.

Accelerated rotarod

The mouse rotarod apparatus (Med associates inc.) consisted of a rubber roller with small grooves running along its turning axis and was performed as previous described (Durieux et al., 2012). Briefly, thirty minutes after the acclimatization to the

room, mice were tested for four consecutive trials. During each trial, animals were placed on the rod rotating at a constant speed (4 r.p.m.), then the rod started to accelerate continuously from 4 to 40 r.p.m. over 300 sec. The latency to fall off the rotarod was recorded. Animals that stayed on the rod for 300 sec were removed from the rotarod and recorded as 300 sec. Between each trial, mice were placed in their home cage for a 15–20 min interval.

Active avoidance

The mice were exposure to the Gemini Avoidance System (San Diego Instruments) for 300 sec with the door between stations opened for habituation. After that period of time the opposite chamber where the animal is, is lighted with a house light (conditional stimulus; CS). Consequently, the animal is in the dark chamber and a foot shock (unconditional stimulus; US) is delivered for a maximum period of time of 4 sec. The animal must then leave the dark side of the test station and enter into the lighted chamber to escape the foot shock. This was repeated for 20 trials per day for 5 consecutive days (i.e., a total of 100 trials). The inter trial interval (ITI) was 40 sec. After the 20 trials the mice were then returned to their home cage and 24 h later, the mice were placed in the test station with an acclimatization period equal to the ITI (i.e., 40 sec) and the latency to exit into the lighted side since the CS starts was measured being proportional of learning the task. The extinction trials were similar to the training trials but without the foot shock (US).

Passive Avoidance

The mice were exposure to the Gemini Avoidance System (San Diego Instruments) for 300 sec with the door between stations opened for habituation. During training the mice were placed into the lighted chamber (with house light) for 60 sec

(acclimatization period), after that the gate between chambers open and the house light (conditional stimulus; CS) is turned on for 10 sec. Most strains of mice are exploratory and prefer a dark to a lighted area, so they quickly move to the dark side of the test station. Once in the dark chamber, the gate between the 2 chambers is closed and a single foot shock (unconditional stimulus; US) is delivered through the grid floor on the dark side for 4 sec. The mice remain in the dark chamber for another 10 sec to allow them to form an association between the properties of the chamber and the foot shock. The animal is then returned to their home cage. 5 h later, the mice were taken from their home cage and placed in the lighted chamber with the dividing gate open between the 2 sides of the test station for acclimatization during 60 sec. After that the test started with the CS for 10 sec and the latency for the mice to enter the dark chamber is measure, being proportional of the animal's memory of its aversive experience from the training.

Working memory

We first assessed working memory in a spontaneous alternation paradigm in a *Y-maze*. Individual mice were placed at the end of one arm and allowed to freely explore the maze for 5 min. The sequence of entrance in each arm was recorded and the number of alternations (sequential entrance in the 3 different arms) was quantified. The percentage of spontaneous alternation consists in the percentage of alternations in the total possible alternations (total number of arms' changes minus 2).

We also assessed working memory in a more sensitive test using an *8 radial arm maze (RAM)* as previously described (Singer et al., 2012). To motivate performance in the RAM memory tasks, the animals were maintained on a food deprivation regime, which was gradually introduced with a progressive reduction of the daily available food, until the animals reached a stable weight of not less than 85 % of their *ad libitum* weight, at which time the food provided was stabilized. The RAM had 8 identical and equally

spaced arms (56 cm long, 12 cm wide) radiating from a central octagonal platform (side-length = 12 cm). The mice were exposed to the maze for 5 min each day with a food reward at the end of each arm. The habituation was performed until the animals finished the task within 5 min. In the *4 baited arms paradigm*, 4 of the 8 arms were randomly set with a food reward and the mice were allowed to freely explore the maze until they ate the 4 food rewards. In the *8 baited arms paradigm*, the 8 arms were set with a food reward and the animals allowed to freely explore the maze until they ate the 8 food rewards. Each time a mouse reentered in an arm where the reward was already ate, a working memory error (WME) was scored. Different groups of animals were used in the 2 RAM experiments.

Recognition memory

Spatial reference memory was assessed using a modified version of the Y-maze, as previously described (Cognato et al., 2010; Singer et al., 2012) to measure the innate tendency of mice to recognize spatial novelty, aided by spatial cues. The test consisted of two phases separated by a variable time interval (delay). Each animal was assigned two arms (start arm and familiar arm) to which they were exposed during the acquisition phase. The remaining third arm constituted the novel arm to be used in the second phase (retrieval test phase). Numerous visual cues were placed on the walls of the testing room and were kept constant throughout the behavioral test. The assay consisted of two 5 min trials separated by a 30 min interval. In the first trial (acquisition), one arm of the Y-maze was closed with a door and the mice were released from the end of the start arm facing the center of the maze. After entering the familiar arm the animal was allowed to freely explore both the start and familiar arms for 5 min. During the second trial (retrieval), animals had free access to the three arms and were allowed

to explore the maze for another 5 min. The time and number of entrances in each arm was recorded and the percentage of time and entrances in the "novel arm" quantified.

Statistical analysis

Results are presented as mean ± SEM. Data with one condition and one variable (e.g. genotype) were analyzed with Student's *t* test. Data with more than one variable (e.g. genotype and time) and condition were analyzed with a two-way ANOVA followed by Bonferroni post-hoc tests. Unless otherwise indicate the significance level was 95%.

4. Results

4.1. $A_{2A}R$ and A_1R binding density are not modified in CD73 KO mice

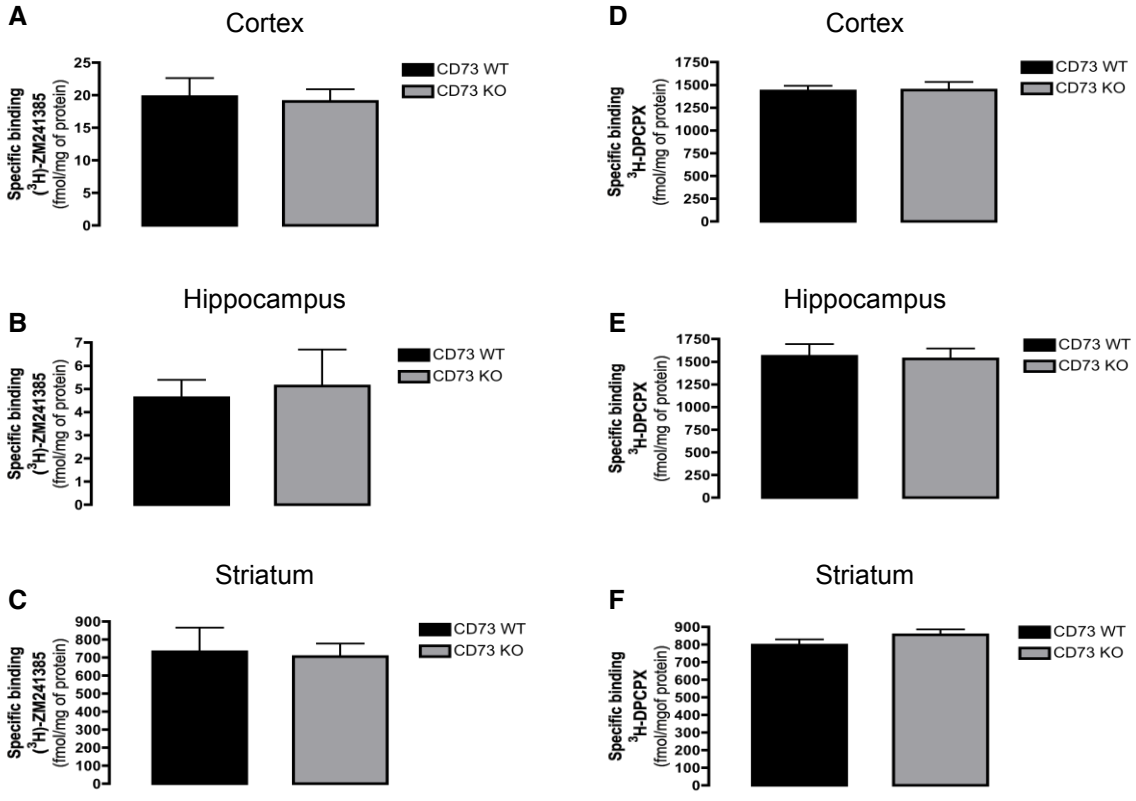

Figure 3.1. CD73 KO mice do not show alterations in $A_{2A}R$ or A_1R binding density. The levels of adenosine receptors in WT and CD73 KO mice, were investigated in total membranes from cortex (A, D), hippocampus (B, E) and striatum (C, F). CD73 KO mice

had an identical binding density for $A_{2A}R$ antagonist ([^{3}H]ZM 241385) when compared with their WT littermates in cortex (A; CD73 WT, n = 7; CD73 KO, n = 7), hippocampus (B; CD73 WT, n = 6; CD73 KO, n = 5) and striatum (C; CD73 WT, n = 6; CD73 KO, n = 5). CD73 KO mice had an identical density of binding sites for A_1R antagonist ([^{3}H]DPCPX) when compared with their WT littermates in cortex (D; CD73 WT, n = 6; CD73 KO, n = 5), hippocampus (E; CD73 WT, n = 6; CD73 KO, n = 5) and striatum (F; CD73 WT, n = 6; CD73 KO, n = 5). The data are mean ± SEM; a Student's *t* test was used.

Since CD73 is able to generate adenosine from AMP, its deletion could produce potential modifications in adenosine receptors that should be analyzed before testing CD73 KO mice. Therefore, we first tested the binding density of a selective $A_{2A}R$ antagonist ([^{3}H]ZM 241385) in total membranes from different brain areas, namely cortex (Fig. 3.1A), hippocampus (Fig. 3.1B) and striatum (Fig. 3.1C) and no changes were found between CD73 KO mice and their WT littermates. Likewise, when the binding density of a selective A_1R antagonist ([^{3}H]DPCPX) was tested in total membranes from the same brain areas, i.e. cortex (Fig. 3.1D), hippocampus (Fig. 3.1E) and striatum (Fig. 3.1F) no changes were found in CD73 KO mice when compared to WT mice.

4.2. D_2R, D_1R and enkephalin levels are not modified in CD73 KO mice

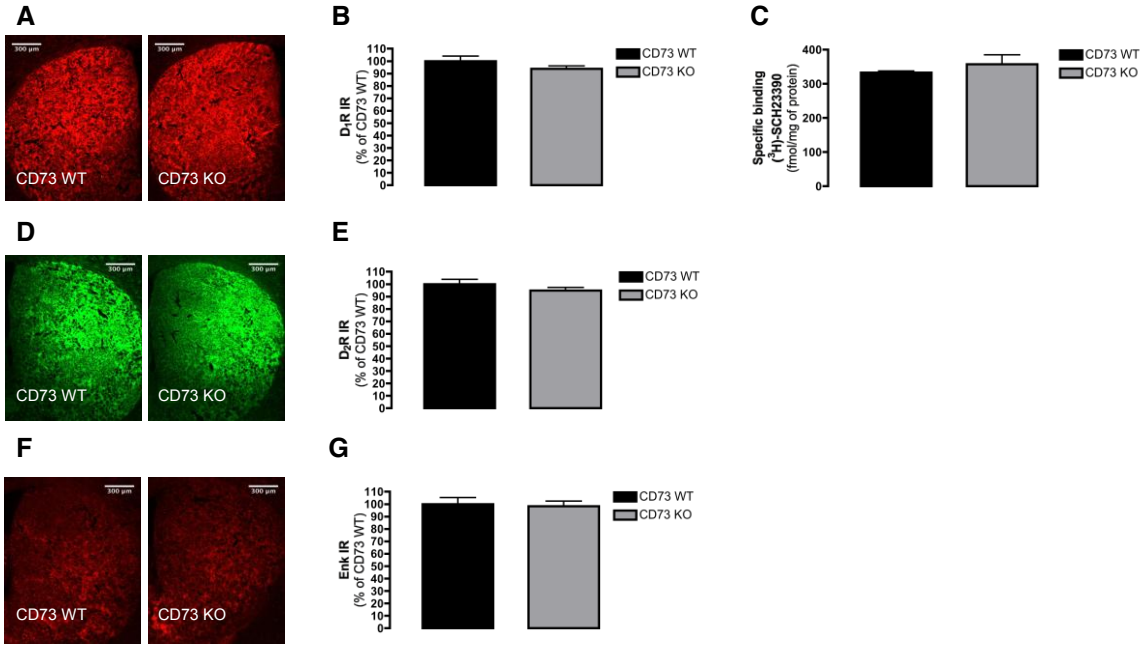

Figure 3.2. CD73 KO mice do not show changes in striatal D_1 or D_2 receptors, along with enkephalin. The levels of dopamine receptors, as well as enkephalin in WT and CD73 KO mice, were investigated in the dorsal striatum. Panel A shows representative images of D_1R immunohistochemistry in dorsal striatum sections from CD73 KO mice (n = 7) and their WT littermates (n = 4). Panel B displays the quantification of D_1R immunoreactivity (IR), showing that D_1R displayed a similar IR between CD73 KO mice and their WT littermates. Panel C shows that the binding density of D_1R antagonist ([^{3}H]SCH23390) is identical in striatal total membranes from CD73 KO mice (n = 5) when compared with their WT littermates (n = 5). Panel D shows representative images of dopamine D_2R immunohistochemistry in dorsal striatum sections from CD73 KO mice (n = 7) and their WT littermates (n = 4). Panel E displays the quantification of D_2R IR, showing a similar IR between CD73 KO mice and their WT littermates. Panel F shows representative images of enkephlin (Enk) immunohistochemistry in dorsal striatum sections from CD73 KO mice (n = 7) and their WT littermates (n = 4). Panel G displays the quantification of the Enk IR, showing a similar IR between CD73 KO mice and their WT littermates. The data are mean ± SEM; a Student's *t* test was used.

Considering that CD73 have a high density in the striatum (see chapter 2), potential modifications from its deletion in this brain area were further analyzed. Thus, we here evaluated dopamine receptors levels and enkephalin immunoreactivity in CD73 KO mice. Immunohistochemistry with anti-D_1R antibody (Fig. 3.2A-B) was able to show that D_1R immunoreactivity in dorsal striatum was similar between CD73 KO and their WT

littermates (CD73 WT, 100.0 ± 4.0 %, n = 4; CD73 KO, 93.8 ± 2.3 %, n = 7, p = 0.159, Student's *t* test). In agreement, no changes were found in the binding density of the selective D_1R antagonist ([^{3}H]SCH23390; Fig. 3.2C) from total striatal membranes of CD73 KO mice compared to WT mice (CD73 WT, 333.3 ± 4.5 fmol/mg of protein, n = 5; CD73 KO, 357.3 ± 27.3 fmol/mg of protein, n = 5; p = 0.395, Student's *t* test). Immunohistochemistry with anti-D_2R antibody (Fig. 3.2D-E) was able to show that D_2R immunoreactivity in dorsal striatum was similar between CD73 KO mice and their WT littermates (CD73 WT, 100.0 ± 3.7 %, n = 4; CD73 KO, 94.8 ± 2.3 %, n = 7, p = 0.159, Student's *t* test). Next we evaluated the levels enkephalin (Enk) in CD73 KO mice. Figure 3.2F-G shows that Enk immunoreactivity in dorsal striatum was similar between CD73 KO mice and their WT littermates (CD73 WT, 100.0 ± 5.2 %, n = 4; CD73 KO, 98.3 ± 4.1 %, n = 7, p = 0.816, Student's *t* test). In conclusion, we here showed that CD73 KO mice have similar levels of D_2R and D_1R (Fig. 3.2A-E), in addition to enkephalin (Enk; Fig. 3.2F-G).

4.3. CD73 KO mice show lower DARPP-32-p(Thr75)

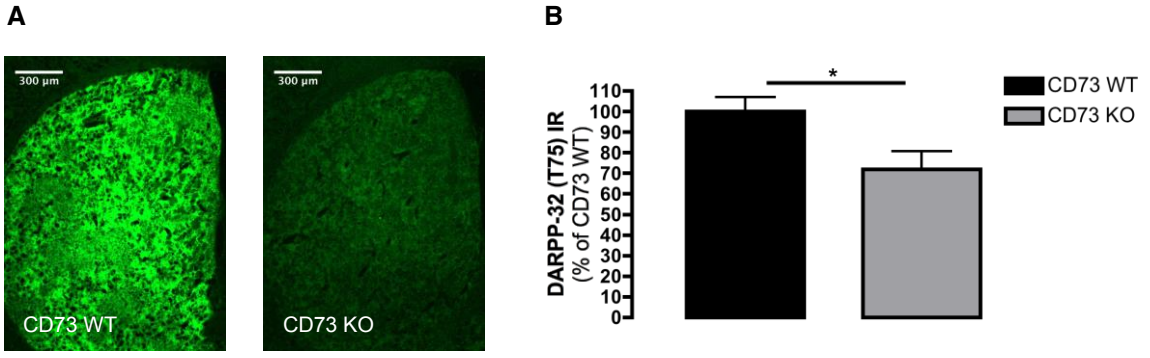

Figure 3.3. CD73 KO mice show lower DARPP-32-p(Thr75). Panel A shows representative images of DARPP-32-p(Thr75) immunohistochemistry in dorsal striatum sections from CD73 KO mice (n = 7) and their WT littermates (n = 4). Panel B displays the quantification of the DARPP-32-p(Thr75) immunoreactivity (IR), showing significantly lower levels in CD73 KO mice compared to their WT littermates (CD73 WT, 100.0 ± 6.9 %, n = 4; CD73 KO, 71.8 ± 8.8 %, n = 7). The data are mean ± SEM. *$p < 0.05$ between genotypes using a Student's *t* test.

Since glutamate levels in the striatum modulate dopamine- and cAMP-regulated phosphoprotein, 32kDa (DARPP-32) phosphorylation at threonine 75 (DARPP-32-p(Thr75)) (Matsuyama et al., 2003; Yamamura et al., 2013) and considering that striatal $A_{2A}R$ at presynaptic nerve terminals are able to control striatal glutamate levels (Popoli et al., 1995; Pintor et al., 2004; Rodrigues et al., 2005) and that CD73 is a key enzyme in the generation of adenosine in the striatum, we next evaluated the levels of DARPP-32-p(Thr75) in CD73 KO. Fig. 3.3A showed that DARPP-32-p(Thr75) immunoreactivity in dorsal striatum was significantly lower in CD73 KO mice when compared with their WT littermates (Fig. 3.3A-B; CD73 WT, 100.0 ± 7.0 %, n = 4; CD73 KO, 71.8 ± 8.8 %, n = 7, $p < 0.05$, Student's *t* test).

4.4. CD73 KO mice show lower striatal D-[^{3}H]aspartate uptake from synaptosomes

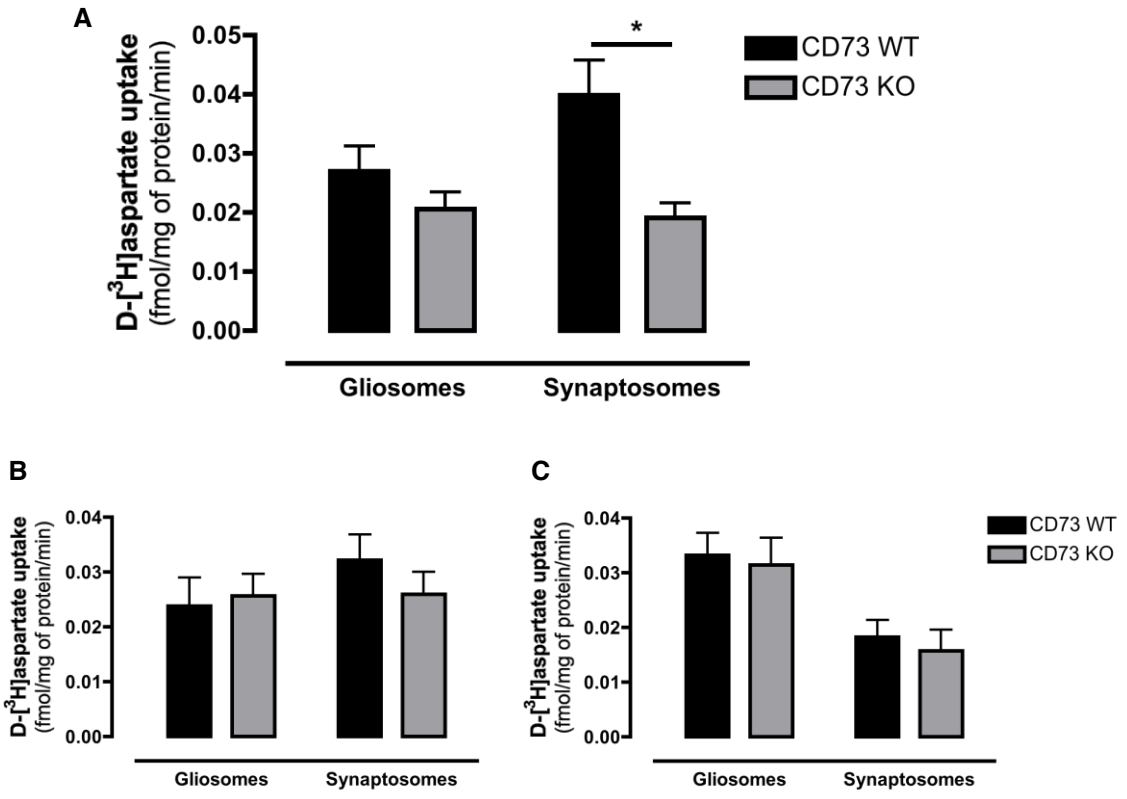

Figure 3.4. CD73 KO mice have a lower D-[^{3}H]aspartate uptake capacity in striatal synaptosomes. Striatal D-[^{3}H]aspartate uptake in gliosomes was similar between CD73

KO and WT mice (A; CD73 WT, 0.027 ± 0.004 fmol/mg of protein/min, n = 5; CD73 KO, 0.021 ± 0.003 fmol/mg of protein/min, n = 5). In striatal synaptosomes D-aspartate uptake was significantly lower in CD73 KO compared to WT mice (A; CD73 WT, 0.040 ± 0.006 fmol/mg of protein/min, n = 5; CD73 KO, 0.019 ± 0.002 fmol/mg of protein/min, n = 5). Hippocampal D-[^{3}H]aspartate uptake in gliosomes was similar between CD73 KO and WT mice (B; CD73 WT, 0.024 ± 0.005 fmol/mg of protein/min, n = 5; CD73 KO, 0.026 ± 0.004 fmol/mg of protein/min, n = 5). In hippocampal synaptosomes D-aspartate uptake was similar between CD73 KO and WT mice (B; CD73 WT, 0.032 ± 0.005 fmol/mg of protein/min, n = 5; CD73 KO, 0.026 ± 0.004 fmol/mg of protein/min, n = 5). Cortical D-[^{3}H]aspartate uptake in gliosomes was similar between CD73 KO and WT mice (C; CD73 WT, 0.033 ± 0.004 fmol/mg of protein/min, n = 5; CD73 KO, 0.032 ± 0.005 fmol/mg of protein/min, n = 5). In cortical synaptosomes D-aspartate uptake was similar between CD73 KO and WT mice (C; CD73 WT, 0.018 ± 0.003 fmol/mg of protein/min, n = 5; CD73 KO, 0.0158 ± 0.004 fmol/mg of protein/min, n = 5). The data are mean ± SEM. *p < 0.01 between genotypes using a Student's t test.

$A_{2A}R$ present at striatal presynaptic nerve terminals are able to control glutamate uptake (Pintor et al., 2004), and since CD73 is a key enzyme in the generation of adenosine in the striatum, we next investigated a potential modulation of glutamate uptake by CD73. We here showed that striatal gliosomes (astrocytic plasmalemmal vesicles) from CD73 KO have similar D-[^{3}H]aspartate uptake to their WT littermattes (Fig. 3.4A; CD73 WT, 0.027 ± 0.004 fmol/mg of protein/min, n = 5; CD73 KO, 0.021 ± 0.003 fmol/mg of protein/min, n = 5, p = 0.217, Student's t test), however striatal synaptosomes (presynaptic nerve terminals) from CD73 KO have lower D-[^{3}H]aspartate uptake to their WT littermattes (Fig. 3.4A; CD73 WT, 0.040 ± 0.006 fmol/mg of protein/min, n = 5; CD73 KO, 0.019 ± 0.002 fmol/mg of protein/min, n = 5, p < 0.01, Student's t test). This change is specific for striatal presynaptic nerve terminals, since no changes were found in hippocampus (Fig. 3.4B) or cortex (Fig.3.4C).

4.5. CD73 KO mice have normal locomotion in home cage

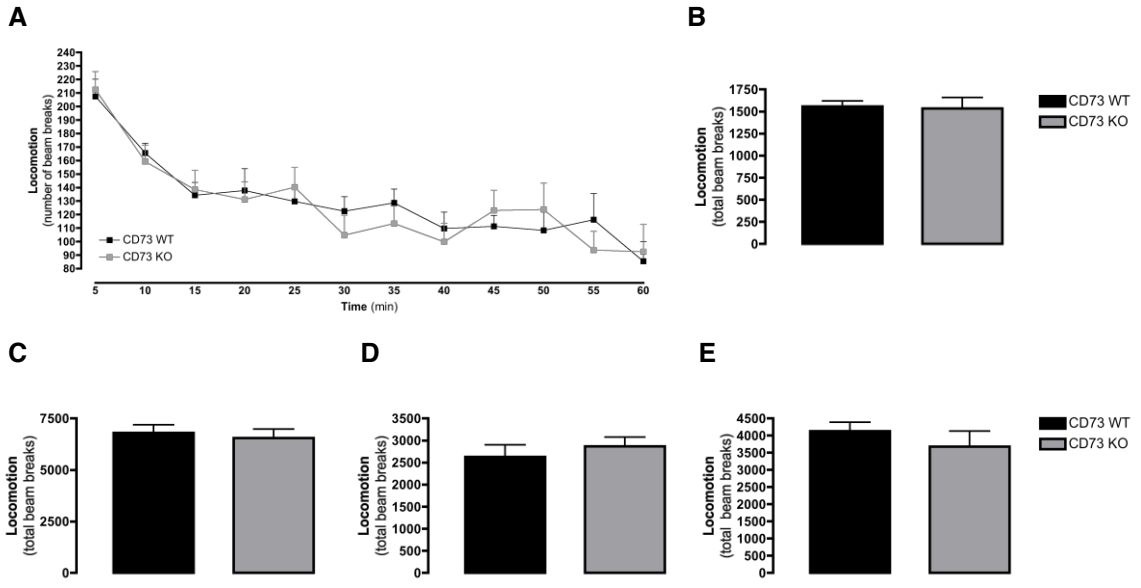

Figure 3.5. CD73 KO mice exhibit a normal locomotion in their home cage. Locomotion during habituation to a new home cage was recorded (A, B). Panel A represents the mean ± SEM of beam breaks per 5 min, whereas panel B represents the mean ± SEM of total number of beam breaks in 60 min in WT (n = 8) and CD73 KO mice (n = 9). Locomotion during 24 hours in home cage was recorded (C-E). Panel C represents the mean ± SEM of total number of beam breaks in 24 hours in WT (n = 8) and CD73 KO mice (n = 9); panel D represents the mean ± SEM of total number of beam breaks during the light period (from 7 a.m. to 7 p.m.) in WT and CD73 KO mice; panel E represents the mean ± SEM of total number of beam breaks during the dark period (from 7 p.m. to 7 a.m.) in WT and CD73 KO mice. The data are mean ± SEM. A two-way ANOVA test was used in A and a Student's *t* test was used in B-E.

Owing to the high density of CD73 in the striatum and the participation of this brain region in controlling locomotion, we next analyzed the locomotion profile of CD73 KO mice in basal conditions. CD73 depletion had no effect on the locomotor response to habituation to a novel home cage in a new environment (Fig. 3.5A-B) or in the 24 hours recorded in home cage (Fig. 3.5C). In addition, no differences were observed when the recording was restricted to the light period (Fig. 3.5D) or the dark period (Fig. 3.5E) of the day.

4.6. CD73 KO mice have hyperlocomotion in the open-field

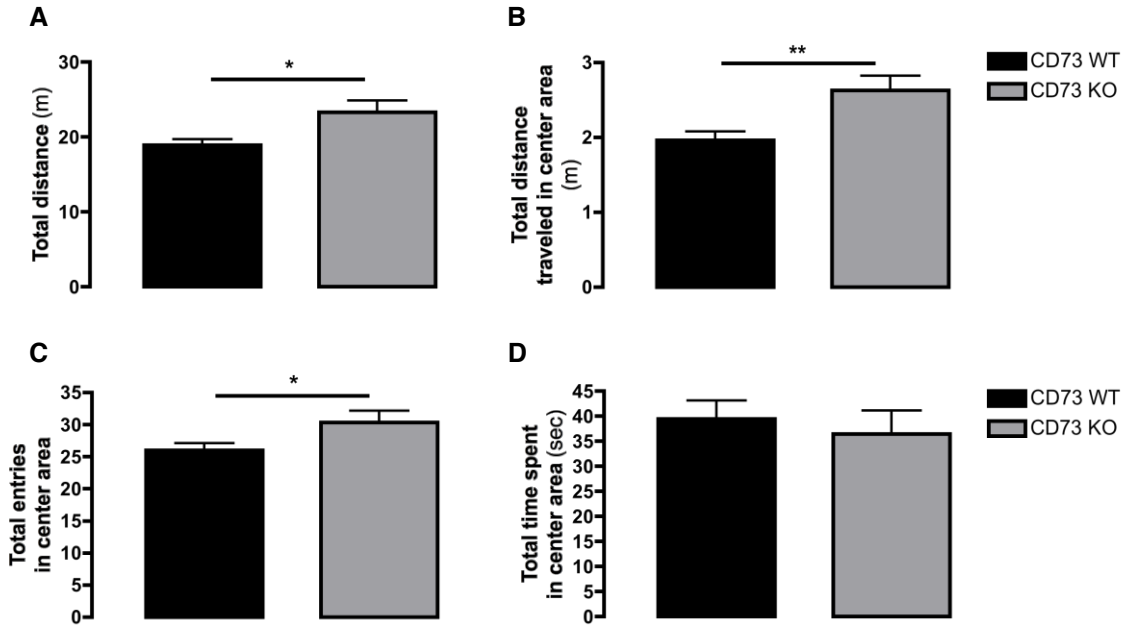

Figure 3.6. Hyperlocomotion of CD73 KO mice in the open-field. Evaluation of spontaneous locomotion recorded in an open field (A-D) shows that the total distance (A) travelled by CD73 KO mice is significantly higher (+ 4.4 ± 1.7 m; CD73 WT, 18.9 ± 0.7 m, n = 17; CD73 KO, 23.3 ± 1.6 m, n = 15). Panel B shows the total distance traveled in the center arena of open-field and panel C represents the total number of entries in the center area. Panel D shows no changes in the time spent in the center arena (CD73 WT, 39.5 ± 3.6 sec., n = 17; CD73 KO, 36.5 ± 4.7 sec., n = 15). The data are mean ± SEM. *$p < 0.05$, **$p < 0.01$ between genotypes using a Student's *t* test.

In order to evaluate a potential locomotion modification associated with a non-familiar and anxiogenic environment, spontaneous locomotion was recorded in a video tracked open field. We here showed that CD73 KO mice have an increased locomotion in a open field (Fig. 3.6A; + 4.4 ± 1.7 m; CD73 WT, 18.9 ± 0.7 m, n = 17; CD73 KO, 23.3 ± 1.6 m, n = 15; $p < 0.05$), and consequently display an increased distance (Fig.3.6B) and number of entries (Fig. 3.6C) in the center arena, but without significant changes in the time spent in the center arena (Fig. 3.6D; CD73 WT, 39.5 ± 3.6 sec., n = 17; CD73 KO, 36.5 ± 4.7 sec., n = 15, $p = 0.613$).

4.7. CD73 KO mice do not show changes in the elevated plus maze

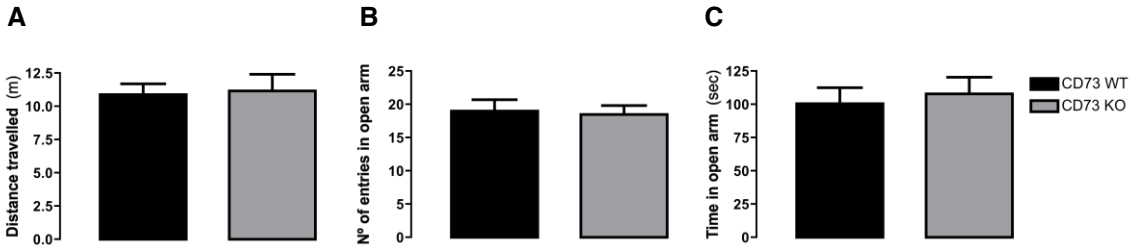

Figure 3.7. CD73 KO mice behavior in the elevated plus maze. Evaluation of spontaneous locomotion recorded in an elevated plus maze (A-C). Panel A shows the total distance travelled in the elevated plus maze by CD73 KO (n = 8) and WT (n = 9) mice; panel B shows the total number of entries in the open arm and panel C shows the total time spent in the open arm. The data are mean ± SEM. Student's *t* test was used.

In order to reinforce the absence of an anxiogenic profile, spontaneous locomotion was then recorded in a video tracked elevated plus maze (Fig. 3.7). We here show that CD73 KO mice have a similar number of entries (Fig. 3.7B) and time (Fig. 3.7C) in the open arm in an elevated plus maze (Fig. 3.7), with a similar travelled distance during the test (Fig. 3.7A).

4.8. CD73 KO mice have impaired motor coordination

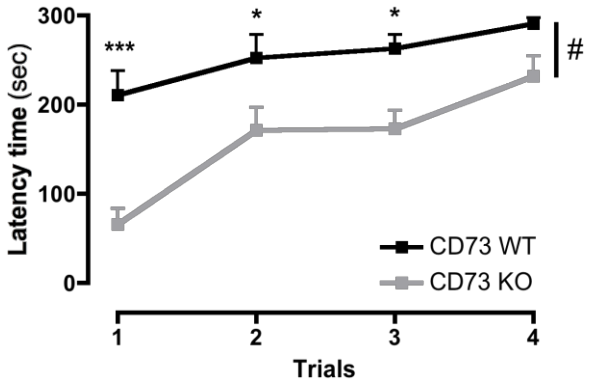

Figure 3.8. CD73 KO mice have a lower latency in the accelerated rotarod. CD73 KO mice have significantly lower latency time during the trials in the accelerated rotarod when compared to their WT littermates (genotype main effect, $F(1,72) = 39.5$, $p < 0.0001$; genotype x trial interaction, $F(3,72) = 1.5$, $p = 0.2196$; n = 11). The data are mean ± SEM. # $p < 0.001$ using a two-way ANOVA followed by Bonferroni *post hoc* test, *$p < 0.05$, ***$p < 0.001$.

We next evaluated the function of CD73 in a motor coordination task (accelerating rotarod; Fig. 3.8). In this task, the mice have to learn a novel sequence of movements to maintain balance on a rotating rod in constant acceleration and receive several consecutive trials (Buitrago et al., 2004). Evaluation of the involvement of CD73 in motor coordination by the accelerating rotarod (Fig. 3.8) showed that CD73 KO mice have lower latency during the trials (CD73 KO n = 11 vs. CD73 WT n = 11; two-way ANOVA followed by Bonferroni *post hoc* test, genotype main effect, $F(1,72) = 39.5$, $p < 0.0001$; genotype x trial interaction, $F(3,72) = 1.5$, $p = 0.2196$).

4.9. CD73 KO mice show improved avoidance learning

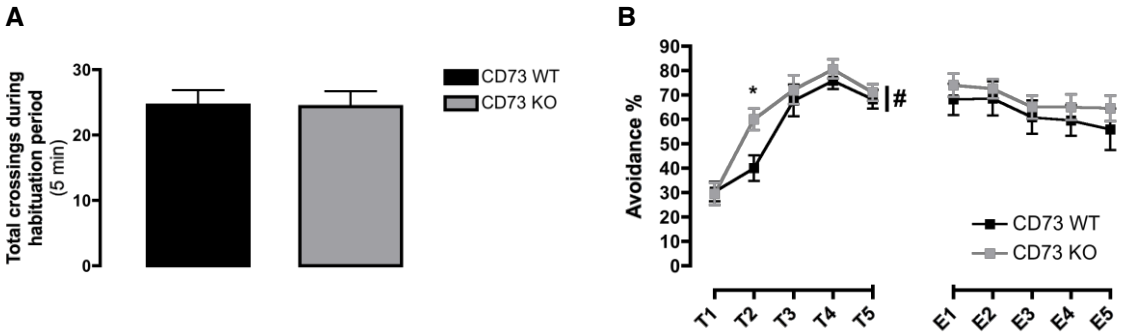

Figure 3.9. CD73 KO mice have an improved avoidance learning capacity in a two-way active avoidance paradigm. Panel A shows the total number of crossings during the habituation period to the chambers of the active avoidance apparatus in CD73 KO (n = 10) and WT (n = 11) mice. Panel B shows the percentage (%) of avoidance during the five training sessions (T1-T5) and during the five extinction sessions (E1-E5). The data are mean ± SEM. Student's *t* test was used in A. # $p < 0.001$ using a two-way ANOVA followed by Bonferroni *post hoc* test, *$p < 0.05$ in B.

Since the different nuclei of the basal ganglia, as well as the central nucleus of amygdala, where CD73 has a high density (see chapter 2), have been implicated in avoidance learning, we tested the role of CD73 in this type of behavior, evaluating the response of CD73 KO mice in the two-way active avoidance paradigm (Fig. 3.9). As observed, CD73 KO mice showed significantly higher avoidance percentage during training, when compared to their WT littermates (Fig. 3.9B, CD73 KO n = 10 vs. CD73

WT n = 10; two-way ANOVA followed by Bonferroni *post hoc* test, genotype main effect, $F(1,90) = 4.3$, $p < 0.05$; genotype x trial interaction, $F(4,90) = 1.4$, $p = 0.2264$), without any changes in the number of crossings between chambers during the habituation period (Fig. 3.9A). No modifications on the avoidance % were observed during extinction (Fig. 3.9B, CD73 KO n = 10 vs. CD73 WT n = 10; two-way ANOVA followed by Bonferroni *post hoc* test, genotype main effect, $F(1,90) = 2.3$, $p = 0.1300$; genotype x trial interaction, $F(4,90) = 0.03$, $p = 0.9987$).

4.10. CD73 KO mice do not show changes in the passive avoidance test

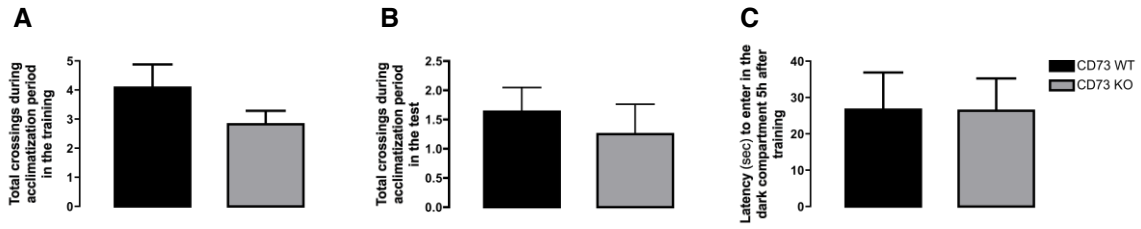

Figure 3.10. CD73 KO mice profile in a passive avoidance paradigm. Panel A shows the total number of crossings during the acclimatization period to the chambers of the passive avoidance apparatus before the training session in CD73 KO (n = 12) and WT (n = 11) mice. Panel B shows the total number of crossings during the acclimatization period before the passive avoidance test session. Panel C shows the latency in seconds (sec) that the mice took to enter in the dark chamber during the test session. The data are mean ± SEM. Student's *t* test was used.

No modifications were observed in the passive avoidance test in CD73 KO mice (Fig. 3.10). No changes were detected in the number of crossings between chambers during the acclimatization period of the training (Fig. 3.10A) or the test (Fig. 3.10B), showing that locomotion and anxiety are not taken into account in this test. More importantly, during the test no changes were observed between CD73 KO mice and their WT littermates in the latency period to enter in the dark chamber during the test (Fig. 3.10C).

4.11. CD73 KO mice display improved working memory

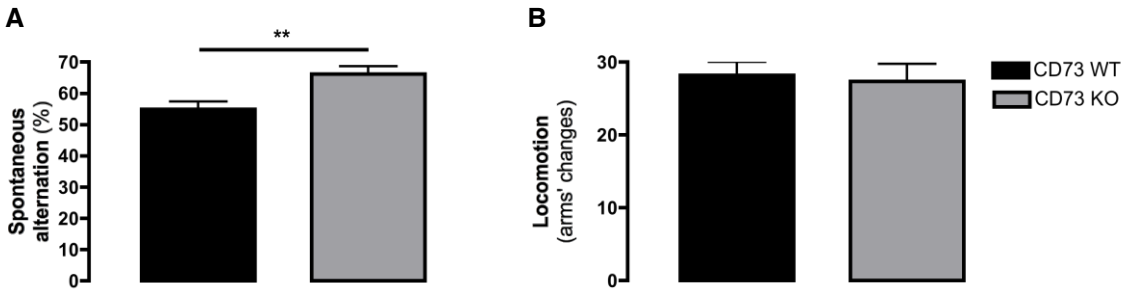

Figure 3.11. CD73 KO mice display an improved working memory in the Y-maze. CD73 KO mice have an improved working memory when tested in a Y-maze paradigm (A) analyzing their spontaneous alternation in comparison with the WT littermates, with no differences in locomotion (B), evaluated by number of arms' changes (n = 11-12). Data are mean ± SEM; ***p* < 0.01 using a Student's *t* test.

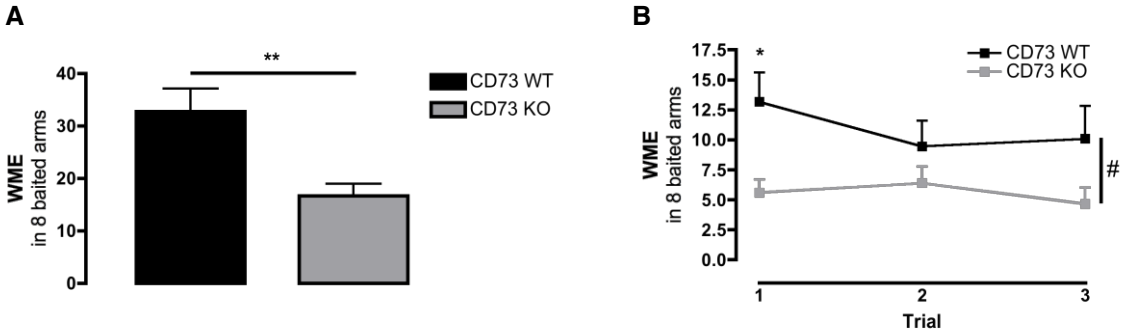

Figure 3.12. CD73 KO mice display an improved working memory in the 8-baited arms paradigm. In the 8 radial arm maze with 8 baited arms (A and B) CD73 KO displayed significantly less working memory errors (WME; scored each time an animal re-enters in a previous visited arm) in comparison with the WT littermates (n = 11-12). Panel A shows the total number of WME in the 3 trials, and panel B shows the total number of WME per trial. Data are mean ± SEM; ***p* < 0.01 using a Student's *t* test in A; #*p* < 0.05 in a two-way ANOVA followed by Bonferroni post-hoc test, **p* < 0.05 in B.

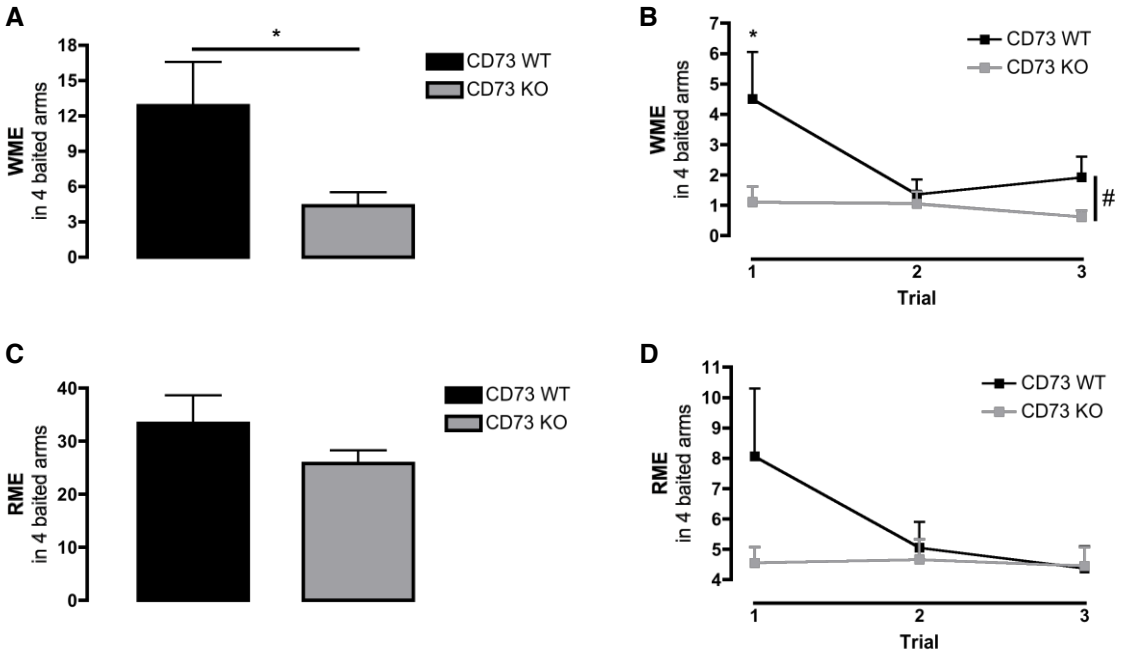

Figure 3.13. CD73 KO mice display an improved working memory in a 4-baited arms paradigm. In the 8 radial arm maze with 4 baited arms CD73 KO mice displayed significantly less working memory errors (A-B; WME; scored each time an animal re-enters in a previously baited arm) in comparison with the WT littermates (n = 8-9). Panel A shows the total number of WME in the 3 trials, and panel B shows the total number of WME per trial. In the same experiment CD73 KO mice displayed similar reference memory errors (C-D; RME; scored each time an animal re-enters in a unbaited arm) in comparison with the WT littermates. Panel C shows the total number of RME in the 3 trials, and panel D shows the total number of RME per trial. Data are mean ± SEM; **p* < 0.05 using a Student's *t* test in A and C; #*p* < 0.05 in a two-way ANOVA followed by Bonferroni post-hoc test, **p* < 0.05 in B and D.

It was previously demonstrated that $A_{2A}R$ control working memory performance. Indeed, it was shown that working memory is deficient in $A_{2A}R$ over-expressing mice (Giménez-Llort et al., 2007) and improved in global $A_{2A}R$ KO as well as in forebrain $A_{2A}R$ KO and striatal $A_{2A}R$ KO mice (Zhou et al., 2009; Wei et al., 2011). We now report that CD73 KO mice display an improved working memory compared to WT mice, when tested in the spontaneous alternation paradigm in the Y-maze (Fig. 3.11A), without changes in their locomotion (Fig. 3.11B). In addition, CD73 KO mice made less working memory errors than WT mice in the 8-baited arms version of the 8 radial arm maze (Fig. 3.12). This result was further validated in the 4-baited arms version of the 8 radial arm

maze with a separate group of mice (Fig. 3.13). In this paradigm it was possible to evaluate reference memory errors, and no changes were observed here (Fig 3.13C-D). Thus, in spite of the limitations of each test in the evaluation of working memory, taken together, these results show a consistent improvement of working memory performance when CD73 is depleted.

4.12. CD73 KO mice show improved recognition memory

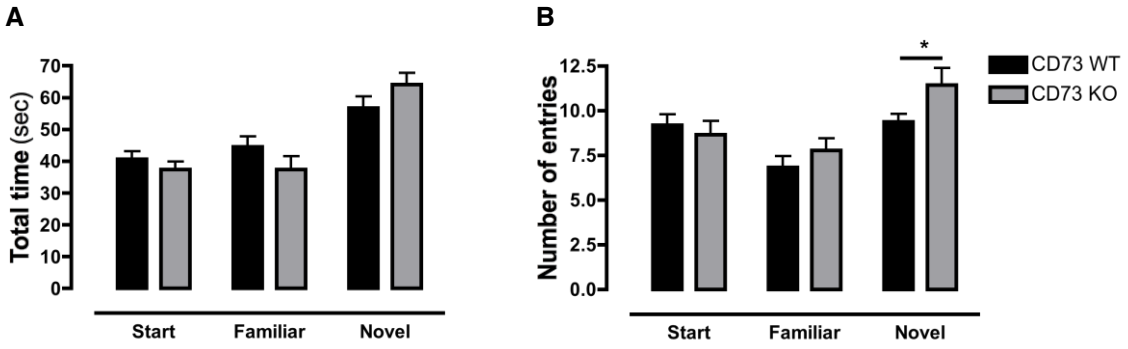

Figure 3.14. CD73 KO mice display an improved recognition memory in the modified Y-maze test. CD73 KO mice have an improved recognition memory when tested in a modified Y-maze paradigm (n = 10-11). Panel A shows the total time in seconds (sec) spent in each arm of the Y-maze during the test. Panel B shows the total number of entries in each arm of the Y-maze during the test. Data are mean ± SEM; **p* < 0.05 using a Student's *t* test.

In order to evaluate the role of CD73 in recognition memory, we next tested the profile of CD73 KO mice in the modified Y-maze paradigm (Fig. 3.14). CD73 KO mice spent a similar time in the novel arm during the test, when compared with their WT littermates (Fig. 3.14A). However, CD73 KO mice made significant more entries in the novel arm during the test, when compared with the WT mice (Fig. 3.14B), revealing an improved recognition memory.

5. Discussion

In this study we provide a characterization of the role of CD73 in physiological

conditions, demonstrating that CD73 can modulate many crucial brain processes, from molecular to behavioral responses. Thus, CD73 KO mice displayed lower DARPP-32-p(Thr75) levels and a decreased neuronal glutamate uptake in the striatum. In addition, these mice exhibited an impaired motor coordination, without modifications in anxiety, and a procognitive profile.

The phenotype of CD73 KO mice was accompanied by a striatal decrease in DARPP-32-p(Thr75) (Fig. 3.3), without changes in adenosine (Fig. 3.1) or dopamine receptors (Fig. 3.2A-E) density or enkephalin immunoreactivity (Fig. 3.2F-G). Striatal functions depend on an activity balance between dopamine and glutamate transmissions that produce opposing physiological effects on DARPP-32 phosphorylation (Greengard, 2001; Chergui et al., 2004). It was showed that tonic activity of the glutamatergic pathway is responsible for maintaining DARPP-32-p(Thr75) in a phosphorylated state (Matsuyama et al., 2003; Yamamura et al., 2013). Thus, our results (Fig. 3.3) suggest lower levels of glutamate in the striatum of CD73 KO mice. Additionally, the lower levels of glutamate can be due to a decreased CD73-dependent activation of presynaptic $A_{2A}R$ on the cortico-striatal pathway, which is know to lead to lower glutamate levels in this brain area (Corsi et al., 2000; Pintor et al., 2001; Popoli et al., 2002; Popoli et al., 2003; Marcoli et al., 2003). Therefore, further studies evaluating glutamate release from the cortico-striatal pathway should be performed in CD73 KO mice in order to better clarify this issue. Nevertheless, the postsynaptic effects observed in CD73 KO mice on medium spiny neurons in the striatum in physiologic conditions, might be mediated instead through modulation of the release of neurotransmitters from presynaptic terminals. The lower striatal levels of glutamate in CD73 KO mice are probably reflected also on the decreased aspartate uptake capacity observed specifically on striatal presynaptic nerve terminals (Fig. 3.4). These findings are particularly important since DARPP-32 regulates the state of phosphorylation and activity of key substrates, including many ion channels,

pumps, neurotransmitter receptors, and transcription factors necessary for altering the physiological status of striatal neurons and, in turn, for altering the function of striatal circuits.

Considering that CD73 is highly expressed in different nucleus of basal ganglia (see chapter 2), alterations on the behavior profile of CD73 KO mice associated with basal ganglia activity were analyzed. Our work shows that CD73 KO mice have an impaired psychomotor coordination (Fig. 3.8). A similar result was observed after specific deletion of A_{2A}R-positive medium spiny neurons in dorsal striatum (Durieux et al., 2012). This suggests that CD73 is critically needed to generate adenosine that activates A_{2A}R in this brain area in order to have a proper learning of the task involved in the accelerated rotarod. In addition, we here revealed that this impairment is specific to balance or motor coordination and not due to an impairment in locomotor functions in general, since we showed that CD73 KO mice do not have changes in basal locomotion, including during adaptation to a new home cage (Fig. 3.5), despite the hyperlocomotion observed in the open-field (Fig. 3.6), which is in consistent with A_{2A}R-positive neurons functions (Durieux et al., 2012). Furthermore, the hyperactivity observed during the open-field test was not associated with anxiety, since no changes were observed in the time spent in the center arena of the open-field, as well as in the elevated plus maze (Fig. 3.7).

CD73 is also highly expressed outside the basal ganglia, namely in central nucleus of amygdala (see chapter 2), where it potentially contributes to the regulation of behavioral avoidance responses. The amygdala comprises several distinct nuclei and plays a critical role in emotional processing. Particularly the central nucleus of the amygdala is a major output region of the amygdaloid complex, and is known for its role in responses to fear stimuli (Pascoe and Kapp, 1985; LeDoux et al., 1988; Hitchcock and Davis, 1991). The two-way active avoidance paradigm requires not only the association

of a cue with a foot shock, but also the learning of a foot-shock avoidance strategy. Recent studies indicate a role for the central nucleus of the amygdala in avoidance learning (Samson and Paré, 2005; Wilensky et al., 2006; Moscarello and LeDoux, 2013). However, the participation of striatum and dopamine in this behavior has also been long established (POSLUNS, 1962; Fibiger et al., 1974; Koob et al., 1984; Da Cunha et al., 2002). In this study CD73 KO mice showed quicker and improved avoidance learning behavior when compared to their WT littermates (Fig. 3.9), without further modifications in the passive avoidance task (Fig. 3.10). The participation of a specific pool (e.g., central nucleus of amygdala or striatal) of CD73 in the improved avoidance is still to be demonstrated.

It was also shown that inactivation of $A_{2A}R$ (Zhou et al., 2009), namely striatal $A_{2A}R$ (Wei et al., 2011), enhances working memory performance, and a similar phenotype was observed in CD73 KO mice (Fig. 3.10, 3.11, 3.12). All together, the parallel modifications of these behavioral responses by eliminating CD73 or $A_{2A}R$ but not A_1R (Giménez-Llort et al., 2002; Giménez-Llort et al., 2005), prompt the conclusion that CD73 is responsible for the formation of the adenosine that activates $A_{2A}R$ in the striatum, which negatively regulates working memory. This proposed activation of $A_{2A}R$ selectively by CD73-mediated formation of adenosine seems to be a more general feature of $A_{2A}R$ not only in the striatum, but also in other tissues and cell types. Indeed, it was shown that the inhibition of CD73 selectively blunts the ability of $A_{2A}R$ to control synaptic plasticity in hippocampal synapses (Rebola et al., 2008) that could participate in the improved recognition memory observed in CD73 KO mice (Fig. 3.13).

The presence of CD73 activity in the CNS outside the striatum has been observed previously, namely in plexus choroid and meninges (Langer et al., 2008; see chapter 2). The meninges and plexus choroid are important in maintaining cerebrospinal fluid (CSF) generation and flow rate, which is absolutely crucial for a normal brain function. In

agreement, it was showed that CD73 has a crucial role in $A_{2A}R$ activation in blood brain barrier permeability, through the plexus choroid, which have an essential role in pathological conditions like experimental autoimmune encephalomyelitis (Mills et al., 2008; Yao et al., 2012), invasion of pathogens to the central nervous system (Mahamed et al., 2012), as well as for drug delivery (Carman et al., 2011). However, the role of CD73 in meninges and plexus choroid in physiological conditions (Xie et al., 2013) were not specifically tested and thus could potentially affect important brain functions, namely some associated with the phenotypes described here. Therefore, alterations in the adenosine concentrations through CD73 activity could have dramatic effects on functions and behavior of the whole CNS.

In conclusion, we here show the role of CD73 in different behaviors, namely motor coordination and cognitive processes, as well as, in striatal molecular functions, like DARPP-32 phosphorylation and glutamate uptake. These results open-up new avenues to explore specific, as well as, broad roles of CD73 in the CNS, including in different pathologies related with motor and cognitive functions, like Parkinson's and Alzheimer's diseases.

6. Acknowledgements

I am thankful to Marco Matos for his help, sharing of knowledge and advice in the D-[^{3}H]aspartate uptake experiments.

CHAPTER 4

THE ROLE OF CD73 IN THE BRAIN IN PATHOLOGICAL CONDITIONS

1. Abstract

Adenosine is a neuromodulator that, acting through adenosine receptors, is able to participate in different pathological conditions. Striatal adenosine acting through adenosine A_{2A} receptors that is highly expressed in the striatum, participates in pathological conditions associated with this brain area and the dopaminergic system, like Parkinson's disease, schizophrenia or drug addiction. We previously showed that ecto-5'-nucleotidase (CD73), the major enzyme able to convert extracellular AMP into adenosine in the brain, is highly expressed in the striatum and therefore we here demonstrate its participation in amphetamine-induced sensitization, MK-801-induced psychomotor activity and MPTP-induced neurodegeneration. This study suggests CD73 as a possible therapeutic target to manipulate striatal dysfunctions that occur in drug addiction or schizophrenia in addition to preventing the neurodegeneration that occurs in Parkinson's disease.

2. Introduction

Adenosine is an important neuromodulator in the central nervous system (CNS), modulating the glutamatergic and dopaminergic systems (Fredholm et al., 2005a) which in turn are tightly associated with different pathological conditions, including Parkinson's disease (PD) (Chen et al., 2001b), drug addiction (Turgeon et al., 1996) and schizophrenia (Malec and Poleszak, 2006; Yee et al., 2007; Shen et al., 2012).

There are different sources of adenosine: it can be generated intracellularlly and potentially directly released through the equilibrative nucleoside transporters (ENT) (Brundege and Dunwiddie, 1998) or by exocytosis (Klyuch et al., 2012); and indirectly as ATP, followed by its extracellular enzymatic catabolism through an ectonucleotidases pathway, from which ecto-5'-nucleotidase (CD73) plays a key role, dephosphorylating adenosine monophosphate (AMP) to adenosine (Zimmermann, 1996). Adenosine

executes its actions through activation of different adenosine receptors (A_1, A_{2A}, A_{2B} or A_3), from which A_1 receptors (A_1R) and A_{2A} receptors ($A_{2A}R$) have a higher abundance in the CNS (Cunha, 2008a).

The participation of these receptors in many pathophysiological conditions has been extensively described (Cunha, 2005; Boison, 2008a), however the source of adenosine in the different conditions it is still debatable and incomplete. Therefore, we here proposed to explore the role of CD73 in different pathological conditions that are distinctly associated with striatal dysfunctions, a brain region where this enzyme has a remarkably higher density (see chapter 2). By taking advantage of CD73 knockout (KO) mice model we went to explore the possible participation of CD73 to striatal-associated brain disorders such as drug addiction, schizophrenia or PD.

3. Materials and methods

Animals

Approval from the Institutional Animal Care and Use Committee at Boston University School of Medicine and the Portuguese Veterinarian Office was granted for all experiments conducted in Boston and Coimbra, respectively. They adhered to the NIH Guide for the Care and Use of Laboratory Animals, the Portuguese Law and Ordinance on Animal Protection, and European Council Directive 86/609/EEC. The global CD73 knockout (CD73 KO) mice used, with a C57Bl/6 genetic background, were previously characterized (Thompson et al., 2004). In all experiments males and females adult (2-3 months old) mice were used.

Drug treatments and locomotor activity

The horizontal locomotor activity of mice was assessed in standard polypropylene cages (15 x 25 cm, i.e. clean home cages) and recorded with infrared photobeams (San

Diego Instrument). Ambulation was quantified as the number of sequential breaks in adjacent beams. All mice were habituated to the test cage for at least 120 min before recording basal locomotion for 60 min in 5 min bins. To assess the motor stimulatory effect of NMDA receptor antagonist dizocilipine (MK-801, Sigma-Aldrich) the animals were injected intraperitoneally (i.p.) at an efficient dose (0.5 mg/kg) and the motor activity recorded for the next 3 hours. In the amphetamine (i.p., 2.5 mg/kg; Sigma) paradigm, the mice were injected in the same environment for 8 consecutive days and the locomotor activity was recorded for the next 80 min.

1-Methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) treatment

The MPTP (Sigma-Aldrich) was dissolved in sterilized 0.9 % NaCl and a single dose (35 mg/kg) was administrated (i.p.) to both groups of mice (CD73 WT and CD73 KO). The control group received an i.p. injection of vehicle. 7 days after treatment the mice were perfused and brain sections were used to evaluate MPTP-induced neurodegeneration.

Immunohistochemistry

Mice were anesthetized with avertin and brain fixation was performed through transcardiac perfusion with 4 % paraformaldehyde in PBS, postfixation overnight in PBS with 4 % paraformaldehyde and cryopreservation in PBS containing 25 % sucrose. Frozen brains were sectioned (30 μm coronal slices) with a Leica CM3050S cryostat (Leica Microsystems). Four of every sixth hippocampal sections (i.e., four sections separated by 150 μm from each other) were selected for independent stainings for quantification. The sections were first rinsed for 5 min with PBS at room temperature and then permeabilized and blocked with PBS containing 0.2 % Triton X-100 and 5 %

donkey serum during 1 h, incubated in the presence of rabbit anti-tyrosine hydroxylase (TH; 1:1000; Millipore) overnight at room temperature, rinsed three times for 10 min in PBS, and then incubated with donkey anti-rabbit secondary antibodies conjugated with a fluorophore (Alexa Fluor 555, 1:200, Invitrogen) for 2 h at room temperature. After rinsing three times for 10 min in PBS, the sections were mounted on slides and allowed to dry. Vectashield mounting medium with DAPI (Vector Laboratories) was applied as well as the cover glass. All sections were examined under a fluorescence Nikon eclipse E600 microscope, with SPOT software 4.7 (Diagnostic instruments, Inc.).

Statistical analysis

Results are presented as mean ± SEM. Data with one condition and one variable (e.g. genotype) were analyzed with Student's *t* test or repeated measures ANOVA. Data with more than one variable (e.g. genotype and time) and condition were analyzed with a two-way ANOVA followed by Bonferroni post-hoc tests. Unless otherwise indicate the significance level was 95 %.

4. Results

4.1. CD73 KO mice display no sensitization to amphetamine

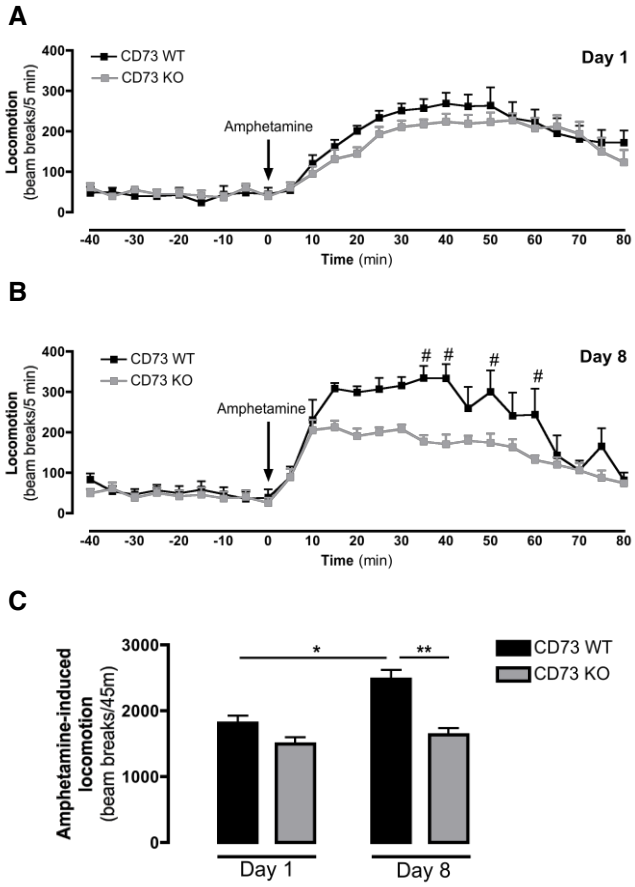

Figure 4.1. CD73 KO mice display no locomotor sensitization to amphetamine. CD73 KO (n = 7) and WT (n = 6) mice showed identical hyperlocomotion induced by an acute single administration of amphetamine (A, C). However, CD73 KO mice showed no sensitization to amphetamine after being challenged for 8 consecutive days, in contrast to the WT that showed a significant increase in locomotion (B, C). Data are mean ± SEM; $^{\#}p < 0.05$, using a two-way ANOVA followed by Bonferroni post-hoc tests in B; $^{*}p < 0.01$, using repeated measures ANOVA and $^{**}p < 0.001$, using a two-way ANOVA followed by Bonferroni post-hoc test in C.

It was previously shown that global $A_{2A}R$ KO mice (Chen et al., 2003), as well as forebrain $A_{2A}R$ KO mice (Bastia et al., 2005) do not develop psychomotor sensitization to amphetamine. In order to test if the source of adenosine responsible for this phenotype is CD73-dependent we tested CD73 KO mice in the same paradigm. CD73 depletion

had no effect on the locomotor response to habituation to a novel environment (see Chapter 3) or to the habituation to a saline injection (data not shown). Furthermore, there was no difference between CD73 KO and WT mice in the locomotor response to the first administration of a low dose (2.5 mg/kg) of amphetamine (Fig. 4.1A, C). However, continuous daily treatment with this low dose of amphetamine markedly enhanced (sensitized) locomotor responses in control WT mice ($p < 0.05$, day 8 *versus* day 1), whereas no sensitization to amphetamine was observed in CD73 KO mice (Fig. 4.1B, C).

4.2. CD73 KO mice exhibit lower MK-801-induced psychomotor activity

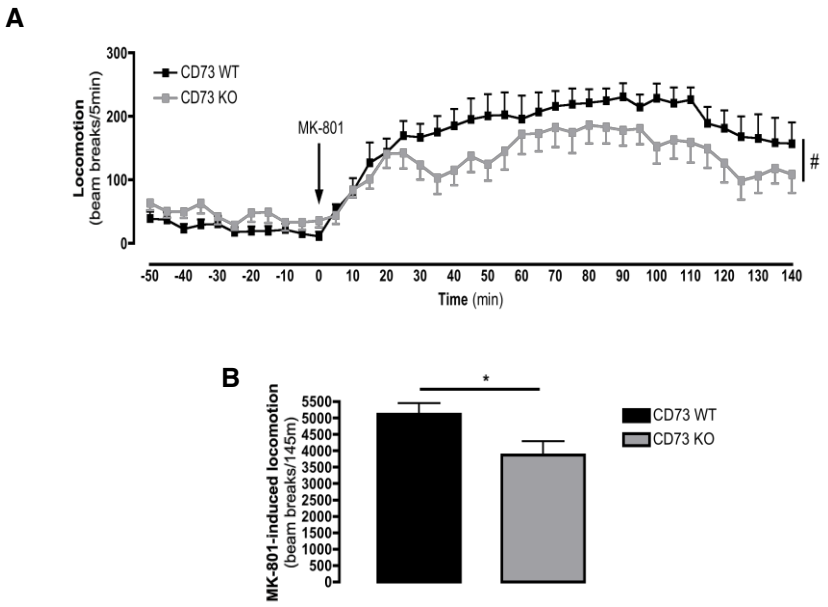

Figure 4.2. CD73 KO mice exhibit lower MK-801-induced locomotion. CD73 KO (n = 9) and WT (n = 10) mice showed identical basal locomotion in a home cage (A). However, CD73 KO mice showed less psychomotor activity when compared to the WT after MK-801 single administration (A, B). Data are mean ± SEM; $^{\#}p < 0.05$, using a two-way ANOVA followed by Bonferroni post-hoc tests in A; $^{*}p < 0.05$, using a Student's *t* test in B.

It was previously shown that adenosine has a crucial role in schizophrenia (Boison et al., 2012), and in order to test CD73 as a source of adenosine that participates in MK-801-induced psychomotor activity we tested the vulnerability of CD73 KO mice to schizophrenia-associated exacerbation of MK-801-induced hyperlocomotion. Indeed, CD73 depletion significantly decreased the psychomotor activity induced by MK-801 (Fig 4.2).

4.3. CD73 KO mice show lower MPTP-induced neurodegeneration

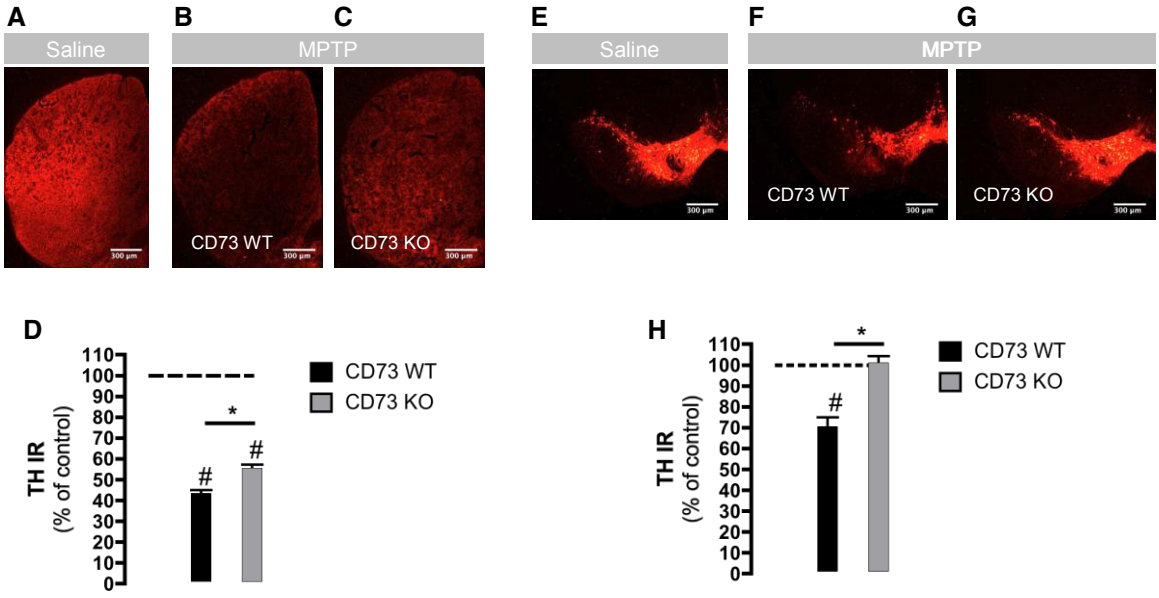

Figure 4.3. CD73 depletion is neuroprotective in a MPTP mouse model. 7 days after an acute treatment with MPTP (35 mg/kg; intraperitoneal) or control (vehicle; intraperitoneal), TH immunoreactivity (IR) was evaluated. Panel A shows a representative image of TH immunoreactivity in dorsal striatum in control group, and panels B and C show representative images of TH immunoreactivity in MPTP treated mice, CD73 WT and CD73 KO mice, respectively. Panel D shows the quantification of TH immunoreactivity (IR) in dorsal striatum (CD73 WT, 43.5 ± 1.4 % of control, n = 5; CD73 KO, 55.5 ± 1.8 % of control, n = 4). Panels E, F and G show representative images of TH immunoreactivity in the substantia nigra in the control mice, MPTP-treated CD73 WT mice and MPTP-treated CD73 KO mice respectively. H is the quantification of TH IR in the substantia nigra pars compacta (CD73 WT, 70.6 ± 4.3 % of control, n = 5; CD73 KO, 101.2 ± 2.9 % of control, n = 4). The data are mean ± SEM. *p < 0.0001 between genotypes using a Student's t test; # p < 0.001, using a two-way ANOVA followed by Bonferroni *post hoc* test in D and H.

The biochemical and cellular modifications that occur after 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) administration are remarkably similar to those observed in idiopathic PD (Gerlach et al., 1991). Therefore, this is a well-established model to study the neurodegeneration that occur in PD in mice. Furthermore, it is known that acute treatments with MPTP can cause a severe loss of TH and dopamine transporters levels and dopamine contents in the striatum of mice, as compared to continuous MPTP treatment (Gao et al., 2011). Thus, in order to study the role of CD73 in the neurodegenerative processes of PD, we took advantage of the acute MPTP treatment model. 7 days after an acute treatment with MPTP (35 mg/kg; intraperitoneal) both groups of mice (CD73 WT and CD73 KO), as well as the control group (saline; intraperitoneal) were perfused and brain sections were used to evaluate MPTP-induced neurodegeneration through TH imunoreactivity. Immunohistochemistry (Fig. 4.3) showed that TH immunoreactivity in dorsal striatum was significantly lower in MPTP treated group of mice; however, the neurodegeneration was significantly lower in CD73 KO mice when compared to their WT littermates (CD73 WT, 43.5 ± 1.4 % of control, n = 5; CD73 KO, 55.5 ± 1.8 % of control, n = 4, $p < 0.0001$, Student's *t* test). TH immunoreactivity in substantia nigra pars compacta was also significantly lower in WT mice after MPTP treatment when compared to the CD73 KO mice (CD73 WT, 70.6 ± 4.3 % of control, n = 5; CD73 KO, 101.2 ± 2.9 % of control, n = 4, $p < 0.0001$, Student's *t* test).

5. Discussion

We previously performed a characterization of the distribution of CD73 (see chapter 2), and of its role in different physiological brain processes (see chapter 3). However, it remained to be determined if CD73-mediated formation of ATP-derived adenosine has a role in pathological brain conditions. In this study we demonstrate the role of CD73 in different disorders that affect the basal ganglia normal function, namely

in the striatum where CD73 has a high density. We here demonstrate that CD73 participates in the synaptic plasticity during drug sensitization and MK-801-induced psychomotor activity and in the neurodegeneration taking place in an animal model of PD.

Despite the extensive characterization of $A_{2A}R$ in basal ganglia on different pathological conditions, its source of adenosine has been unclear. Remarkably, deleterious brain conditions trigger an enhancement of the extracellular levels of ATP (Di Virgilio, 2000). Since it has already been shown that the extracellular conversion of AMP (which usually derives from ATP) into adenosine seems to be wiped-out in CD73 KO mice (Klyuch et a., 2012; Lovatt et al., 2012; Zhang et al., 2012), with no compensation of alternative enzymatic activities such as alkaline phosphatase (Langer et al., 2008), it is tempting to consider the possibility that manipulation of CD73 might afford a similar benefit to that observed by $A_{2A}R$ blockade (Cunha, 2005; Chen et al., 2007), due to their similar distributions.

The presence of CD73 in the nucleus accumbens (see chapter 2) suggested a possible role of this enzyme in drug sensitization, which was further supported by the role of adenosine receptors in this type of behavior (Chen et al., 2003; Bastia et al., 2005). The behavioral sensitization is characterized by the augmented motor-stimulant response that occurs with repeated intermittent exposure to the drug (Paulson and Robinson, 1991). Consequently, this addictive behavioral dysfunction is commonly assessed by monitoring the motor activity, although it can also be assessed via conditioned place preference or drug self-administration. In addition, several studies have shown that DARPP-32 participates in the generation and expression of behavioral sensitization to psychostimulants. Chronic treatment with cocaine (Bibb et al., 2001; Scheggi et al., 2004) or methamphetamine (Lin et al., 2002; Chen and Chen, 2005) decreased Thr34 and increased Thr75 phosphorylation. This suggests that the

decreased phosphorylation of DARPP-32 at threonine 75 (DARPP-32-p(Thr75)) in CD73 KO mice previously observed (see Chapter 3) may provide a buffer effect to the development of amphetamine sensitization. In addition, it is known that dopaminergic and glutamatergic transmissions participate in psychostimulant-induced behavioral sensitization (Wolf, 1998) and consequently, $A_{2A}R$ have emerged as an attractive therapeutic target as a modulator of both of these systems in behavioral sensitization. Actually, studies with $A_{2A}R$ antagonists and genetic deletion (Chen et al., 2003; Bastia et al., 2005) showed that the behavioral sensitization to repeated treatments with amphetamine does not develop when $A_{2A}R$ is blockade or deleted. However, a facilitative role of $A_{2A}R$ in sensitization is still controversial since other reports have shown that the $A_{2A}R$ agonist CGS21680 attenuates the development of behavioral sensitization induced by methamphetamine (Shimazoe et al., 2000; Hobson et al., 2012). Nevertheless, the possibility that $A_{2A}R$ antagonists could provide a rational pharmacological intervention for the treatment on addictive disorders was further supported by the demonstration that $A_{2A}R$ participate in the development rather than the expression of amphetamine sensitization (Bastia et al., 2005). This suggests that $A_{2A}R$ prevent or delay the maladaptive neuroplasticity that contributes to the induction or maintenance phases of some addictive behaviors. However, the source of adenosine that activates $A_{2A}R$ during drug sensitization is unknown. Since we here showed a similar phenotype after CD73 deletion (prevention of amphetamine-induced sensitization), is tempting to suggest that the amphetamine sensitization is CD73- and $A_{2A}R$-dependent. This is in agreement with the increased adenosine and AMP after amphetamine administration (Pintor et al., 1995). Still, a molecular correlation, for example through delta FosB imunoreactivity in the striatum, would strengthen the behavior observed in CD73 KO mice. Thus, further studies ought to be performed.

Schizophrenia is a mental disorder characterized by a spectrum of positive, negative and cognitive symptoms (van Os and Kapur, 2009). The positive symptoms of the disease seem to be associated with a dopaminergic hyperfunction (Snyder, 1976). On other hand, the negative and cognitive symptoms are supported by a glutamatergic hypofunction (Ranganath et al., 2008). This latter hypothesis is based on the observation that psychotomimetic agents such as phencyclidine and dizocilpine (MK-801) induce psychotic and cognitive disturbances in human and animals similar to those observed in schizophrenia patients, by blocking N-methyl-D-aspartate receptors (NMDAR) (Moghaddam and Javitt, 2012). In agreement, MK-801-induced hyperlocomotion has been consistently used as a mouse model for schizophrenia, based on the notion that the enhanced motor activity triggered in rodents is a faithful indicator of the propensity of a drug to elicit or enhance psychosis in humans (Moghaddam and Javitt, 2012). Adenosine is a network regulator, being suitable to modulate both dopaminergic and glutamatergic neurotransmissions (Chen et al., 2013). Actually, adenosine is considered to play a key integrative role in controlling the expression of schizophrenia-related psychomotor and cognitive endophenotypes (Boison et al., 2012; Shen et al., 2012). In agreement, we here showed that CD73 deletion decreased MK-801-induced psychomotor activity, a phenotype similar to that observed in forebrain $A_{2A}R$ KO mice (unpublished data, Catherine Wei PhD thesis). In addition, working memory impairment is a key endophenotype of schizophrenia (Amann et al., 2010), and both CD73 (see Chapter 3) and forebrain $A_{2A}R$ (see Chapter 7; Wei et al., 2011) KO mice display an improved working memory performance, which could provide a further support for adenosine as a therapeutic target for schizophrenia.

The presence of CD73 in the substantia nigra and in the striatum (see chapter 2) suggested a possible role of this enzyme in PD. MPTP is a neurotoxin that causes permanent symptoms of PD by destroying dopaminergic neurons in the substantia nigra,

being suitable as a neurodegenerative mice model for PD. On the other hand, adenosine acting through $A_{2A}R$ is established to participate in neurodegeneration in animal models of PD. $A_{2A}R$ antagonists in dopamine-depleted mice induce motor enhancement (Popoli et al., 2000) and are neuroprotective (Chen et al., 2001b) . However, both the mechanism (Ferré et al., 1991; Mori et al., 1996; Fuxe et al., 1998; Richardson et al., 1999; Aoyama et al., 2000; Chen et al., 2001a) as well as the cellular pool of $A_{2A}R$ behind the neuroprotective mechanism (Yu et al., 2008) are still debatable. Despite that, CD73 depletion mimics the phenotype of $A_{2A}R$ KO, enabling a neuroprotective benefit in an acute MPTP mice model. In addition, different reports showed that a rodent model of PD with deficient striatal dopamine do not change DARPP-32 phosphorylation at threonine 34, but significantly increased the DARPP-32-p(Thr75) (Brown et al., 2005; Santini et al., 2007), which was observed also in the MPTP mice model (Yamamura et al., 2013). These findings suggest that the glutamate/DARPP-32-Thr75 pathway may be important to further take into account in the pathophysiology of PD, further proposing that the lower DARPP-32-p(Thr75) observed in CD73 KO mice (see Chapter 3) may prompt them with an advantage against MPTP-mediated neurodegeneration.

In summary, the present study suggests new therapeutic avenues, providing the first demonstration that CD73 activity is responsible for the formation of the adenosine involved in different basal ganglia-associated pathologies, like drug addiction, schizophrenia and PD. Therefore, our work points CD73 as a new target to manipulate activity-dependent synaptic adaptation and neurodegeneration.

CHAPTER 5

THE ROLE OF CD73 IN STRIATAL $A_{2A}R$ ACTIVATION

1. Abstract

Adenosine is a neuromodulator acting through inhibitory A_1 receptors (A_1R) and facilitatory A_{2A}R, which have similar affinities for adenosine. It has been shown that the activity of intracellular adenosine kinase preferentially controls the activation of A_1R, but the source of the adenosine activating A_{2A}R is unknown. We now show that ecto-5'-nucleotidase (CD73), the major enzyme able to convert extracellular AMP into adenosine, co-localizes with A_{2A}R in the basal ganglia. Notably, CD73 co-immunoprecipitated with A_{2A}R and proximity ligation assays confirmed the close proximity of CD73 and A_{2A}R in the striatum. Accordingly, the cAMP formation in striatal synaptosomes, as well as the hypolocomotion induced by a novel A_{2A}R prodrug that requires CD73 metabolization to activate A_{2A}R were observed in wild type mice, but not in CD73 knockout (KO) mice or A_{2A}R KO mice. These results show that CD73-mediated formation of extracellular adenosine is responsible for the activation of striatal A_{2A}R function. This study points CD73 as a new target that can fine-tune A_{2A}R activity, and a novel therapeutic target to manipulate A_{2A}R-mediated control of striatal function and neurodegeneration.

2. Introduction

Adenosine is a neuromodulator that fine-tunes brain neurotransmission mainly acting through inhibitory A_1R and facilitatory A_{2A}R (Fredholm et al., 2005a). A_1R are abundantly expressed throughout the brain, controlling synaptic transmission (Dunwiddie and Masino, 2001). A_1R activation depends on the tissue workload (Cunha, 2001a) and adenosine kinase activity is a key regulator of endogenous adenosine activating A_1R (Boison, 2011). In accordance with their inhibitory role curtailing excitatory transmission, bolstering A_1R activation through inhibition of adenosine kinase affords neuroprotection against brain damage involving glutamate excitotoxicity (Fredholm et al., 2005a), namely

upon epilepsy and brain ischemia (Boison, 2006). Importantly, the manipulation of the metabolic pathways associated with A_1R activation is more promising than the direct A_1R activation to control neurodegeneration, since the former locally enhances adenosine where activity is disrupted whereas the latter also activates peripheral A_1R causing marked cardiovascular effects (Cunha, 2005).

The brain distribution of A_{2A}R is different from that of A_1R: A_{2A}R are most abundant in the basal ganglia (Schiffmann et al., 1991b), but are also present at lower density throughout the brain (Rosin et al., 2003). Like A_1R, A_{2A}R are mostly located at synapses (Rebola et al., 2003a; 2005a) but they fulfill different roles. Thus, A_{2A}R are selectively engaged to assist the implementation of synaptic plastic changes in excitatory synapses (Cunha, 2008a), by facilitating NMDA receptor-mediated responses (Rebola et al., 2008), by increasing glutamate release (Rodrigues et al., 2005) and by desensitizing presynaptic inhibitory modulation of systems like A_1R (Lopes et al., 2002; Ciruela et al., 2006a) or cannabinoid CB_1R (Martire et al., 2010). Therefore, A_{2A}R play a key role in modulating the plasticity of neuronal circuits, such as upon learning and memory (Zhou et al., 2009; Wei et al., 2011) or drug addiction (Chen et al., 2003). Notably, neurodegenerative conditions are accompanied by an up-regulation of A_{2A}R (Cunha, 2005), justifying that A_{2A}R blockade controls the burden of Parkinson's (Chen et al., 2001b) or Alzheimer's diseases (Canas et al., 2009b).

The source of the adenosine activating A_{2A}R is poorly characterized. We have previously shown that different sources of adenosine activate A_1R and A_{2A}R (Cunha et al., 1996a) and that A_{2A}R are selectively activated upon extracellular catabolism by ecto-nucleotidases of ATP (Cunha et al., 1996a; Rebola et al., 2008). We have also shown that the ATP-derived formation of adenosine by ecto-nucleotidases is limited and controlled by ecto-5'-nucleotidase (CD73) activity (Cunha, 2001b), the only enzyme able to dephosphorylate extracellular AMP into adenosine in the brain (Lovatt et al., 2012). In

agreement with this proposed functional association between CD73 and A_{2A}R, CD73 activity displays a brain distribution similar to A_{2A}R, both being higher in the basal ganglia (Langer et al., 2008).

Using mice deficient in either CD73 or A_{2A}R, coupled with a novel A_{2A}R agonist prodrug requiring a CD73-mediated activation and a proximity ligation assay, we now explored if CD73 and A_{2A}R are co-localized and physically associated in the striatal neurons and if CD73 provides the particular pool of extracellular adenosine selectively responsible for activating striatal A_{2A}R.

3. Materials and methods

Animals

Approval from the Institutional Animal Care and Use Committee at Boston University School of Medicine and the Portuguese Veterinarian Office was granted for all experiments conducted in Boston and Coimbra, respectively. They adhered to the NIH Guide for the Care and Use of Laboratory Animals, the Portuguese Law and Ordinance on Animal Protection, and European Council Directive 86/609/EEC. The knockout (KO) mice used, both with a C57Bl/6 genetic background, were previously characterized, namely global CD73 KO (CD73 KO) (Thompson et al., 2004), as well as the global A_{2A}R KO mice (A_{2A}R KO) (Chen et al., 1999). In all experiments males and females adult (2-3 months old) mice were used.

Separation of total membranes

Mice were euthanized by decapitation after deep anesthesia with isoflurane, and the brain tissues were homogenized in sucrose (0.32 M) solution containing 1 mM EDTA, 10 mM HEPES, 1 mg/mL bovine serum albumin (BSA; Sigma), pH 7.4 at 4 ºC. The homogenates were centrifuged at 3,000 *g* for 10 min at 4 °C and the supernatants

then centrifuged at 14,000 *g* for 10 min at 4 °C. The pellets were washed in Krebs-HEPES-Ringer (KHR) solution containing 140 mM NaCl, 1 mM EDTA, 10 mM HEPES, 5 mM KCl, 5 mM glucose, pH 7.4 at 4 ºC and further centrifuged at 14,000 *g* for 10 min at 4 ºC. The pellets were resuspended in Phosphate Buffered Saline (PBS; 140 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) for Co-immunoprecipitation (Co-IP) analysis.

Co-immunoprecipitation (Co-IP)

Co-IP was performed as previously described (*e.g.* Ciruela et al., 2001). Briefly, total membranes from the striatum (1 mg) were prepared as described above, washed in PBS and centrifuged at 14,000 *g* for 10 min at 4 ºC. The pellets were either resuspended in the immunoprecipitation buffer (IPB; containing 20 mM Tris, pH 7.0, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 µM okadaic acid, 0.1 mM PMSF and 1:1000 protease inhibitor cocktail) with 1 % Triton X-100, sonicated for 30 sec on ice and further spun down for 10 min to remove insoluble materials. A sample was collected to determine the protein concentration using the bicinchoninic acid (BCA) assay (Thermo Scientific), another was stored at -20 ºC as input (positive control) and the rest of the sample was processed for IP at a dilution of 0.5 mg/mL. Protein A sepharose beads were incubated with the sample for 1 h at 4 ºC under rotation to pre-absorb any protein that non-specifically binds to the protein A sepharose beads. The supernatant was recovered by centrifugation and 3 µg of anti-$A_{2A}R$ antibody (Millipore) or irrelevant IgG (for negative control) were added and incubated for 3 h at 4 ºC under rotation. To pull-down the immune complexes, samples were then incubated with protein A sepharose beads for 2 h at 4 ºC and centrifuged. The pellets were washed twice in IPB with 1 % Triton X-100, 3 times in IPB with 1 % Triton X-100 and 500 mM NaCl and twice in IPB. The input (5 % of the initial sample), 20 % of the supernatant of both pull-

downs, as well as 100 % of the immunoprecipitates were resolved in RIPA buffer, and Western blots were performed with anti-A_{2A}R or anti-CD73 antibodies (see Western blot).

Western blot

Western blotting was performed as previously described (Rebola et al., 2005a), using non-reducing conditions for rabbit anti-murine CD73. Incubation with the primary antibodies, namely mouse anti-A_{2A}R (1:1,000, Millipore), and rabbit anti-murine CD73 (1:1,000, (Fausther et al., 2012), all diluted in Tris-buffered saline (137 mM NaCl and 20 mM Tris–HCl, pH 7.6) with 0.1 % Tween (TBS-T) and 5 % BSA (fatty acid free), was carried out overnight at 4 ºC. After washing twice with TBS-T, the membranes were incubated with appropriate IgG secondary antibodies conjugated with alkaline phosphatase (Amersham) for 2 h at room temperature. After washing, the membranes were revealed using an ECF kit (Amersham) and visualized with an imaging system (VersaDoc 3000, Bio-Rad) and the densiometric analysis of protein bands was performed using the Quantity One software (Bio-Rad).

cAMP measurement

Striatal synaptosomes were prepared as previously described (Rebola et al., 2003a). Briefly, the synaptosomal fraction was resuspended in KHR with ADA (4 U/mL, Roche) and DPCPX (50 nM, Tocris) and incubated for 10 min at 37 ºC, to eliminate endogenous adenosine and eliminate putative A_1R-mediated effects. The mixture was then incubated with PSB-12404 (50 nM) or PSB-12405 (50 nM) for 10 min at 37 ºC and the cAMP levels were measured as previously described (Chen et al., 2010). Briefly, the reaction was terminated by addition of 5 % ice-cold trichloroacetic acid (Ricca Chemical Company) and centrifuged for 10 min at 600 *g* to pellet the debris after homogenization.

The trichloroacetic acid was extracted from the supernatant with water-saturated ether (Alfa Aesar). The aqueous extract was dried overnight and reconstituted in assay buffer. The samples were acetylated and the levels of cAMP accumulated in synaptosomes were determined using a cAMP Complete ELISA kit (Assay designs) according to the manufacturer's instruction.

Inorganic free phosphate measurement

The activity of ecto-5'-nucleotidase was evaluated by measuring the formation of inorganic phosphate upon addition of AMP to striatal synaptosomes (Chan et al., 1986). Briefly, the synaptosomal fraction, prepared as previously described (Rebola et al., 2003a), was resuspended in KHR and incubated for 5 min at 37 ºC to stabilize, followed by incubation with the tissue-nonspecific alkaline phosphatase inhibitor (TNAB-I, 10 μM; Calbiochem) and/or adenosine 5'-monophosphate (AMP, 1 mM; Acros Organics) for 30 min at 37 ºC. The mixture was then centrifuged at 14 000 *g* at 4 ºC for 12 min and the inorganic free phosphate levels were measured from the supernatant using a Malachite Green Phosphate Assay kit (Cayman Chemical Company) according to the manufacturer's instructions.

Immunohistochemistry

Mice were anesthetized with avertin and brain fixation was performed through transcardiac perfusion with 4 % paraformaldehyde in PBS, postfixation overnight in PBS with 4 % paraformaldehyde and cryopreservation in PBS containing 25 % sucrose. Frozen brains were sectioned (30 μm coronal slices) with a Leica CM3050S cryostat (Leica Microsystems). The sections were first rinsed for 5 min with PBS at room temperature and then permeabilized and blocked with PBS containing 0.2 % Triton X-100 and 5 % donkey serum during 1 h, incubated in the presence of the rabbit anti-

murine CD73 antibody (1:500, Fausther et al., 2012) and mouse anti-A_{2A}R antibody (1:500; Millipore) overnight at room temperature, rinsed three times for 10 min in PBS, and then incubated with donkey anti-mouse and donkey anti-rabbit secondary antibodies conjugated with a fluorophore (Alexa Fluor 488 or Alexa Fluor 555, 1:200, Invitrogen) for 2 h at room temperature. After rinsing three times for 10 min in PBS, the sections were mounted on slides and allowed to dry. Vectashield mounting medium with DAPI (Vector Laboratories) was applied as well as the cover glass. All sections were examined under a fluorescence Nikon eclipse E600 microscope, with SPOT software 4.7 (Diagnostic instruments, Inc.).

Proximity Ligation Assay (PLA)

The PLA was performed as previously described (Trifilieff et al., 2011) in brain sections prepared as described above. The sections were first rinsed in TBS (0.1 M Tris, pH.7.4, and 0.9 % w/v NaCl) at room temperature and then permeabilized and blocked with TBS with 1 % BSA and 0.5 % Triton X-100 for 2 h at room temperature. The slices were incubated with the primary antibodies, namely rabbit anti-murine CD73 (1:300, Fausther et al., 2012) and anti-A_{2A}R (1:300; Millipore) overnight at room temperature. After wash four times (30 min each) in TBS with 0.2 % Triton X-100, the slices were incubated for 2 h at 37 °C with the PLA secondary probes (1:5; Olink Bioscience) under gentle agitation. After washing twice for 5 min with Duolink II Wash Buffer A (Olink Bioscience) with agitation at room temperature, the slices were incubated with the ligation-ligase solution (Olink Bioscience) for 30 min at 37 °C. After washing twice for 2 min with Duolink II Wash Buffer A with agitation at room temperature, the slices were incubated with polymerase (1:40; Olink Bioscience) in the amplification solution (Olink Bioscience) for 100 min at 37 ºC under gentle agitation. After washing in decreasing concentrations (2x, 1x, 0.2x, 0.02x; 10 min each) of SSC buffers (Olink Bioscience),

slices were mounted on slides, allowed to dry and coverslips were placed with Duolink Mounting Medium (Olink Bioscience). Fluorescence images were acquired on an Axiovert 200M inverted confocal microscope (Carl Zeiss Microscopy) using a 40x objective and the PLA puncta signals quantified with the ImageJ software, using a manual threshold to discriminate PLA puncta from background fluorescence. The built in macro "Analyze Particles" was then used to count and characterize all objects in the thresholded image. Objects larger than 5 μm^2 were rejected, thereby effectively removing nuclei. The remaining objects were counted as PLA puncta.

Drug treatments and locomotor activity

The horizontal locomotor activity of mice was assessed in standard polypropylene cages (15 x 25 cm, i.e. clean home cages) and recorded with infrared photobeams (San Diego Instrument). Ambulation was quantified as the number of sequential breaks in adjacent beams in 5 min bins. The mice were habituated to the new room and new cage for at least 2 h. 2 h before the intraperitoneal (i.p.) administration of SCH58261 (3 mg/kg; Tocris), the animals were challenged with vehicle (i.p., 75 % saline, 15 % dimethylsulfoxide, 10 % castor oil). In the experiment with the A_{2A}R agonist *drug* or *prodrug* (see below), the mice were anesthetized with isoflurane and oxygen and stereotaxically injected bilaterally in nucleus accumbens (anterio-posterior = +1 mm from bregma, medio-lateral = ± 0.8 mm from midline, dorso-ventral = 4.4 mm from the skull surface) with 1 µL of 2 mM (2 nmol) per side at rate of 0.2 µL/min. The activity was recorded after the mice recovered from the surgery.

The A_{2A}R agonist PSB-12404 ((*2R,3R,4S,5R*)-2-(6-amino-2-(2-cyclohexylethylthio)-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol) and its phosphate prodrug PSB-12405 ((*2R,3S,4R,5R*)-5-(6-amino-2-(2-cyclohexylethylthio)-9H-

purin-9-yl)-3,4-dihydroxy-tetrahydro-furan-2-yl)methylphosphoric acid triethylammonium salt) were synthesized as previously described (El-Tayeb et al., 2009).

Working memory

When testing the impact of the A_{2A}R and A_1R antagonists, SCH58261 and DPCPX (Tocris) respectively, the drugs or its vehicle solution were intraperitoneally (i.p.) administered to the animals 30 min before they were placed in the maze. We first assessed working memory in a spontaneous alternation paradigm assessed in a *Y-maze*. Individual mice were placed at the end of one arm and allowed to freely explore the maze for 5 min. The sequence of entrance in each arm was recorded and the number of alternations (sequential entrance in the 3 different arms) was quantified. The percentage of spontaneous alternation consists in the percentage of alternations in the total possible alternations (total number of arms' changes minus 2).

We also assessed working memory in a more sensitive test using an *8 radial arm maze (RAM)* as previously described (Singer et al., 2012). To motivate performance in the RAM memory tasks, the animals were maintained on a food deprivation regime, which was gradually introduced with a progressive reduction of the daily available food, until the animals reached a stable weight of not less than 85 % of their *ad libitum* weight, at which time the food provided was stabilized. The RAM had 8 identical and equally spaced arms (56 cm long, 12 cm wide) radiating from a central octagonal platform (side-length = 12 cm). The mice were exposed to the maze for 5 min each day with a food reward at the end of each arm. The habituation was performed until the animals finished the task within 5 min. In the *8 baited arms paradigm*, the 8 arms were set with a food reward and the animals allowed to freely explore the maze until they ate the 8 food rewards. Each time a mouse reentered in an arm where the reward was already ate, a working memory error (WME) was scored.

Statistical analysis

Results are presented as mean ± SEM. Data with one condition and one variable (e.g. genotype) were analyzed with Student's *t* test. Data from more than one condition were analyzed with one-way ANOVA followed by a Tukey's multiple comparison post-hoc test or followed by a Dunnett's multiple comparison post-hoc test (for comparison with specific controls). Data with more than one variable (e.g. genotype and time) and condition were analyzed with a two-way ANOVA followed by Bonferroni post-hoc tests. Unless otherwise indicate the significance level was 95 %.

4. Results

4.1. Co-localization of CD73 and $A_{2A}R$ in the striatum

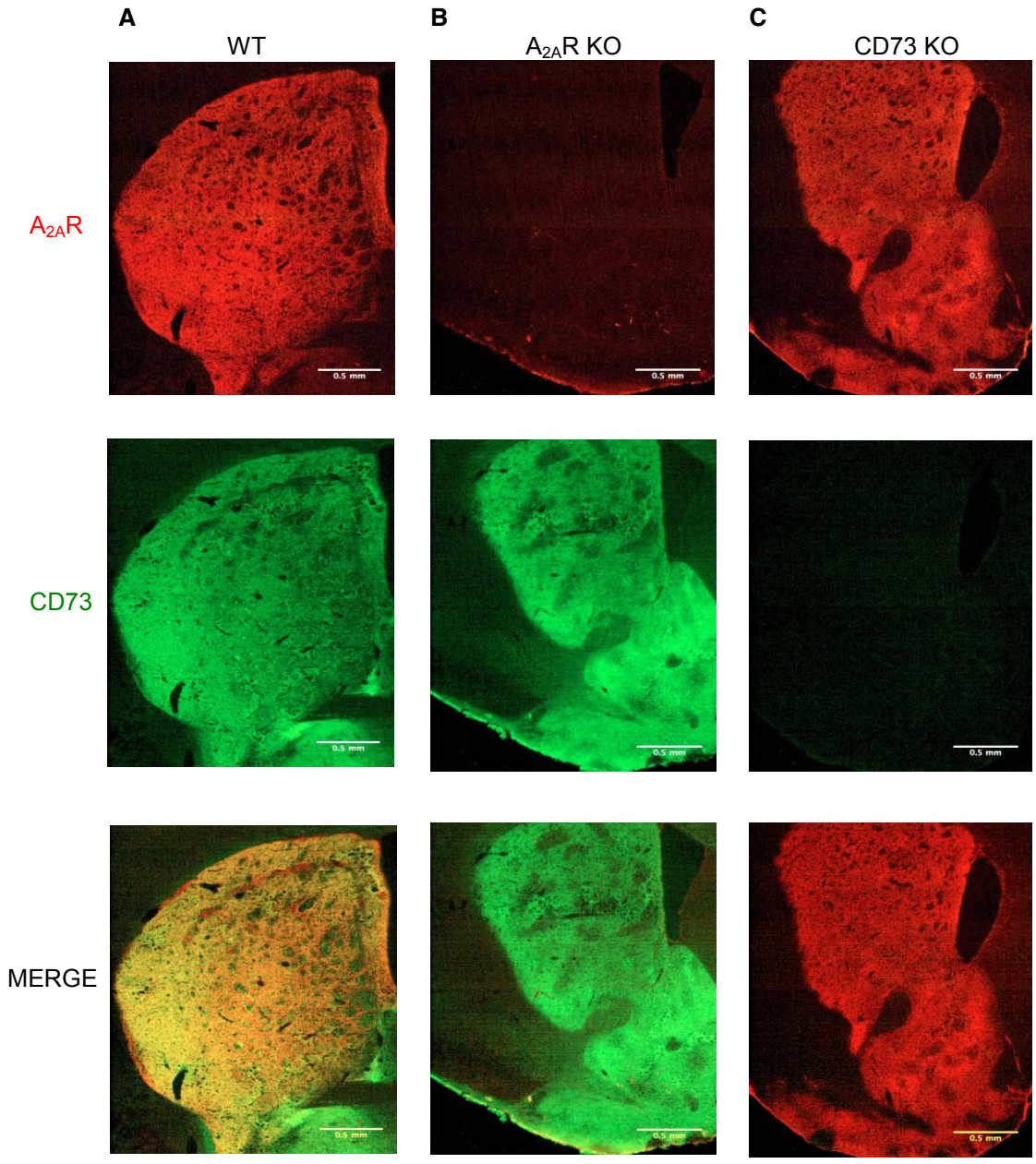

Figure 5.1. Co-localization of CD73 and $A_{2A}R$ in the striatum. Macroscopic immunohistochemical co-localization of CD73 and $A_{2A}R$ in striatal slices (A; n = 5), with no immunoreactivity for CD73 in CD73 KO mice (B; n = 5), and no immunoreactivity for $A_{2A}R$ in $A_{2A}R$ KO mice (C; n = 5).

The observed similar greater abundance of CD73 and $A_{2A}R$ in the striatum together with the proposed functional association between ATP-derived adenosine and the activation of $A_{2A}R$ (Cunha et al., 1996a; Rebola et al., 2008), led us to investigate the association between CD73 and $A_{2A}R$. Double immunohistochemistry analysis showed that CD73 co-localized with $A_{2A}R$ in the striatum (Fig. 5.1A). We confirmed the selectivity of each antibody labeling by showing the absence of the putative $A_{2A}R$ signal in $A_{2A}R$ KO mice (Fig. 5.1B) and the absence of the putative CD73 signal in CD73 KO mice (Fig. 5.1C); furthermore, there does not seem be overt changes of CD73 immunoreactivity in $A_{2A}R$ KO mice (Fig. 5.1B) nor of $A_{2A}R$ signal in CD73 KO mice (Fig. 5.1C).

5

4.2. Physical association of CD73 and $A_{2A}R$ in the striatum

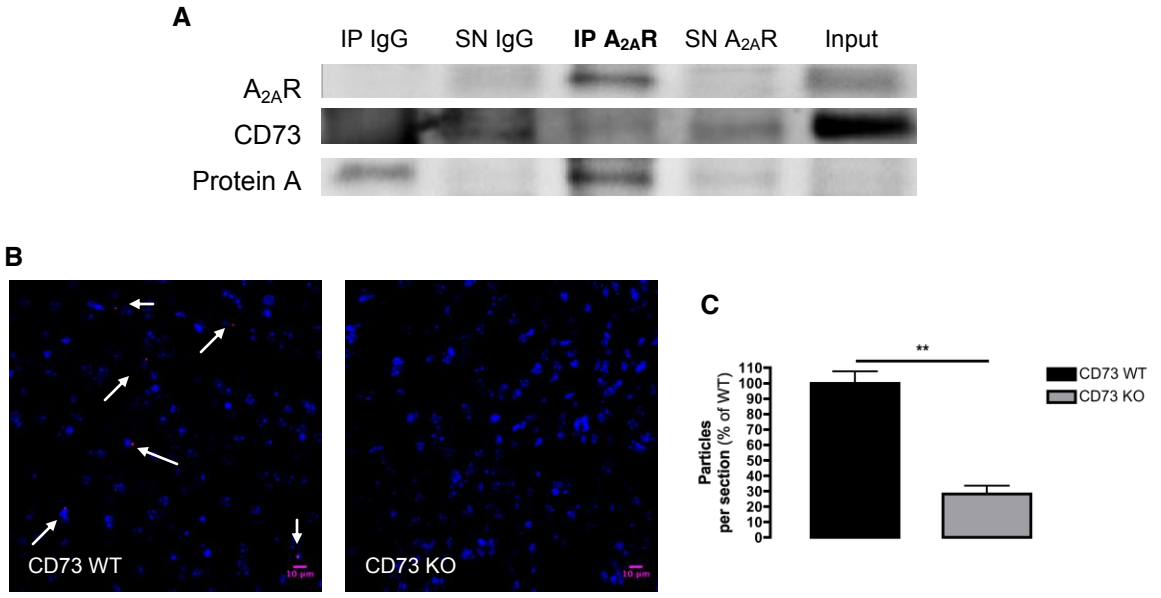

Figure 5.2. Physical association of CD73 and $A_{2A}R$ in the striatum. The pull-down of $A_{2A}R$ from striatal tissue revealed the co-immunoprecipitation of CD73 (A); indeed, the pull-down of $A_{2A}R$ (IP $A_{2A}R$) revealed the presence of $A_{2A}R$ (top strip of blots), CD73 (middle strip of blots) and protein A (bottom strip of blots), which was also present in the pull-down of IgG (IP IgG), however this condition (IP IgG) does not show immunoreactivity for $A_{2A}R$ (top strip of blots) or CD73 (middle strip of blots). The input represent 5% of the sample before the pull-down, SN $A_{2A}R$ and SN IgG represents 20% of the supernatant of $A_{2A}R$ and IgG's pull down, respectively (results representative of n = 3). The intimate proximity between $A_{2A}R$ and CD73 was further shown by the signal

recorded in a proximity ligation assay (PLA) in sections of the striatum from WT mice but not from CD73 KO mice (B and C). Panel B shows representative PLA images of striatal sections from WT mice and CD73 KO mice (representative from n = 3), showing PLA positive signals (red) in the WT. Panel C displays the quantification of the PLA experiments, with the WT having 100.0 ± 7.8 % of positive signals per slice and the CD73 KO having significant less PLA signal (28.3 ± 5.3 %). The data are mean ± SEM; $^{**}p < 0.001$ using a Student's *t* test.

The physical interaction between A_{2A}R and CD73 was prompted by the observation that the pull-down of striatal A_{2A}R revealed a co-immunoprecipitation with CD73 (Fig. 5.2A). To consolidate this suggested association between CD73 and A_{2A}R in the striatum, we used a PLA approach that showed a selective physical proximity (≤ 16 nm) between A_{2A}R and CD73 in WT but not CD73 KO mice (Fig. 5.2B, C).

4.3. CD73-derived adenosine is required for striatal A_{2A}R activation

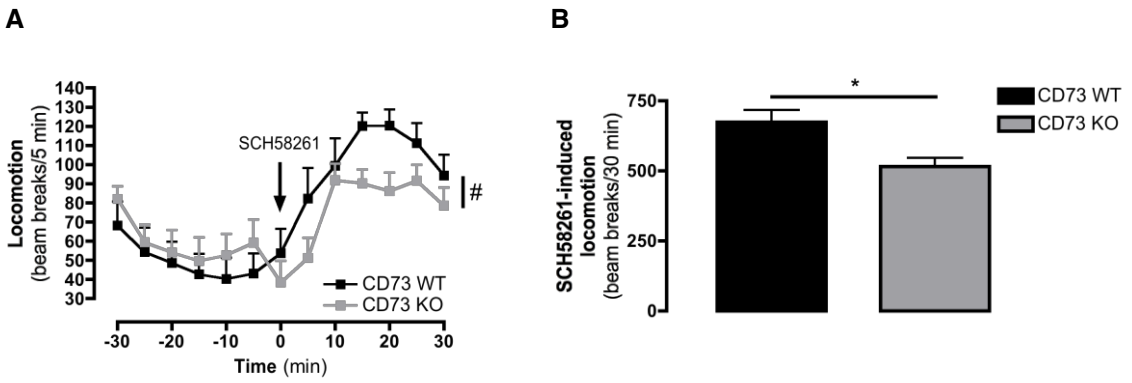

Figure 5.3. CD73 activity provides the adenosine that produce hypolocomotion. Acute administration of the A_{2A}R antagonist SCH58261 (3 mg/kg, i.p.) induced hyperlocomotion (A, B). Panel A represents the mean ± SEM of beam breaks per 5 min, whereas panel B represents the mean ± SEM of total number of beam breaks in 30 min after SCH58261 administration in WT and CD73 KO mice (n = 10-11). The data are mean ± SEM. $^{\#}p < 0.05$ using a two-way ANOVA test followed by a Bonferroni post-hoc test in A; $^{*}p < 0.05$ using a Student's *t* test in B.

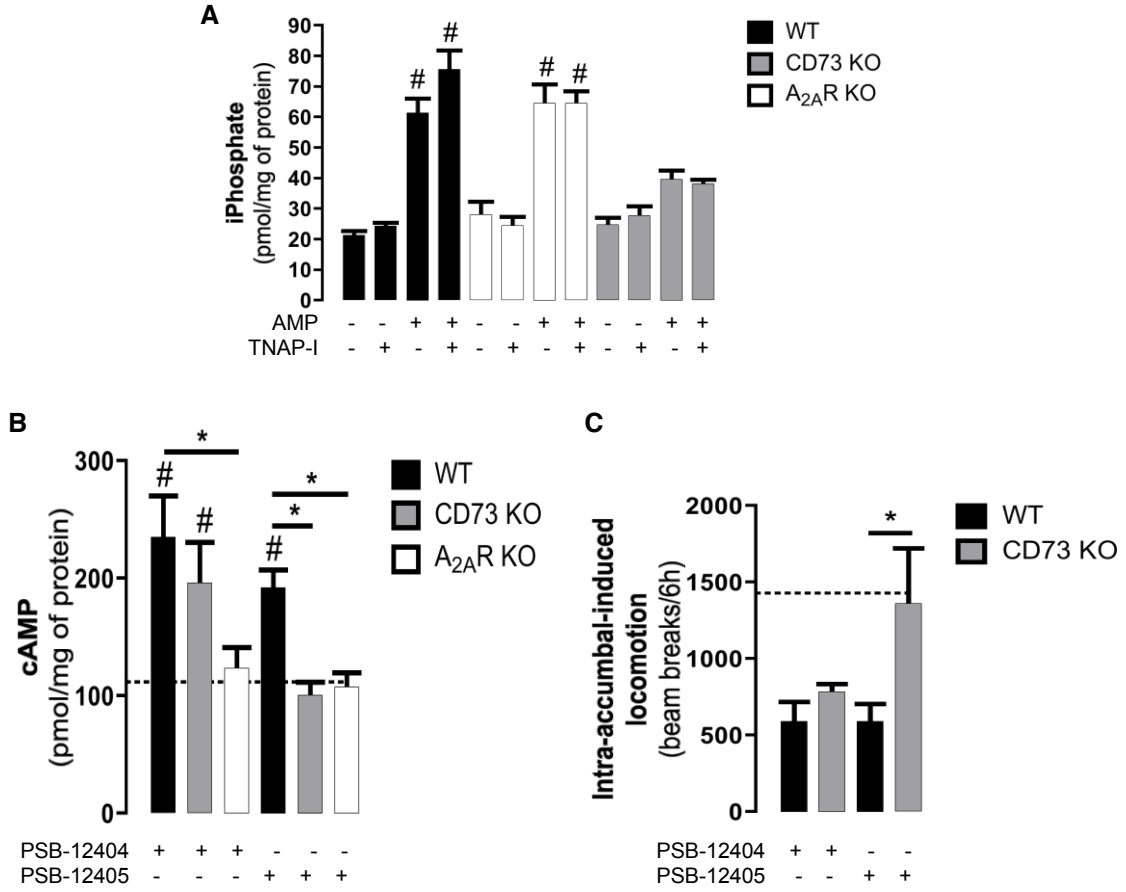

Figure 5.4. CD73 activity provides the adenosine activating striatal A_{2A}R. Panel A shows that striatal synaptosomes from both WT and A_{2A}R KO mice can dephosphorylate AMP (1 mM), whereas the striatal synaptosomes from CD73 KO mice cannot (n = 4-7); furthermore, the inhibitor of tissue non-specific alkaline phosphatases (TNAP-I, 10 μM) failed to affect the extracellular catabolism of AMP. Panel B shows that both PSB-12405 (50 nM, a prodrug that requires CD73 activity to become an A_{2A}R agonist) and PSB-12404 (50 nM, an A_{2A}R agonist) enhanced cAMP levels in comparison to control (111.7 ± 8.3 pmol/mg of protein, n = 5, represented by the horizontal dash line) in striatal synaptosomes from WT mice, whereas only the drug but not prodrug enhanced cAMP in striatal synaptosomes from CD73 KO mice and neither PSB-12404 nor PSB-12405 were effective in A_{2A}R KO mice (n = 4-6). Panel C shows that the bilateral intra-accumbal administration of the pro-drug (PSB-12405, 2 nmol) induced hypolocomotion in WT when compared with CD73 KO mice, whereas the drug (PSB-12404, 2 nmol) induced hypolocomotion in both groups (n = 5-6). The horizontal dashed line indicates the spontaneous locomotion in control mice (1427.9 ± 107.1 bean breaks/6h, n = 17). The data are mean ± SEM. *p < 0.05 between genotypes (as indicated by the upper bars) using a Student's t test in C or using a one-way ANOVA test followed by a Dunnett's multiple comparison test to WT as control in B; # indicates differences from control, *i.e.* no added drugs, with p < 0.001 using a one-way ANOVA test followed by a Tukey's multiple comparison test in A; and p < 0.05 using a one-way ANOVA test followed by a Dunnett's multiple comparison test to control in B.

Since it is well documented that A_{2A}R antagonists trigger a hyperlocomotion through the blockade of striatopallidal A_{2A}R (Shen et al., 2008), we now tested if CD73 would be responsible for the formation of the adenosine tonically activating this population of A_{2A}R. In accordance with this hypothesis, we report that the hyperlocomotion triggered by the selective A_{2A}R antagonist SCH58261 (3 mg/kg, i.p.) had significantly ($p < 0.01$) lower amplitude in CD73 KO mice when compared with their WT littermates (Fig. 5.3A,B). This is probably due to the lack of AMP-derived adenosine to tonically activate striatal A_{2A}R since we now report that striatal synaptosomes from WT and A_{2A}R KO mice were able to dephosphorylate AMP, as gauged from the formation of inorganic phosphate, whereas this did not occur in striatal synaptosomes from CD73 KO mice (Fig. 5.4A). This extends to striatal preparations the conclusion reached in other brain preparations that CD73 is the predominant activity responsible for the formation of adenosine from extracellular AMP, as further confirmed by the lack of impact of the alkaline phosphatase inhibitor TNAP-I (10 μM) on the extracellular catabolism of AMP in striatal synaptosomes from either WT or CD73 KO mice (Fig. 5.4A).

To re-enforce the direct relation between CD73 and A_{2A}R in the striatum, we took advantage of a novel A_{2A}R pro-agonist (PSB-12405), which needs to be dephosphorylated by CD73 to generate the active form of the A_{2A}R agonist, PSB-12404 (El-Tayeb et al., 2009; Flögel et al., 2012). We first confirmed in striatal synaptosomes that the pro-agonist indeed activated A_{2A}R in a CD73-dependent manner, by comparing the ability of the drug and prodrug to enhance cAMP levels, an established measure of A_{2A}R activity in the striatum (e.g. Svenningsson et al., 1998; Corvol et al., 2001). We found that the prodrug increased cAMP levels in striatal synaptosomes from WT, but neither from CD73 KO nor from A_{2A}R KO mice (Fig. 5.4B), whereas the A_{2A}R agonist (PSB-12404) increased cAMP levels in WT and CD73 KO, but not in A_{2A}R KO mice (Fig.

5.4B). This shows that PSB-12405 requires CD73 activity to activate striatal A_{2A}R, which allows using this pro-agonist to test if CD73 is responsible for generating the adenosine that specifically controls the impact of A_{2A}R on striatal-related behavioral responses.

In agreement with the previously reported hypolocomotor effect of A_{2A}R agonists directly injected in the nucleus accumbens (Hauber and Münkle, 1997; Nagel et al., 2003), the bilateral intra-accumbal injection of PSB-12405 reduced locomotion in WT mice to an extent greater that in the CD73 KO mice (Fig. 5.4C). Instead, when PSB-12404 was injected no differences were found between the two genotypes (Fig. 5.4C).

4.4. Improved working memory in CD73 KO mice is mimicked by genetic and pharmacological inactivation of A_{2A}R

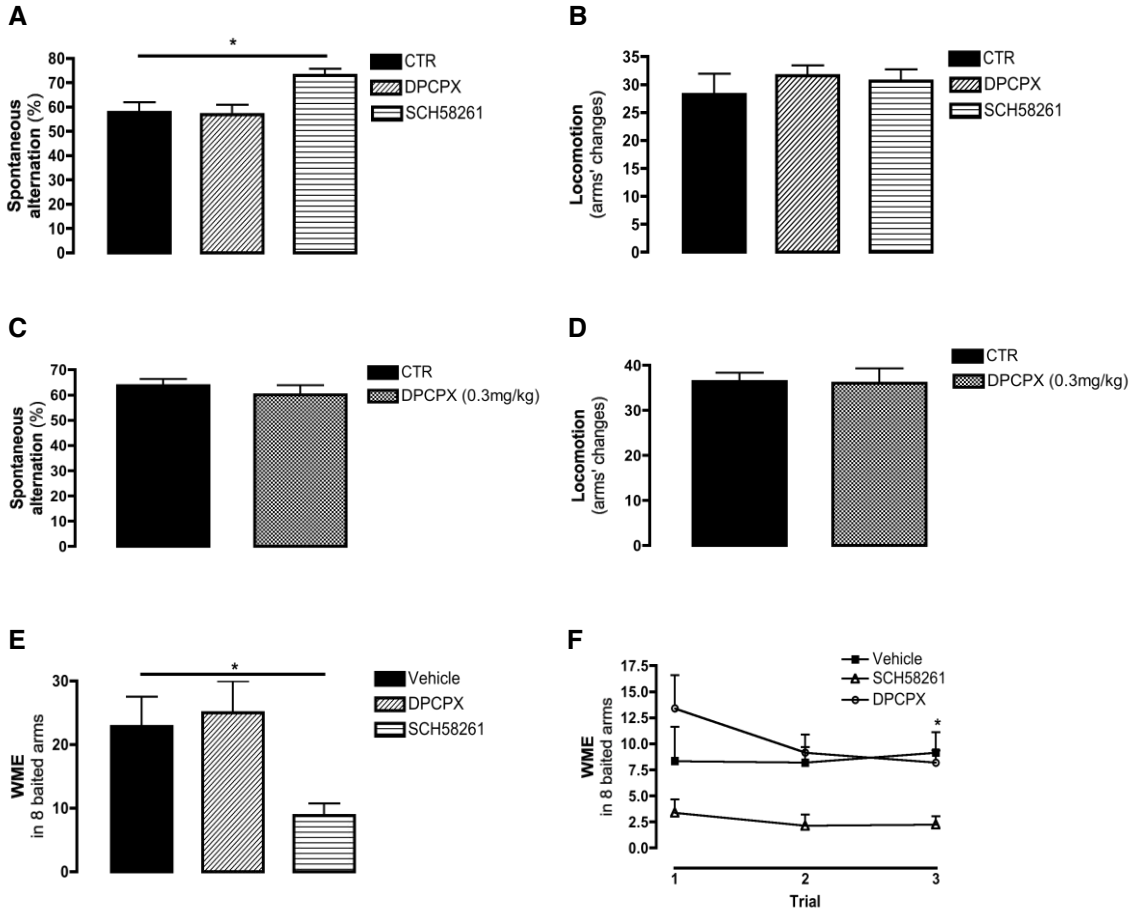

Figure 5.5. Improved working memory in CD73 KO mice is rescued by the acute blockade of A_{2A}R, but not A_1R. Mice acutely treated with a selective A_{2A}R antagonist (SCH58261, 0.03 mg/kg, i.p.) had an improved working memory when tested in a Y-maze paradigm, analyzing their spontaneous alternation in comparison with the control group (vehicle, i.p.), with no differences in the mice treated with a selective A_1R antagonist (DPCPX, 0.03 mg/kg, i.p.) (A), and no differences in locomotion (B), evaluated by number of arms' changes (n = 8). In a separate group of mice acutely treated with a higher dose of DPCPX (0.3 mg/kg, i.p.) no differences were observed when tested in the Y-maze paradigm (C and D, n = 8). In the 8 radial arm maze with 8 baited arms (E, F) SCH58261-treated mice displayed significantly less working memory errors in comparison with the control group, with no differences in the mice treated with a selective A_1R antagonist (n = 8). Data are mean ± SEM; **p* < 0.05 using either a Student's *t* test (C and D), a one-way ANOVA test followed by a Dunnett's multiple comparison test to control (A, B and E) or a two-way ANOVA followed by Bonferroni post-hoc test (F). A Student's *t* test was used in C and D.

It was previously shown that A_{2A}R control working memory performance: indeed it is deficient in A_{2A}R over-expressing mice (Giménez-Llort et al., 2007) and improved in global A_{2A}R KO as well as forebrain A_{2A}R KO and striatal A_{2A}R KO mice (Zhou et al., 2009; Wei et al., 2011). We previously reported that CD73 KO mice displayed an improved working memory compared to WT mice, without changes in their locomotion (see chapter 3). In order to discount developmental changes in the KO lines, we tested the effect of an acute blockade of A_{2A}R in two different paradigms of working memory, using a selective A_{2A}R antagonist (SCH58261, 0.03 mg/kg, i.p.; the same dose that was able to blunt amphetamine sensitization without changing basal locomotion (Bastia et al., 2005)). In order to discard the involvement of the other main adenosine receptors in the phenotype, we also tested the effect of an acute blockade of A_1R using a selective A_1R antagonist (DPCPX, 0.03 mg/kg, i.p.) in the same paradigms. The mice that received SCH58261 30 min prior to testing displayed an improved working memory compared to the mice that received either vehicle (control) or DPCPX, when tested in the spontaneous alternation paradigm in the Y-maze test (Fig. 5.5A), without changes in their locomotion (Fig. 5.5B). A similar result was obtained with a higher dose of DPCPX (0.3 mg/kg, i.p.; Fig. 5.5C-D). In addition, mice treated with SCH58261 made less

working memory errors than either control or DPCPX-treated mice in the 8-baited arms (Fig. 5.5E and 5.5F) version of the 8 radial arm maze. This phenotype resulting from the pharmacological blockade of A_{2A}R and not A_1R is super-imposable to that of A_{2A}R KO mice and also parallels that of CD73 KO mice, further strengthening our contention that CD73 is responsible for the formation of the adenosine that tonically activates striatal A_{2A}R.

5. Discussion

We here showed that the activity of CD73, the major enzyme dephosphorylating AMP to adenosine in the central nervous system, and therefore responsible for the last enzymatic step for the formation of extracellular ATP-derived adenosine, has a crucial role in the activation of striatal A_{2A}R. The intrinsic relation between CD73 and A_{2A}R is supported by their anatomical localization and physical proximity in the striatum. The co-localization of CD73 and A_{2A}R in the striatum is demonstrated by their similar distribution patterns in the basal ganglia (Fig. 5.1), as well as, by the postsynaptic enrichment of these two molecules (see chapter 2) and by their physical proximity documented by co-immunoprecipation and proximity ligation assays (Fig. 5.2). After showing that the deletion of CD73 abolished the extracellular dephosphorylation of AMP (Fig. 5.4), the functional association between CD73 activity and the activation of striatal A_{2A}R was validated by the abolishment of *ex vivo* (i.e. cAMP formation) as well as *in vivo* effects (hypolocomotor) of a novel prodrug for A_{2A}R agonism (El-Tayeb et al., 2009; Flögel et al., 2012) either in CD73 KO or in A_{2A}R KO mice (Fig. 5.4).

The functional association between CD73 activity and the activation of striatal A_{2A}R was further confirmed *in vivo,* in three major behavioral responses that have previously been shown to involve A_{2A}R activation, i.e. hypolocomotion (Fig. 5.3), decreased working memory (see chapter 3) and behavioral sensitization to psychoactive drugs (see chapter

4). Thus, we here showed that CD73 KO mice display a reduced hyperlocomotor response to a supra-maximal dose of a selective A_{2A}R antagonist (SCH58261), which indicates that CD73 KO mice have less adenosine that is selectively activating striatal A_{2A}R responsible for the hyperlocomotor effect (Yu et al., 2008). We are not suggesting that CD73 KO mice have in general lower levels of adenosine since they display a normal A_1R-mediated control of synaptic transmission (Zhang et al., 2012); instead these data indicate that CD73 KO mice have lower levels of adenosine near CD73-A_{2A}R complexes. It was also shown that inactivation of A_{2A}R (Zhou et al., 2009), namely striatal A_{2A}R (Wei et al., 2011), enhances working memory performance, a similar phenotype as now observed after A_{2A}R antagonist administration, as well as in CD73 KO mice (see chapter 3), but not after A_1R antagonist administration. All together, the parallel modifications of the behavioral responses by eliminating CD73 or A_{2A}R but not A_1R (Giménez-Llort et al., 2002; 2005), as well as upon acute A_{2A}R, but not A_1R blockade, prompt the conclusion that CD73 is responsible for the formation of the adenosine that activates A_{2A}R in the striatum.

This proposed activation of A_{2A}R selectively by CD73-mediated formation of adenosine seems to be a general feature of A_{2A}R not only in the striatum, but also in other tissues and cell types. Indeed, it was shown that the inhibition of CD73 selectively blunts the ability of A_{2A}R to control synaptic plasticity in hippocampal synapses (Rebola et al., 2008) or synaptic adaptation at the neuromuscular junction (Correia-de-Sá et al., 1996; Cunha et al., 1996a), as well as the control of glutamate-induced toxicity in cultured granular cells (Boeck et al., 2007). Furthermore, the control by A_{2A}R of the vascular tone (Koszalka et al., 2004; Zernecke et al., 2006) and of the immune-inflammatory system has also been shown to strictly depend on the activity of CD73 (Deaglio et al., 2007; Peng et al., 2008; Flögel et al., 2012). The tight association between CD73 and A_{2A}R is further heralded by the observation that several conditions

trigger a coordinated induction or repression of CD73 and A_{2A}R expression (Napieralski et al., 2003; Deaglio et al., 2007), strongly supporting the view that these two molecules are tightly interconnected.

Notably, there seems to be a selective association of CD73-mediated formation of adenosine with the activation of facilitatory A_{2A}R rather than with the more abundant inhibitory A_1R in the nervous system (Fredholm et al., 2005a). Indeed, several groups concluded that the inhibition or genetic deletion of CD73 failed to affect the modulation of synaptic transmission by A_1R either in physiological or pathological conditions (Brundege and Dunwiddie, 1996; Cunha et al., 1996a; Lloyd et al., 1993; Lovatt et al., 2012; Zhang et al., 2012), in contrast to the conclusions derived from a transgenic mouse with hampered release of gliotransmitters (Pascual et al., 2005). This dissociation between CD73 activity and A_1R activation is further supported by the different localization of CD73 and A_1R throughout the brain (Lee et al., 1986; Fastbom et al., 1987). This is in general agreement with the idea that the activation of A_1R results from the activity-dependent metabolic control of adenosine kinase (Diógenes et al., 2012) producing a direct outflow of adenosine as such (Lloyd et al., 1993; Brundege and Dunwiddie, 1998; Frenguelli et al., 2003). However, it cannot be excluded that ATP-derived adenosine might also activate A_1R in particular systems, such as in the control of tubuloglomerular feedback (Thomson et al., 2000) or of nociception, which requires the participation of alkaline phosphatase (Zylka et al., 2008; Sowa et al., 2010), which we now ruled out to contribute for the extracellular catabolism of AMP in striatal synapses.

This selective activation of A_{2A}R by CD73-mediated adenosine formation provides direct support to the previous proposal to understand the differential activation of inhibitory A_1R and facilitatory A_{2A}R according to the functional needs of neuronal circuits (Cunha, 2008a). Thus, it is proposed that the activation of synaptic A_{2A}R (Rebola et al., 2005a) is designed for local adaptive functional changes that are driven by activity-

dependent experience (Cunha, 2008a); therefore, the source of the adenosine designed to activate A_{2A}R should be locally produced, solely within the recruited synapses. The presently observed localization of CD73 within synapses (see also Cunha et al., 2000b), mainly at the postsynaptic density, contributes for this main aim of converting the activity-dependent ATP release from synapses (Cunha et al., 1996b; Pankratov et al., 2006; Wierazko et al., 1989) into the adenosine responsible for the local activation of A_{2A}R. In addition to astrocytic release of ATP (Halassa, et al. 2009; Schmitt et al., 2012), the localization of the newly identified vesicular nucleotide transport (VNUT) within synapses, namely at the nerve terminal (Larsson et al., 2012), heralds our proposal of a local synaptic release of ATP as the possible source of neuronal CD73-mediated adenosine signaling acting through A_{2A}R. The present study only focused on the relation between adenosine formation and A_{2A}R activation; it remains to be explored if the clearance of adenosine by the large family of nucleoside transporters (Parkinson et al., 2011), which activity is controlled by A_{2A}R in synapses (Duarte-Pinto et al., 2005), might also play a role in restraining CD73-generated adenosine for the activation of A_{2A}R, as recently proposed (Nam et al., 2013).

In addition to the association between CD73-mediated formation of ATP-derived adenosine and the activation of A_{2A}R observed under near-physiological conditions it was also observed in pathological brain conditions (see Chapter 4). Indeed, A_{2A}R blockade is established to afford a robust neuroprotection in animal models of brain diseases ranging from Alzheimer's or Parkinson's diseases to epilepsy or ischemia (Chen et al., 1999; 2001b; Canas et al., 2009b; El Yacoubi et al., 2008). However, despite the extensive characterization of the role of A_{2A}R, its source of adenosine has been unclear. Remarkably, noxious brain conditions trigger an enhancement of the extracellular levels of ATP (di Virgilio, 2000). Since we confirmed that the extracellular conversion of AMP into adenosine seems to be wiped out in CD73 KO mice (Klyuch et

al., 2012; Lovatt et al., 2012; Zhang et al., 2012), with no compensation of alternative enzymatic activities such as alkaline phosphatase (Langer et al., 2008), it is tempting to consider the possibility that the manipulation of CD73 might afford a benefit similar to that observed for A_{2A}R blockade (Cunha, 2005; Chen et al., 2007; see Chapter 4). This might eventually provide a functional role for the localization of CD73 in astrocytic membranes (Kreutzberg et al., 1978), now also confirmed to be present in gliosomes (see Chapter 2), which joins the proposed role of glial A_{2A}R in neurodegeneration (Yu et al., 2008; Matos et al., 2012b).

In summary, the present study provides the first molecular and behavioral demonstration that CD73 activity is responsible for the formation of the adenosine that activates striatal A_{2A}R. Therefore, our work points CD73 as a new target that can fine-tune A_{2A}R activity, paving the way to consider CD73 as a potentially alternative target to A_{2A}R to manipulate activity-dependent synaptic adaptation and neurodegeneration.

6. Acknowledgements

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CHAPTER 6

THE ROLE OF HIPPOCAMPAL CD73 AND $A_{2A}R$ IN EPILEPSY

1. Abstract

Adenosine is widely recognized as an endogenous anticonvulsant via activation of adenosine A_1 receptors (A_1R). However, the clarification of the molecular source of adenosine and the relevance of other adenosine receptors, like A_{2A}R, in the progression of this pathology are yet debatable. In addition, recent observations suggest a differential function of neuronal and astrocytic A_{2A}R activity, particularly in the modulation of glutamatergic transmission. We here show that CD73, the enzyme that generates adenosine from extracellular AMP, does not participate on A_1R but instead on A_{2A}R activation in a kainic acid-induced of mesial temporal lobe epilepsy. Mice with a genetic deletion of CD73 exhibited a typical convulsive profile, albeit with a decreased hippocampal neuronal loss. In addition, mice with selective deletion on either neuronal or astrocytic A_{2A}R, displayed opposite phenotypes, with an anticonvulsive and neuroprotective profile for neuronal A_{2A}R knockout (KO) mice and a proconvulsive and neurotoxic profile in astrocytic A_{2A}R KO mice. Notably, our data also suggest that a dysfunctional glutamate uptake caused by the selective deletion of A_{2A}R in astrocytes is at the basis of the different phenotypic patterns and imply a differential and delicate coordinated action between astrocytic A_{2A}R and neuronal A_{2A}R to fine-tune glutamatergic transmission that is apparently disrupted in epilepsy. In conclusion, CD73 does not generate the anticonvulsive adenosine and neuronal and astrocytic A_{2A}R have crucial, but opposite roles in epilepsy.

2. Introduction

Epilepsy is a common group of neurological disorders characterized by the periodic and unpredictable occurrence of seizures, affecting 1-2% of the population (McNamara, 1999). Despite decades of research, about 20-40% of patients continue to have frequent seizures during treatment (French, 2007). This is particularly true for

mesial temporal lobe epilepsy (MTLE), which is the most common form of medical intractable refractory epilepsy (Wiebe, 2000) and consequently, with an urgent requirement of new therapies. Human MTLE is characterized by seizures with origin within the mesial aspects of the temporal regions, particularly in the hippocampus (Engel, 2001), being associated with hippocampal sclerosis (Babb et al., 1984), i.e. pyramidal cell loss in the hippocampus, reactive gliosis, granule cells dispersion in the dentate gyrus and mossy fibre reorganization (Babb et al., 1984; 1991; Mathern et al., 1995). A similar histological pattern was described in an adult mice model after injection of kainate (KA) into the dorsal hippocampus (Suzuki et al., 1995; Bouilleret et al., 1999).

Notably, adenosine has been described as the brain's endogenous anticonvulsant (Dragunow, 1986). Local levels of adenosine increase during seizure activity, supposedly as a negative feedback mechanism to terminate seizures (During and Spencer, 1992), through activation of adenosine A_1 receptors (A_1R) (Wiesner et al., 1999), which provide a global inhibitory tone on glutamate release (Fastbom and Fredholm, 1985) and induce neuronal hyperpolarization (Fredholm et al., 2005b). Therefore, A_1R agonists are effective in various animal seizure models, including limbic seizures (Gouder et al., 2003), however, the side effect profile of these agonists is discouraging (Dunwiddie and Worth, 1982; Malhotra and Gupta, 1997). An alternative strategy followed by different groups through the years is to enhance adenosine, which can theoretically be reached by preventing the uptake and/or the metabolism of adenosine (Foster et al., 1994). The extracellular levels of brain's adenosine depend on different intermediary steps that generically comprise: i) ATP release, ii) rapid degradation of ATP into adenosine through NTPDases and ecto-5'-nucleotidase (CD73), iii) reuptake of adenosine through equilibrative nucleoside transporters and iv) phosphorylation by adenosine kinase (ADK) or v) deamination by adenosine deaminase (Boison, 2008a). Overall, inhibitors of adenosine deaminase or adenosine transport have

demonstrated limited effectiveness in regulating extracellular adenosine levels (Wiesner et al., 1999). On the other hand, although ADK inhibitors exert substantial higher regional selectivity by altering the site- and event-specific surge of adenosine, they are still restricted by some side effects (Wiesner et al., 1999). In addition, despite the general evidences supporting a beneficial role for adenosine-enhancing strategies in epilepsy, the involvement of CD73 and other adenosine receptors, like A_{2A}R, in the progression of this pathology is yet to be defined (Jones et al., 1998a; Yacoubi et al., 2001).

We recently showed that A_{2A}R activation is CD73-dependent (Augusto et al., 2013), and the modifications induced by seizures in the purinergic system, with increased density of A_{2A}R (Rebola et al., 2005c) and activity of CD73 (Schoen et al., 1999; Bonan et al., 2000) on one hand, and decreased density of A_1R (Glass et al., 1996; Ochiishi et al., 1999; Ekonomou et al., 2000; Rebola et al., 2003c) and increase of ADK levels (Gouder et al., 2004) on the other, all reinforce the hypothesis that ATP-derived adenosine leads to a preferential activation of facilitatory A_{2A}R rather than inhibitory A_1R (Cunha et al., 1996a). In addition, A_{2A}R are able to modulate both neuronal glutamate release (Popoli et al., 1995) as well as astrocytic glutamate uptake (Matos et al., 2012b), linking this adenosinergic mediator to the glutamatergic network dysfunctions characteristic of epilepsy (Eid et al., 2008).

In order to define and characterize the involvement of the CD73 mediated formation of adenosine and the activation of different pools of A_{2A}R in epilepsy, we here compared mice models with selective cellular deletions of A_{2A}R (neuronal or astrocytic) and mice deficient in CD73 on a mice model of MTLE. The genetic deletion of CD73 does not change the convulsive profile; however it decreased neuronal loss in the CA3 hippocampal region. Notably, mice with a neuronal-selective A_{2A}R deletion (Fb A_{2A}R KO) exhibited a low mortality rate, in addition to lower neuronal death and astrogliosis. By

contrast, when A_{2A}R deletion was restricted to astrocytes (Gfa2 A_{2A}R KO), the mice showed an abnormally high mortality rate and higher convulsion profile, together with exacerbated neuronal death and astrogliosis. In addition, our results are suggestive of an association between increased astrocyte glutamate transporter densities with neuronal death. This report of the participation A_{2A}R on convulsions-induced brain damage and death, suggest that the complete elucidation of the cellular and molecular mechanisms of A_{2A}R-modulation of seizure activity may lead to novel antiepileptic drug therapies.

3. Materials and methods

Animals

Approval from the Institutional Animal Care and Use Committee at Boston University School of Medicine was granted for all experiments conducted. They adhered to the NIH Guide for the Care and Use of Laboratory Animals. The knockout (KO) mice used, with different genetic background, were previously characterized, namely: i) global CD73 KO (CD73 KO) with C57Bl/6J genetic background for both CD73 KO and CD73 wild-type (WT) were generated as previously detailed (Thompson et al., 2004); global A_{2A}R KO mice (A_{2A}R KO) with C57Bl/6J genetic background for both A_{2A}R KO and A_{2A}R WT were generated as previously detailed (Chen et al., 1999); neuronal (i.e., CaMKIIα)-specific A_{2A}R KO (Fb A_{2A}R KO), which are CaMKIIα-Cre[+]$A_{2A}R^{flox/flox}$, with C57Bl/6J genetic background for both Fb A_{2A}R KO and Fb A_{2A}R WT, which are CaMKIIα-Cre[-] $A_{2A}R^{flox/flox}$ were generated as previously detailed (Bastia et al., 2005); the astrocytic-specific A_{2A}R KO (Gfa2 A_{2A}R KO), which are Gfa2-Cre[+]$A_{2A}R^{flox/flox}$, with C57Bl/6J genetic background for both Gfa2 A_{2A}R KO and Gfa2 A_{2A}R WT, which are Gfa2-Cre[-] $A_{2A}R^{flox/flox}$ were generated as previously detailed (Matos et al., 2012b). Experiments

were conducted on animals housed in cages in 12 hours light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) with food and water provided ad libitum. In all experiments males and females adult (3-6 months old) mice were used.

Mouse model of temporal lobe epilepsy (TLE)

Under general anesthesia using vaporized isoflurane supplemented with oxygen, the mice were stereotactically injected as previous described (Gouder et al., 2004) with 50 nl of a 20 mM solution of kainic acid (KA) in 0.9 % NaCl (i.e., 1 nmol of KA) into the right dorsal hippocampus [coordinates with bregma as reference: anteroposterior (AP) -2.0, mediolateral (ML) -1.5, dorsoventral (DV) -1.8 mm] using a stainless steel cannula connected to a microsyringe (Hamilton). Each injection was performed over a period of 1 min. At the end of the injection, the cannula was left in place for an additional period of 8-10 min to limit reflux along the cannula track.

Convulsions evaluation

After the injections the animals were placed in individual cages kept at room temperature and monitored continuously for 3 h to score the kainate-induced convulsions according to a previously established six-point seizure scale (Schauwecker and Steward, 1997) adapted from a five-point scale for rats (Racine, 1972): stage 1: immobility; stage 2: forelimb and/or tail extension, rigid posture; stage 3: repetitive movements, head bobbing; stage 4: rearing and falling; stage 5: continuous rearing and falling; stage 6: severe tonic-clonic seizures.

Cresyl violet staining of Nissl bodies

7 days after KA injection the mice were anesthetized with avertin and brain fixation was performed through transcardiac perfusion with 4 % paraformaldehyde in PBS,

postfixation overnight in PBS with 4 % paraformaldehyde and cryopreservation in PBS containing 25 % sucrose. Frozen brains were sectioned (30 μm coronal slices) with a Leica CM3050S cryostat (Leica Microsystems). Four of every sixth hippocampal sections (i.e., four sections separated by 150 μm from each other) were mounted on slides, allowed to dry at room temperature and stained with cresyl violet to determine neuronal cell loss. Briefly, sections were rehydrated through 100 % and 95 % alcohol for 1 min each, and then to distilled water. Sections were incubated for 10 min in pre-warmed (40 ºC) cresyl violet solution (IHCWorld) and then rinsed quickly in distilled water, follow by dehydration in 95 % alcohol for 2 min, twice in 100 % alcohol for 5 min each, cleared twice with xylene for 5 min each, and mounted with DPX mounting medium (Sigma). All sections were examined under a fluorescence Nikon eclipse E600 microscope, with SPOT software 4.7 (Diagnostic instruments, Inc.). The evaluation of damage was performed twice by two independent subjects, as previously described (Cho et al., 2006), using the following bias: 0, no observable damage; +1, < 10 %; +2, 11-25 %; +3, 26-50 %; +4, 51-75 %; +5, 76-90 %; +6 > 91 % of observable cell loss .

Immunohistochemistry

Brain sections were obtained as previously described (see Chapter 2). Four of every sixth hippocampal sections (i.e., four sections separated by 150 μm from each other) were selected for independent stainings. The sections were first rinsed for 5 min with PBS at room temperature and then permeabilized and blocked with PBS containing 0.2 % Triton X-100 and 5 % donkey serum during 1 h, incubated in the presence of the mouse anti-GFAP-Cy3 antibody (1:200; Sigma) or mouse anti-glutamate transporters 1 (GLT-I)/ excitatory amino acid transporters type 2 (EAAT2; 1:300; Millipore) overnight at room temperature, rinsed three times for 10 min in PBS, and then incubated with donkey

anti-mouse secondary antibodies conjugated with a fluorophore (Alexa Fluor 555, 1:200, Invitrogen) for 2 h at room temperature. After rinsing three times for 10 min in PBS, the sections were mounted on slides and allowed to dry. Vectashield mounting medium with DAPI (Vector Laboratories) was applied as well as the cover glass. All sections were examined under a fluorescence Nikon eclipse E600 microscope, with SPOT software 4.7 (Diagnostic instruments, Inc.).

4. Results

4.1. CD73-dependent adenosine is not anticonvulsive but confers neuroprotection

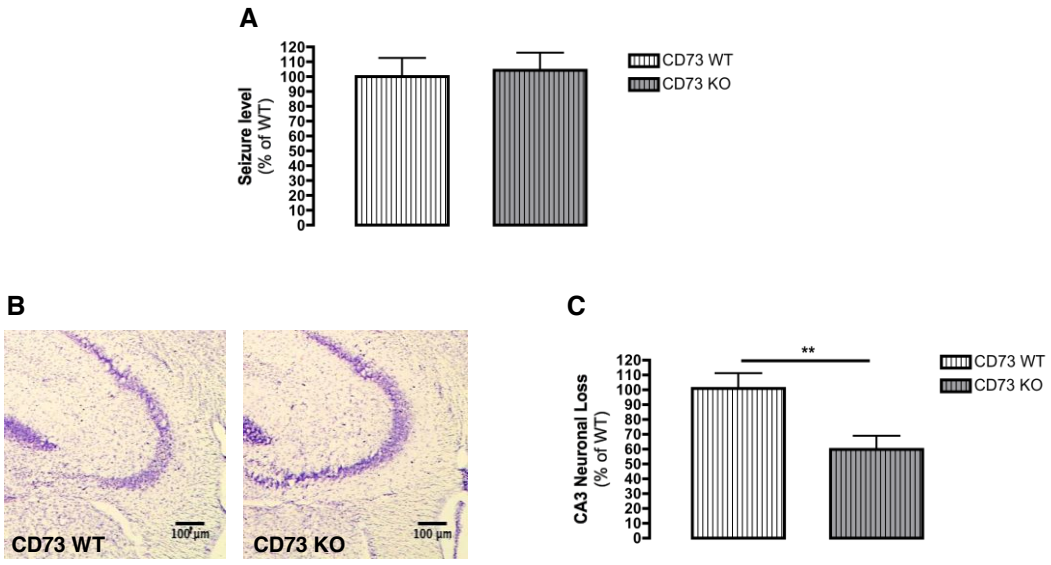
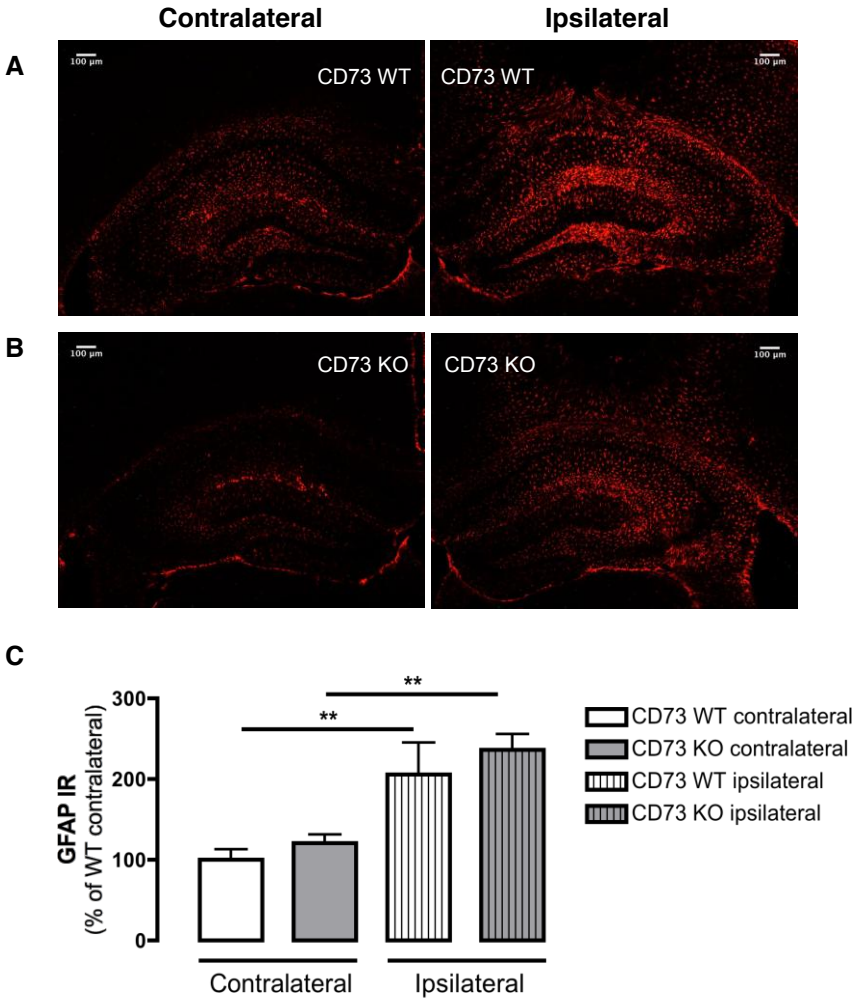


Figure 6.1. Anticonvulsive adenosine is not CD73-dependent in a MTLE model. The level of seizure (n = 7) after intrahippocampal injected KA (1 nmol) was identical between WT and CD73 KO mice (A). Panel B shows representative images (n = 6) of cresyl violet staining 7 days after injection showing the neuronal loss in CA3 in the ipsilateral side from CD73 KO and WT mice. Panel C is the quantification of the neuronal loss in CA3, which is lower in CD73 KO mice when compared to their WT littermates. Data are mean ± SEM of 6 mice and a *t* test was used; $^{**}p < 0.01$.

It is well known that the anticonvulsive adenosine is ADK- and A_1R-dependent (Boison, 2008b). However, the role of CD73-mediated adenosine formation in epilepsy is still unknown. In order to explore if the anticonvulsive adenosine is also CD73-

dependent in the hippocampus, we tested a mice model of MTLE in CD73 KO mice and analyzed the behavior, as well as the hippocampal changes in the silent phase of the pathology. Administration of KA (1 nmol) into the dorsal hippocampus generates a mice model of human MTLE and the convulsive profile of mice was evaluated according to a previously established six-point seizure scale (Schauwecker and Steward, 1997). The stage of convulsion reached by mice was similar between CD73 KO and their WT littermates (Fig. 6.1A), corroborating the results with cortical seizures (Lovatt et al., 2012). However, the neuronal loss in the ipsilateral hippocampus evaluated 7 days post-KA injection showed a significant lower neurodegeneration in the CA3 area of CD73 KO mice (- 40.2 ± 9.0 %; $p < 0.01$) when compared to WT mice (Fig. 6.1B-C).

4.2. CD73 KO mice showed no changes in astrocytic parameters 7 days after KA injection



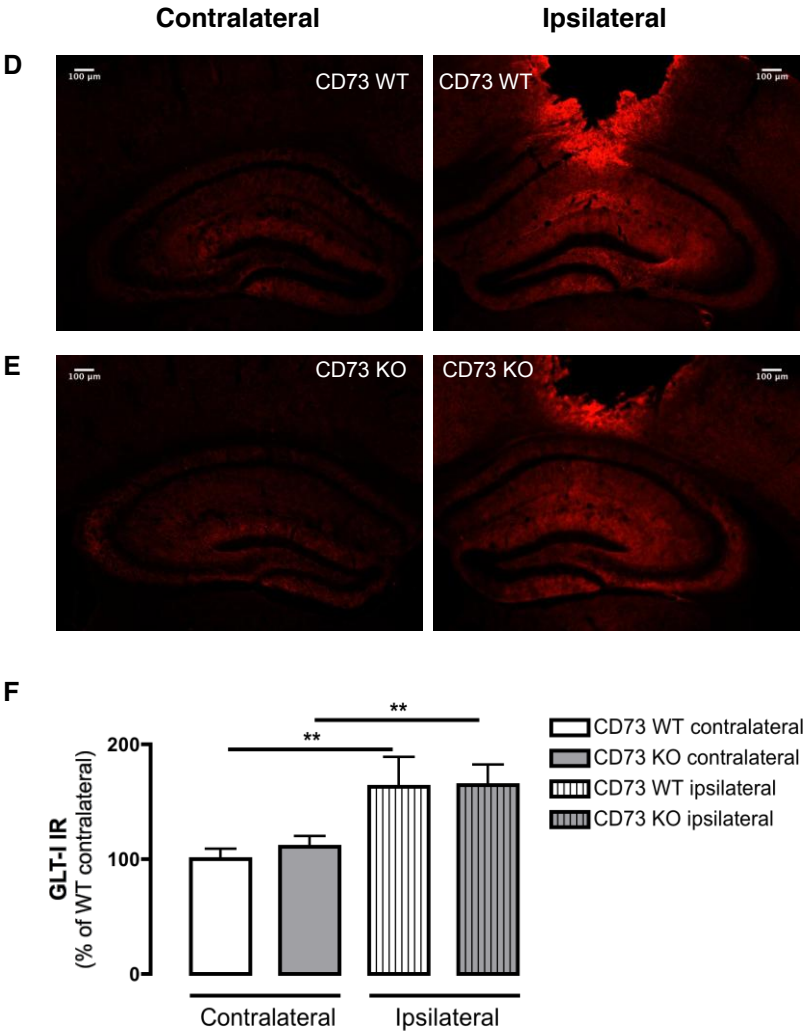


Figure 6.2. Astrocytic modifications 7 days after KA injection were similar between WT and CD73 KO. Panels A and B show representative images (n = 6) of hippocampal GFAP immunoreactivity in the contralateral and ipsilateral sides 7 days after KA injection into the dorsal hippocampus of WT (A) and CD73 KO mice (B). C is the quantification of GFAP immunorectivity (IR), with a significant increase in the ipsilateral side when compared to the contralateral side, which was similar in both groups, WT and CD73 KO mice. GFAP IR was identical between CD73 KO and their WT littermates in the contralateral side and also in the ipsilateral side. Panels D and E show representative images (n = 6) of hippocampal GLT-I immunoreactivity in the contralateral and ipsilateral sides 7 days after KA injection into the dorsal hippocampus of WT (D) and CD73 KO mice (E). F is the quantification of GLT-I IR, with a significant increase in the ipsilateral side when compared to the contralateral side, which was similar in both groups, WT and CD73 KO mice. GLT-I IR was identical between CD73 KO and their WT littermates in the contralateral side and also in the ipsilateral side. Data are mean ± SEM of 6 mice and a one-way ANOVA test was used, followed by a Newman-Keuls multiple comparison test; $^{**}p < 0.01$.

Despite, the neuroprotection observed in CD73 KO mice, no changes were observed in GFAP and GLT-I immunoreactivity in the ipsilateral side 7 days post-KA injection when compared with their WT littermattes (Fig. 6.2). Interestingly, the increased immunoreactivity of GFAP observed in the ipsilateral side was similar between CD73 KO (+ 136.2 ± 19.5 %; $p < 0.01$) and CD73 WT mice (+ 105.7 ± 39.3 %; $p < 0.01$) when compared to the contralateral side of CD73 WT mice (Fig. 6.2A-C). In the contralateral side the GFAP immunoreactivity was similar between CD73 KO and CD73 WT mice (Fig. 6.2A-C). GLT-I immunoreactivity had a similar pattern (Fig. 6.2D-F), with increased immunoreactivity in the ipsilateral side similar between CD73 KO (+ 64.7 ± 17.5 %; $p < 0.01$) and CD73 WT mice (+ 63.2 ± 25.7 %; $p < 0.01$) when compared to the contralateral side of CD73 WT mice. In the contralateral side the GLT-I immunoreactivity was also similar between CD73 KO and CD73 WT mice (Fig. 6.2D-F).

4.3. Neuronal A_{2A}R deletion is anticonvulsive and neuroprotective, while astrocytic A_{2A}R deletion is proconvulsive and neurodegenerative in a MTLE model

A

% of Mortality	WT	KO	KO
Fb A_{2A}R	12.5 %	0.0 %	↓
Gfa2 A_{2A}R	15.4 %	42.0 %	↑

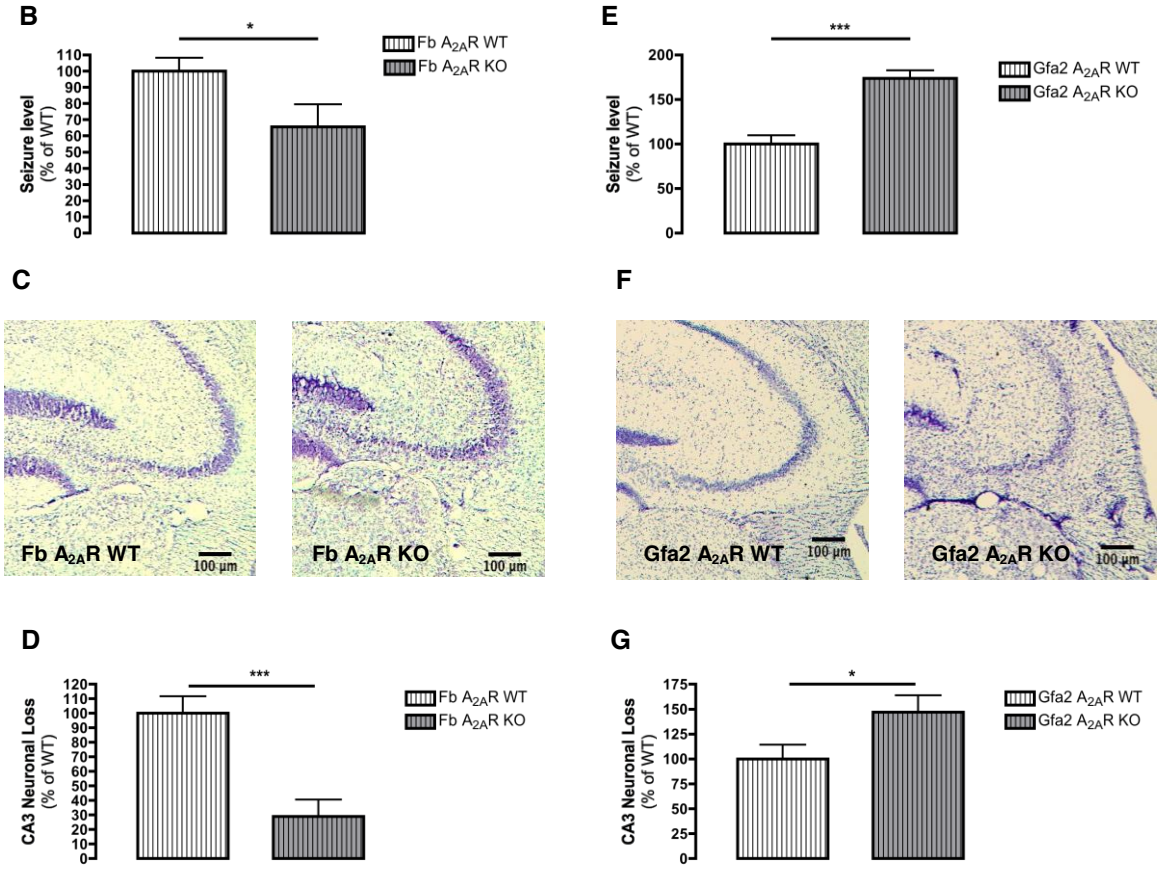


Figure 6.3. Fb A_{2A}R KO and Gfa2 A_{2A}R KO have opposite phenotypes in a MTLE model. A shows increased mortality in Gfa2 A_{2A}R KO mice and decreased mortality in Fb A_{2A}R KO mice, after intrahippocampal injected KA (1 nmol). Panel B shows the seizure intensity (n = 6-9) after intrahippocampal injected KA (1 nmol), which was significantly lower in Fb A_{2A}R KO mice when compared to their WT littermates. Panel C shows representative images (n = 5-6) of cresyl violet staining 7 days after KA injection showing the neuronal loss in CA3 in the ipsilateral side from Fb A_{2A}R KO mice and their WT littermates. Panel D is the quantification of the neuronal loss in CA3 in the ipsilateral side, which was significantly lower in Fb A_{2A}R KO mice when compared to their WT

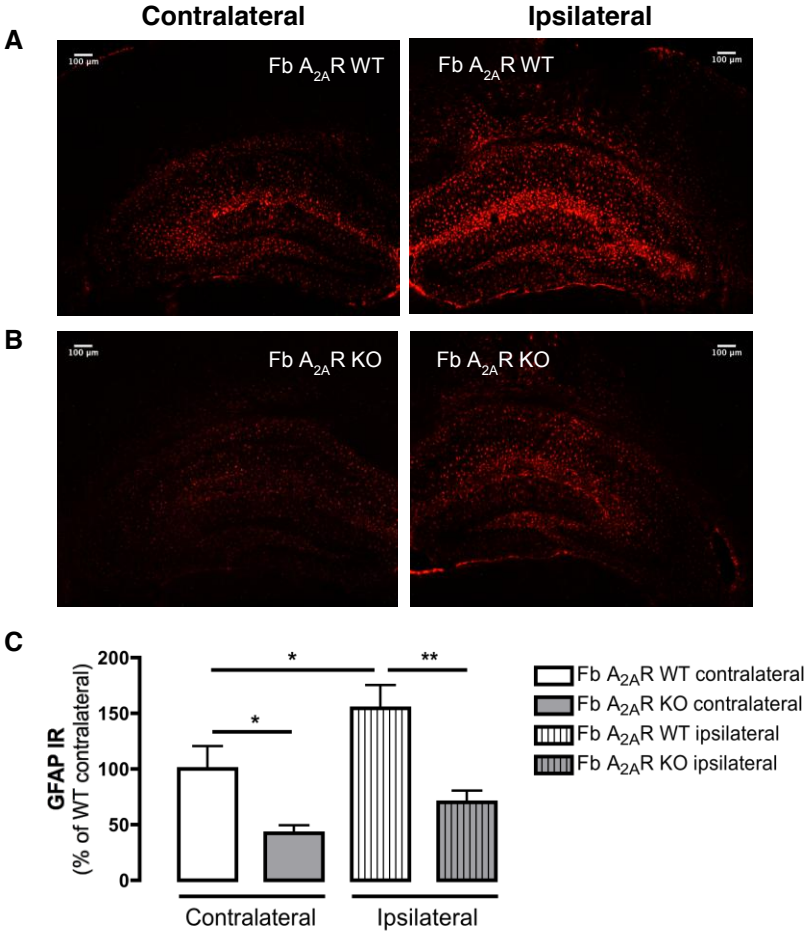
littermates. Panel E shows the level of seizure (n = 10-12) observed after intrahippocampal injected KA (1 nmol), which was significantly higher in Gfa2 A_{2A}R KO mice when compared to their WT littermates. Panel F shows representative images (n = 6-7) of cresyl violet staining 7 days after injection showing the neuronal loss in CA3 in the ipsilateral side from Gfa2 A_{2A}R KO mice and their WT littermates. Panel G is the quantification of the neuronal loss in CA3 in the ipsilateral side, which was significantly higher in Gfa2 A_{2A}R KO mice when compared to their WT littermates. Data are mean ± SEM and a Student's *t* test was used; **p* < 0.05; ****p* < 0.001.

It was shown that blockade or genetic deletion of A_{2A}R was neuroprotective in several mice models of neurodegeneration (Chen et al., 1999; 2001). In order to test if the neuroprotection observed in CD73 KO mice after KA-induced seizure was due to the lower activation of neuronal A_{2A}R and to explore the role of CD73-derived adenosine in the activation of A_{2A}R in epilepsy, we subjected the neuronal specific A_{2A}R KO mice (Fb A_{2A}R KO) to the MTLE mice model. The differences between Fb A_{2A}R KO and their WT littermates were evident immediately after intrahippocampal injection of KA, since the percent of mortality in the Fb A_{2A}R KO mice was 0.0 %, while in the Fb A_{2A}R WT mice it was 12.5 % (Fig. 6.3A). This result was the outcome of a significantly lower level of seizure post-KA injection in Fb A_{2A}R KO mice (- 34.4 ± 13.8 %; $p < 0.05$) compared to their WT littermates (Fig. 6.3B), showing that neuronal A_{2A}R activation is proconvulsive. The neuronal loss in the ipsilateral side evaluated from cresyl violet staining 7 days post-KA injection showed also a significant lower neurodegeneration in the CA3 region of Fb A_{2A}R KO mice (- 71.0 ± 11.4 %; $p < 0.001$) when compared to WT (Fig. 6.3C-D).

Astrocytic A_{2A}R can regulate glutamate uptake (Matos et al., 2012b), which can have a crucial role in epilepsy (Takahashi et al. 2010). In order to explore the role of the astrocytic A_{2A}R in epilepsy, we subjected the astrocytic specific A_{2A}R KO mice (Gfa2 A_{2A}R KO) to the MTLE mice model. As with the Fb A_{2A}R KO mice, the differences between Gfa2 A_{2A}R KO and Gfa2 A_{2A}R WT mice were evident immediately after intrahippocampal injection of KA, since the percent of mortality was higher in Gfa2 A_{2A}R

KO mice (42.0 %) when compared to Gfa2 A_{2A}R WT mice (15.4%; Fig. 6.3A). This higher mortality in Gfa2 A_{2A}R KO mice was the outcome of a significantly higher level of seizure post-KA injection in this group of mice (+ 73.5 ± 9.2 %; $p < 0.001$) when compared to their WT littermates (Fig. 6.3E), showing that astrocytic A_{2A}R activation is anticonvulsive. The neuronal loss in the ipsilateral side evaluated from cresyl violet staining 7 days post-KA injection showed also a significant higher neurodegeneration in CA3 in Gfa2 A_{2A}R KO (+ 82.566 ± 25.497 %; $p < 0.05$) when compared to WT mice (Fig. 6.3F-G).

4.4. Neuronal or astrocytic A_{2A}R deletion generate opposite astrogliosis profiles on a MTLE model



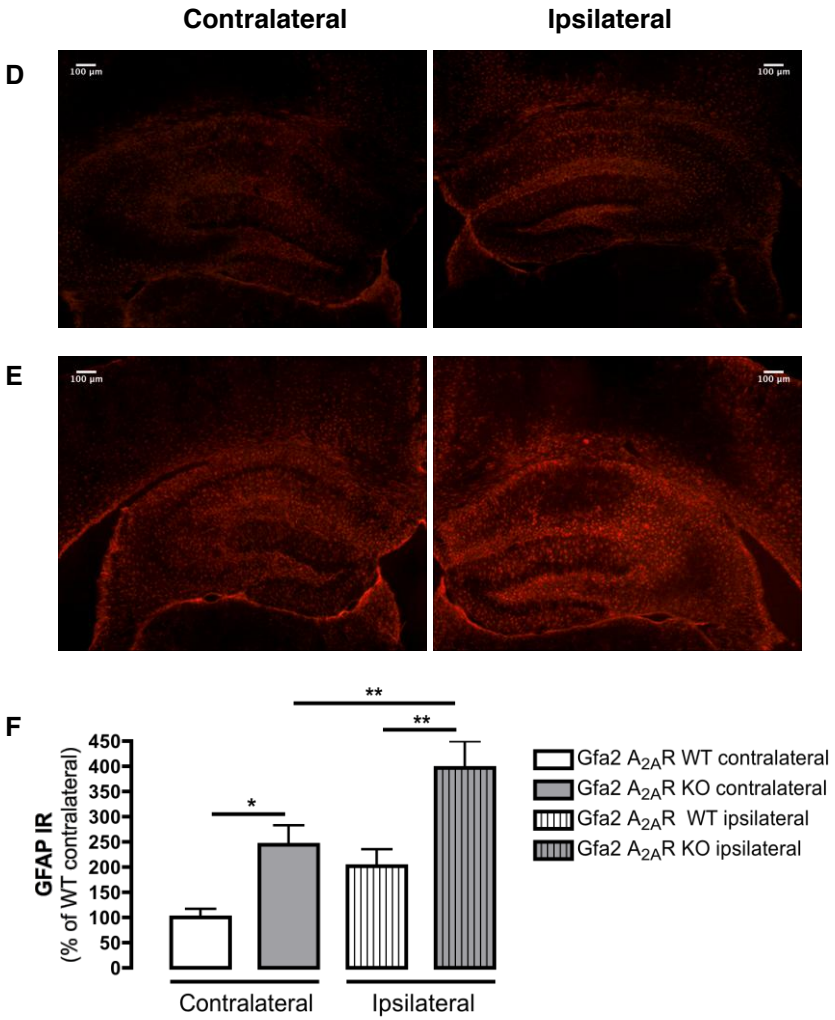
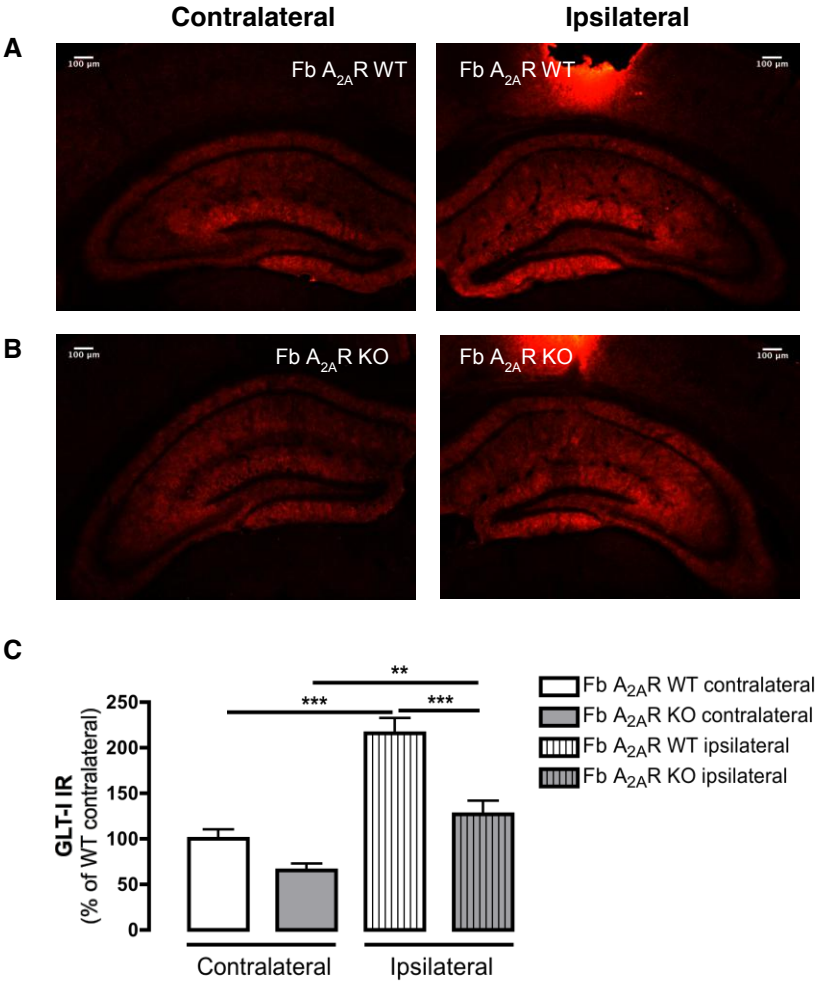


Figure 6.4. Modifications of GFAP in Fb A_{2A}R KO and Gfa2 A_{2A}R KO mice 7 days after KA injection. Panels A and B are representative images (n = 5-6) of hippocampal GFAP immunoreactivity in the contralateral and ipsilateral sides 7 days after KA injection into the dorsal hippocampus of WT (A) and Fb A_{2A}R KO mice (B). Panel C shows the quantification of GFAP immunorectivity (IR), with a significant increase in the ipsilateral side when compared to the contralateral side in WT but not in Fb A_{2A}R KO mice. GFAP IR was lower in Fb A_{2A}R KO when compared to their WT littermates in the contralateral side and also in the ipsilateral side. Panels D and E show representative images (n = 4-6) of hippocampal GFAP IR in the contralateral and ipsilateral sides 7 days after KA injection into the dorsal hippocampus of WT (D) and Gfa2 A_{2A}R KO mice (E). Panel F shows the quantification of GFAP IR, with a significant increase in the ipsilateral side when compared to the contralateral side in Gfa2 A_{2A}R KO mice but not in the WT mice. GFAP IR was higher in Gfa2 A_{2A}R KO mice when compared to their WT littermates in the contralateral side and also in the ipsilateral side. Data are mean ± SEM and a one-way ANOVA test was used, followed by a Newman-Keuls multiple comparison test; *p < 0.05; **p < 0.01.

The neurodegeneration in the ipsilateral side in the Fb A_{2A}R WT mice was accompanied by increased GFAP immunoreactivity (+ 54.6 ± 20.7 %; $p < 0.05$) when compared with the contralateral side (Fig. 6.4A-C). On the other hand, the ipsilateral side of Fb A_{2A}R KO mice did not show increased GFAP immunoreactivity after KA injection (Fig. 6.4A-C). Interestingly, GFAP immunoreactivity was decreased in Fb A_{2A}R KO mice in the contralateral side (- 57.5 ± 6.9 %; $p < 0.05$) when compared to the WT littermates (Fig. 6.4B-C). However, the decreased GFAP immunereactivity between Fb A_{2A}R KO and Fb A_{2A}R WT mice was even more evident in the ipsilateral side (- 84.3 ± 10.4 %; $p < 0.01$).

On other hand the neurodegeneration in the ipsilateral side in Gfa2 A_{2A}R KO mice was accompanied by an increased immunoreactivity in GFAP (Fig. 6.4D-F). The higher neurodegeneration in the ipsilateral side of Gfa2 A_{2A}R KO mice (Fig. 6.3G) was accompanied by a significant increase in GFAP immunoreactivity (+ 195.2 ± 52.7 %; $p < 0.01$) when compared with the WT mice in the same condition (Fig. 6.4D-F). Interestingly, the ipsilateral side of Gfa2 A_{2A}R KO mice showed an increased GFAP immunoreactivity after KA injection (+ 152. 3 ± 52.7 %; $p < 0.01$) when compared with contralateral (Fig. 6.3F). However, GFAP immunoreactivity was increased in Gfa2 A_{2A}R KO in the contralateral side (+ 144.5 ± 37.7 %; $p < 0.05$) when compared to the contralateral side of WT littermates (Fig. 6.4F).

4.5. Neuronal or astrocytic A_{2A}R deletion produce opposite modifications of GLT-I on a MTLE model



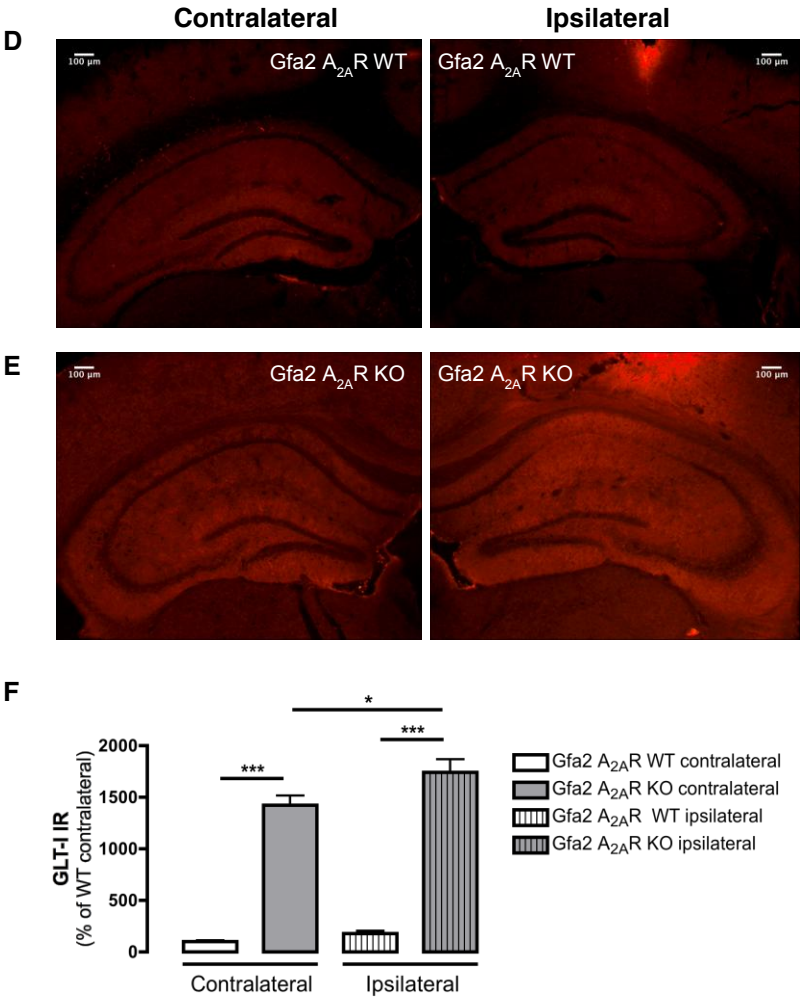


Figure 6.5. Modifications of GLT-I in Fb A_{2A}R KO and Gfa2 A_{2A}R KO mice 7 days after KA injection. Panels A and B show representative images (n = 5-6) of hippocampal GLT-I immunoreactivity in the contralateral and ipsilateral sides 7 days after KA injection into the dorsal hippocampus of WT (A) and Fb A_{2A}R KO mice (B). Panel C shows the quantification of GLT-I IR, with a significant increase in the ipsilateral side when compared to the contralateral side in both groups, WT and Fb A_{2A}R KO mice. GLT-I IR was identical between Fb A_{2A}R KO mice and their WT littermates in the contralateral side but was significantly lower in the ipsilateral side from Fb A_{2A}R KO when compared to the WT in the same condition. Panels D and E are representative images (n = 4-6) of hippocampal GLT-I IR in the contralateral and ipsilateral sides 7 days after KA injection into the dorsal hippocampus of WT (D) and Gfa2 A_{2A}R KO mice (E). Panel F shows the quantification of GLT-I IR, with a significant increase in the ipsilateral side when compared to the contralateral side in Gfa2 A_{2A}R KO mice but not in WT littermates. GLT-I IR was significantly higher in Gfa2 A_{2A}R KO mice when compared to their WT littermates in both, contralateral and ipsilateral sides. Data are mean ± SEM and a one-way ANOVA test was used, followed by a Newman-Keuls multiple comparison test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The changes observed with GFAP immunoreactivity were also extended to GLT-I immunoreactivity, with the ipsilateral side of Fb A_{2A}R WT mice showing increased GLT-I immunoreactivity (+ 115.8 ± 16.6 %; $p < 0.001$) when compared with the contralateral side (Fig. 6.5A-C). The ipsilateral side of Fb A_{2A}R KO mice also showed a significant increased GLT-I immunoreactivity after KA injection (+ 61.5 ± 15.1 %; $p < 0.01$) when compared to the contralateral side, however the increase was smaller in this group of mice than WT mice (Fig. 6.4C). GLT-I immunoreactivity was decreased in Fb A_{2A}R KO mice in the contralateral side (- 34.9 ± 7.7 %) when compared to the WT littermates (Fig. 6.4C). The decreased GLT-I immunereactivity between Fb A_{2A}R KO mice and Fb A_{2A}R WT mice was more evident in the ipsilateral side (- 89.1 ± 15.1 %; $p < 0.01$). These results show that deletion of neuronal A_{2A}R induced changes in astrocytic GLT-I, that could be due to the lack of control in the glutamatergic synapses, where neuronal A_{2A}R have a higher density and activity (Rebola et al., 2005b; 2008).

The changes observed in GFAP immunoreactivity were also observed with GLT-I in Gfa2 A_{2A}R KO mice. The ipsilateral side of Gfa2 A_{2A}R KO mice showed a significant increased GLT-I immunoreactivity at 7 days post-KA injection (+ 1561.0 ± 128.8 %; $p < 0.001$) when compared to the WT mice in the same condition (Fig. 6.5D-F). The ipsilateral side of Gfa2 A_{2A}R KO mice also showed a significant increased GLT-I immunoreactivity at 7 days post-KA injection (+ 318.2 ± 128.8 %; $p < 0.01$) when compared to the contralateral side (Fig. 6.5F). However, GLT-I immunoreactivity was significantly increased in Gfa2 A_{2A}R KO mice in the contralateral side (+ 1321.0 ± 93.2 %; $p < 0.001$) when compared to the contralateral side of WT littermates (Fig. 6.5F). These results show that deletion of astrocytic A_{2A}R induced major changes in astrocytes, that could be due to the lack of control in the density and activity of glutamate transporters, which we showed is under a tight control by astrocytic A_{2A}R (Matos et al., 2012b). These changes could be underlying the proconvulsive phenotype observed in

Gfa2 A_{2A}R KO mice.

5. Discussion

Adenosine is an endogenous modulator on the nervous system, fine-tuning on-going synaptic transmission, particularly upon stressful or acute disorders like epilepsy (Chen et al., 2013). The importance of the adenosinergic system to the development of epilepsy is emphasized by several observations during the last decade showing an increase on the adenosine levels predominantly during seizure termination (During and Spencer, 1992), an observation that has placed this metabolite as a promising anti-convulsive target for the treatment of epilepsy. The protective role of adenosine has been partially attributed to the activation of neuronal inhibitory A_1R (Zhang et al., 1993), which provide a global inhibitory tone on glutamate release (Fastbom and Fredholm, 1985) and neuronal hyperpolarization (Fredholm et al., 2005b). In addition, increases in the levels of ADK, an astrocyte-specific enzyme responsible to the metabolism of adenosine, have been suggested to play a key role in the higher susceptibility to seizure disorders (Boison, 2008b).

Despite these various achievements, the relevance of other key molecular and cellular intermediates of the adenosine cycle to the processes leading to epilepsy has been less investigated. Particularly, the role of the facilitatory high-affinity adenosine receptor subtype - A_{2A}R - in the processes leading to epilepsy has been more inconclusive and less explored (Pagonopoulou et al., 2006). Importantly, the neuroprotective role ascribed to A_1R during cortical seizures has not been confirmed to be due to ATP-dependent adenosine (Lovatt et al., 2012) in contrast to the previous view (Pascual et al., 2005). However, the previous study (Pascual et al., 2005) did not manipulate CD73 activity, the proposed last step of the endogenous control of the ATP-derived adenosine, prior to A_1R activation. Finally, recent observations demonstrated the

crucial modulatory role of astrocytic A_{2A}R in the glutamate extracellular levels (Popoli et al., 1995; Matos et al., 2012b), in addition to the array of different processes by which these cells have been recognized as potential therapeutic targets for the treatment of epilepsy (Seifert and Steinhäuser, 2013). Overall, these findings underscore the necessity of extending the investigation of the role of adenosine activity in epilepsy to astrocytes.

We had previously shown the presence of CD73 in hippocampus and its preferential postsynaptic location (see Chapter 2). This subcellular distribution profile is corroborated by a previous study showing that postsynaptic activation of A_{2A}R in the CA3 region is CD73-dependent (Rebola et al., 2008). In addition, we here showed that CD73-dependent adenosine is not anticonvulsive in a MTLE mice model, which is in agreement with the work by Lovatt and colleagues on a model of cortical seizures (Lovatt et al., 2012). Instead, the global CD73 KO mice showed less neuronal loss in CA3 in our MTLE model. The neurodegeneration potentiated by CD73 is probably due to A_{2A}R activation, since we recently showed that striatal A_{2A}R activation is CD73-dependent (Augusto et al., 2013) and the neuroprotection afforded by global A_{2A}R deletion or blockade in different types of neurodegenerative diseases is now well known (Chen et al., 1999; 2001; Canas et al., 2009b). Overall, these data suggest a beneficial therapeutic potential in the suppression of CD73 activity, further implying that blockade of A_{2A}R might afford neuroprotection in the pathogenesis of epilepsy.

As previously mentioned, the involvement of adenosine receptors in epilepsy other than A_1R, particularly the high-affinity A_{2A}R, is still controversial. Previous observations have demonstrated that genetic deletion (Yacoubi et al., 2001; 2009) or selective pharmacological blockade (Jones et al., 1998b; Yacoubi et al., 2008; D'Alimonte et al., 2009; Moschovos et al., 2012) of A_{2A}R can afford protection against seizures. However, these data contradict other studies showing that A_{2A}R agonists can also be

neuroprotective in different *in vivo* epilepsy models (Adami et al., 1995; Jones et al., 1998a; Huber et al., 2002). Nevertheless, the neuroprotective effects of A_{2A}R agonists have been partially attributed to the activation of non-neuronal A_{2A}R (Dai et al., 2010), while the neuroprotective effects of A_{2A}R antagonists have been associated to the blockade of neuronal A_{2A}R (Yacoubi et al., 2009). Thus, the opposing effects of A_{2A}R in different cell types clearly represent major caveat and challenge in purinergic drug development (Chen et al., 2013). In addition, conventionally epilepsy has been considered a disorder of the neuronal function, with drug targeting focusing on this neurocentric hypothesis, while overlooking the clear potential in the astrocytic counterpart (Seifert and Steinhäuser, 2013). It is recognized the major role of excitatory glutamatergic modifications in epilepsy, by directly and indirectly modulating the initiation and spread of seizure activity (Coulter and Eid, 2012). Therefore, the increased awareness of the role of A_{2A}R in modulating neuronal glutamate release and astrocytic uptake (Popoli et al., 1995; Matos et al., 2012b) in addition to astrogliosis (Boison et al., 2010), a classical hallmark of TLE (Ortinski et al., 2010), highlights the requirement to investigate the differential role of A_{2A}R in astrocytes and neurons.

Our data reinforce the idea of an opposing neuromodulatory functions mediated by neuronal and astrocytic A_{2A}R, since the suppression of neuronal A_{2A}R activity was shown to be neuroprotective while the selective deletion of astrocytic A_{2A}R potentiated KA-induced damage. Indeed, when A_{2A}R deletion was restricted to neurons (Fb A_{2A}R KO) the mice showed lower mortality rates, milder convulsion profile, less neuronal death and astrogliosis, when compared with the WT littermates at 7 days after KA administration. On the other hand, when A_{2A}R deletion was restricted to astrocytes (Gfa2 A_{2A}R KO mice) the mice showed an abnormally high mortality rate and convulsion profile, together with an exacerbated neuronal death and astrogliosis post-KA injection. In addition, our results are suggestive of an association between an increased astrocyte

glutamate transporters density with neuronal death. Indeed, Gfa2 A_{2A}R KO mice exhibited a much higher GLT-I immunoreactivity, exacerbated in the ipsilateral side, with an opposite phenotype in the Fb A_{2A}R KO mice. The importance of these findings is underlined by the recognition that disrupting the expression or activity of GLT-I, the major glutamate transporters in the adult brain, results in excessive activation of glutamate receptors, abnormal neuronal activity, and eventual excitotoxic neuronal death (Benarroch, 2010). Therefore, several studies have analyzed the involvement of astrocyte glutamate transporters in various models of epilepsy. However, the results have been generally contradictory which may reflect the specific characteristics of the experimental epilepsy model preferred. Indeed, in generalized tonic-clonic models like genetically epilepsy-prone rodents, decreased levels or activity of glutamate transporters have been commonly associated with seizure activity and brain injury (Tanaka et al., 1997; Akbar et al., 1998; Dutuit et al., 2002). However, a different array of studies involving chemical convulsants in experimental models of complex focal epilepsy like ours have generally shown increased expression, density and activity of glutamate transporters (Simantov et al., 1999; Ueda and Willmore, 2000; Takahashi et al., 2010; Moreira et al., 2011). In addition, in human TLE brain samples, both decreases in glutamate transporters immunoreactivity in hippocampal sclerotic formations and increases in non-sclerotic affected sites were mutually detected (Mathern et al., 1999; Proper et al., 2002), suggesting that increased glutamate uptake may occur prior to the onset of neuron loss. Our data showing a simultaneous increase of astrocytic GLT-I and neuronal death in Gfa2 A_{2A}R KO mice after KA-administration suggests the validity in this contention. Thus, the suppression of astrocytic A_{2A}R, which is a negative regulator of glutamate uptake, triggers a deregulated increase on the levels and activity of GLT-I (Matos et al., 2012b). Decreased glutamate levels in perisynaptic regions due to exacerbated astrocyte uptake may lead to compensatory unrestrained increases in

synaptic areas due to increased presynaptic glutamate release (unpublished data), thus providing an additional excitatory drive for the seizure activity. As a result, local oscillations on the glutamate levels may lead to severe modifications in the glutamatergic network, conferring higher susceptibility to KA-induced damage (Duncan et al., 2010) as seen in other models (Omrani et al., 2009). In addition, hyperactive levels of GLT-I may result in glutamate-reversed transport (Kawahara et al., 2002; Selkirk et al., 2005), paving to way to excitotoxicity and neuronal cell death.

The somewhat continuous range of different phenotypes exhibited by the different mice-models used - from high neuroprotection in Fb A_{2A}R KO mice, to the mild-neuroprotection in CD73 KO mice and high neurotoxicity in Gfa2 A_{2A}R KO mice - suggests that when the general A_{2A}R activity is impaired by CD73 suppression, the overall phenotype is mostly due to loss of neuronal, and not astrocytic, A_{2A}R activity. This is in agreement with the comparatively much lower levels of A_{2A}R in astrocytes (Boison, 2008b), whose activity, as expected, is overridden by the neuronal A_{2A}R. Significantly, the fairly mixed phenotype of global CD73 KO mice in comparison with Fb A_{2A}R KO and Gfa2 A_{2A}R KO, also suggests that the molecular source of both neuronal and astrocytic A_{2A}R activation derives from AMP catabolism by CD73.

Taken together, our data show an important unappreciated role of CD73-derived adenosine on A_{2A}R activation and higher KA-induced susceptibility, implying the necessity to investigate if the pharmacological inhibition of CD73 can be therapeutically relevant in epilepsy. In addition, they imply a differential and delicate coordinated action of astrocytic and neuronal A_{2A}R to fine-tune glutamatergic transmission, raising awareness to the necessity to discriminate between the two cellular compartments during the development of adenosinergic-based therapeutic strategies for epilepsy and other disorders of the central nervous system.

6. Acknowledgements

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CHAPTER 7

THE PHYSIOLOGICAL ROLE OF HIPPOCAMPAL $A_{2A}R$

1. Abstract

The hippocampus plays pivotal roles in different cognitive functions. Despite the relative low expression of adenosine A_{2A} receptors ($A_{2A}R$) in this brain area, their participation in important molecular processes and cognitive functions is well established. In addition, the density of hippocampal $A_{2A}R$ increases in noxious conditions, including in pathological processes associated with memory impairment, specifically at the presynaptic nerve terminals. In agreement, we identified the presence of $A_{2A}R$ mRNA and its local synthesis in presynaptic nerve terminals in the hippocampus, after stimulation. Moreover, we explored the direct participation of hippocampal $A_{2A}R$ in learning and memory behaviors by taking advantage of an hippocampal $A_{2A}R$ knockdown generated through local injection of a vector that express Cre into the dorsal hippocampus of $A_{2A}R$-floxed mice. Here we demonstrate that hippocampal $A_{2A}R$ knockdown does not originate modifications in motor learning or anxiety (in opposite to forebrain $A_{2A}R$ knockout (Fb $A_{2A}R$ KO) that displayed an anxiolytic phenotype), but leads to improved working memory (in agreement with Fb $A_{2A}R$ KO phenotype) and impaired context (but not tone) fear conditioning.

2. Introduction

The hippocampus plays pivotal roles in different cognitive processes, namely memory that is an essential function of the brain and allows the encoding, storage and retrieval of information from the outside world. Memory formation requires a well-organized orchestration of different mechanisms, namely the activation of neuronal pathways, neurotransmitter(s) release followed by activation of postsynaptic receptors which in turn, trigger the activation of intracellular signaling pathways (Malenka and Bear, 2004), gene transcription and/or protein synthesis (Kandel, 2001) as well as epigenetic mechanisms (Zovkic et al., 2013).

It is known that many neurotransmitter systems are involved in the mechanisms of learning and memory, namely glutamatergic, dopaminergic and cholinergic systems (Myhrer, 2003). Adenosine acting through adenosine A_{2A} receptors ($A_{2A}R$) is able to modulate neurotransmitter systems, neuronal excitability and synaptic plasticity in brain regions relevant for learning and memory (Cunha, 2008a). Presynaptic $A_{2A}R$ are well known to modulate the release of multiple neurotransmitters, including glutamate, acetylcholine and dopamine, in brain regions important for memory like the hippocampus (Sebastião and Ribeiro, 1992; Cunha et al., 1994a; 1994b; 1994c; Latini et al., 1996; Ribeiro, 1999; Lopes et al., 2002). At the postsynaptic level $A_{2A}R$ participate in modulating different forms of plasticity in the hippocampus, including long-term potentiation (LTP) (Rebola et al., 2008; Fontinha et al., 2009). On the other hand, $A_{2A}R$ is also able to control the levels of extracellular glutamate by modulating the activity of glutamate transporters in astrocytes (Nishizaki et al., 2002; Matos et al., 2012b). Thereby, $A_{2A}R$ are in the center of a neuromodulatory network, in a position to affect a wide range of cognitive and memory processes by interacting and integrating several neurotransmitter systems (Dunwiddie and Masino, 2001; Fredholm et al., 2005b).

Several recent pharmacological and genetic studies suggest a potential modulatory role of $A_{2A}R$ activity on cognitive processes. Though, the initial studies using antagonists and agonists of $A_{2A}R$ have been somewhat inconsistent. It was shown that $A_{2A}R$ agonists impaired and antagonists improved the performance in different memory tasks (Kopf et al., 1999; Pereira et al., 2005; Prediger et al., 2005c; 2005d; Takahashi et al., 2008). However, it was also reported that $A_{2A}R$ agonists alone were able to generate paradoxical effects on memory performance (Hooper et al., 1996; Pereira et al., 2005; Prediger and Takahashi, 2005). It is possible that these mixed responses reflect differences on the tested dose, the schedule and timing of administration and the mode of administration (locally or peripherally), which may reflect the different contributions of

the distinct brain regions, as well as the specificity of the different tested tasks. Instead, transgenic and knockout (KO) animals studies have recently provided some of the direct evidence that $A_{2A}R$ are major players on the adenosinergic control of memory in physiological conditions. Transgenic mice overexpressing $A_{2A}R$ showed memory deficits in different tasks (Giménez-Llort et al., 2007). In agreement, $A_{2A}R$ KO mice displayed improved memory performance, namely in spatial reference memory (Wang et al., 2006) and working memory (Zhou et al., 2009; Wei et al., 2011). However, recent studies using brain region-specific $A_{2A}R$ KO mice are only beginning to reveal the complexities and vastness of $A_{2A}R$ functions in cognition (Singer et al., 2013; Wei et al., 2013), and provide the first set of tools to begin dissecting the contribution of $A_{2A}R$ in the different brain regions on several types of learning and memory processes.

Despite their relative low expression in the hippocampus, the participation of $A_{2A}R$ in important molecular processes in this brain area is well established. However, the direct evidence between specific hippocampal $A_{2A}R$ functions and significant outcomes on cognitive functions are lacking, largely due to the inability to specifically control hippocampal $A_{2A}R$ activation in freely behaving animals without associated developmental adaptations. In order to provide a greater regional specificity of $A_{2A}R$ inactivation in the hippocampus to adequately dissect the nature of $A_{2A}R$ in this brain area, we here developed hippocampal $A_{2A}R$ knockdown by local injection of a vector that express Cre into the dorsal hippocampus of $A_{2A}R$-floxed mice and explored its phenotype in different behavior paradigms. In addition, the levels of hippocampal $A_{2A}R$ promptly increase after different types of deleterious stimulation, namely in pathological conditions associated with memory impairment, specifically at the presynaptic nerve terminals (Canas et al., 2009b). Based on this and others evidences obtained by our group (unpublished data) corroborating an exceptional swiftness on the $A_{2A}R$ response

to particular stimuli, we decided to explore if $A_{2A}R$ could be locally synthesized in presynaptic terminals of the hippocampus.

3. Materials and methods

Animals

Approval from the Institutional Animal Care and Use Committee at Boston University School of Medicine and the Portuguese Veterinarian Office was granted for all experiments conducted in Boston and Coimbra, respectively. They adhered to the NIH Guide for the Care and Use of Laboratory Animals, the Portuguese Law and Ordinance on Animal Protection, and European Council Directive 86/609/EEC. In all experiments males and females adult (2-3 months old) mice or rats were used. Forebrain-specific $A_{2A}R$ KO (Fb $A_{2A}R$ KO), which are CaMKIIα-Cre[+]$A_{2A}R^{flox/flox}$, with C57Bl/6J genetic background for both Fb $A_{2A}R$ KO and Fb $A_{2A}R$ WT, which are CaMKIIα-Cre[-]$A_{2A}R^{flox/flox}$ were generated as previously detailed (Bastia et al., 2005).

Synaptosomes purification and treatment

After the homogenization of the hippocampus, synaptosomes were obtained using a discontinuous Percoll gradient (3, 10, and 23 % v/v of Percoll in a medium containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM DTT, pH 7.4), as previously described (Dunkley et al., 2008). The mixture was centrifuged at 2,000 *g* for 3 min at 4 ºC and the supernatant is centrifuged at 9,500 *g* for 13 min at 4 ºC. The pellet was placed on the top of the percoll gradient and centrifuged at 25,000 *g* for 11 min at 4 ºC without break. The layer between 10 and 23 % of Percoll (synaptosomal fraction) were collected, washed in 20 mL of HEPES buffered medium (HBM) containing 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4,

and further centrifuged at 22,000 *g* for 11 min at 4 ºC. The pellet was washed in HBM and further centrifuged at 22,000 *g* for 11 min at 4 ºC and incubated in 1 ml of HBM with Ca^{2+} and kainate (5 μM) for 2 h at 37 ºC in an incubator supplied with 5 % of CO_2. The synaptosomes were washed and additionally incubated with HBM for 2 h at 37 ºC in the incubator. For the mRNA experiments the synaptosomes were incubated with RNase for 30 min at 37 ºC, followed by the 'qPCR analysis in synaptosomes' protocol. For specific binding density the synaptosomes were incubated with kainate or cycloheximide (4-[(2*R*)-2-[(1*S*,3*S*,5*S*)-3,5-Dimethyl-2-oxocyclohexyl]-2-hydroxyethyl]piperidine-2,6-dione) plus kainate and the respective vehicles as control, followed by the 'Membrane binding analysis' protocol.

qPCR analysis in synaptosomes and axonal fraction

Total RNA was isolated from striatal tissue, hippocampal synaptosomes and different fractions of cultured neurons using the Magna Pure Compact RNA isolation kit (Roche). The complementary DNA (cDNA) from each sample was subsequently synthesized by reverse transcription using Transcriptor First Strand cDNA Synthesis kit (Roche) with random hexamer primer in a GeneAmp PCR system 2400 thermal cycler (Perkin Elmer). The amplification of cDNA by real-time PCR was carried out on SmartCycler system (Cepheid, Izasa) using FastStart DNA MasterPLUS SYBR Green I kit (Roche) customized for amplification of the target cDNA's. Primers used for real-time PCR for cDNA amplification were synthesized with 100 % homology to the rat sequence by homology search through the NCBI BLAST program. The two set of primers to amplify $A_{2A}R$ mRNA were 5'- GGG GCA AAC TCT GAA GAC CAT G; 5'- CAT CCT CTC CCA CAG CAA CTC, that produced a 420-bp amplicon; and 5'- GGA GTG GAA TTC GGA TGG C; 5'- GCC TGC TTT GTC CTG GTC C, that produced a 90-bp amplicon. The two set of primers to amplify histone 1 mRNA were 5'- ACC CAT TGT TCA AGG

ACA GC; 5'- ATC AGG TCC CCC AAC TTA CC, that produced a 325-bp amplicon; and 5'- CCA CGG ACC ACC CCA AGT ATT CAG; 5'- CTT GGC TTT GGG CTT CAC GGG TTT, that produced a 487-bp amplicon. The two set of primers to amplify β-actin mRNA were 5'- CGA CGA GGC CCA GAG CAA GAG A; 5'- TCC AGG GCA ACA TAG CAC AGC TT, that produced a 487-bp amplicon; and 5'- AGC CAT GTA CGT AGC CAT CC; 5'- CTC TCA GCT GTG GTG GTG AA, that produced a 227-bp amplicon. Samples, together with SYBR Green I reaction mix, were run for 10 min at 95 ºC, followed by 45 cycles of amplification, each composed by denaturation at 95 ºC for 10 sec; primer annealing for 5 sec at 62ºC ($A_{2A}R$ with 420 bp amplicon), at 56 ºC ($A_{2A}R$ – 90 bp amplicon), at 68 ºC (β-actin with 497-bp amplicon), at 57 ºC (β-actin with 227-bp amplicon), at 56 ºC (histone 1 with 325-bp amplicon), at 71 ºC (histone 1 with 487-bp amplicon); and extension at 72 ºC for 17 sec for $A_{2A}R$ (with 420 bp amplicon), 4 sec for $A_{2A}R$ (with 90 bp amplicon), 20 sec for β-actin (with 497-bp amplicon), 10 sec for β-actin (with 227-bp amplicon), 15 sec for histone 1 (with 325-bp amplicon), 20 sec for histone 1 (with 487-bp amplicon). All primers produced responses, whose specificity were checked in 2 % agarose gels (Schmittgen and Livak, 2008).

Membrane binding analysis

Membranes from hippocampal synaptosomes were first incubated with 2 U/ml adenosine deaminase for 30 min at 37 °C, to remove endogenous adenosine. The mixture was then centrifuged at 14,000 *g* for 10 min at 4 °C and the pellets resuspended in the Tris-Mg solution (containing 50 mM Tris and 10 mM $MgCl_2$, pH 7.4). Binding of [^{3}H]-7-(2-phenylethyl)-5-amino-2-(2-furyl)pyrazolo[4,3e][1,2,4]triazolo [1,5c]pyrimidine ([^{3}H]SCH 58261) was for 1 h at room temperature with 200–300 g of protein in a final volume of 0.2 ml in the incubation solution containing 4 U/ml adenosine deaminase, as previously described (Alfaro et al., 2004; Lopes et al., 2004). Specific binding was

determined by subtraction of the nonspecific binding, which was measured in the presence of 1 M 8-{4-[(2-aminoethyl)amino] carbonylmethyloxyphenyl}xanthine (XAC), a mixed A1/A2 receptor antagonist. All binding assays were performed in duplicate. The binding reactions were stopped by vacuum filtration through glass fiber filters (GF/C filters) using a 24 well Brandel harvester. The filters were then placed in scintillation vials and 4 ml of scintillation liquid (Ready Safe, Pharmacia) added. Radioactivity was determined after at least 12 h with a counting efficiency of 55–60%. The protein concentration was determined using the Bio-Rad protein assay based on Bradford dye-binding procedure. The specific binding from saturation experiments was fitted by non-linear regression to one binding site equation using the Raphson-Newton method, performed with a commercial software (GraphPad) to determine the binding parameters.

Primary cultures of hippocampal neurons in microfluid platforms

Hippocampal neurons were cultured from 17- to 19-d-old Wistar rat embryos, as previously described (Silva et al., 2007), and plated on microfluid platforms as previously described (Taylor et al., 2005). Neurons were grown for 2 weeks at 37 °C in a 5 % CO_2 humidified atmosphere in Neurobasal medium with B-27 supplement, glutamate (25 mM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml).

Intra-hippocampal injection of AAV5-Cre into conditional (floxed)-$A_{2A}R$ mice

To investigate the role of the selective deletion of $A_{2A}R$ in the hippocampus on different behaviors, we performed bilateral AAV5-CMV-Cre-GFP injection into the hippocampi of conditional (floxed)-$A_{2A}R$ mice. Specifically, either AAV5-CMV-Cre-GFP (Vector BioLabs, 2 μl of 1 x 10^{12} genome copies/mL) or its control viral vector (AAV5-CMV-GFP) were injected stereotaxically into both hippocampi of floxed-$A_{2A}R$ adult mice (AP = -2.5 mm from bregma, ML = ± 2.0 mm from the midline, and DV = - 1.5 mm from

the skull surface). Three weeks later, the mice were examined for the development of different behaviors responses.

Fluorescence immunohistochemistry

To confirm the injection site and expression of AAV5-GFP after local injection into the hippocampus, mice were anesthetized with Avertin-HCl [2 % 2,2,2-tribromoethanol and 1 % amyl alcohol], and the brains were fixed by transcardiac perfusion with 4 % paraformaldehyde in PBS, post-fixed, and cryopreserved. 30 μm coronal sections (Leica Microsystems) were incubated for 1 h in PBS containing 0.25 % Triton X-100 and 5 % donkey serum, followed by incubation with mouse anti-mouse NeuN (1:1,000; Millipore) and rabbit anti-GFP (1:1,000; Abcam) antibodies overnight at 4 °C. Next, sections were rinsed 3x for 10 min each in PBS and subsequently incubated with donkey anti-mouse and donkey anti-rabbit secondary antibodies conjugated with a fluorophore (Alexa Fluor 488 and Alexa Fluor 555; 1:200; Invitrogen) for 1 h at room temperature. Sections were again rinsed and then mounted with Vectashield mounting medium for examination under a fluorescence Nikon eclipse E600 microscope.

qPCR analysis after AAV5-Cre injection

To confirm the Cre-induced deletion of the $A_{2A}R$ gene, mice were euthanized by decapitation, the hippocampi were homogenized in trizol, and the RNA was isolated according to the protocol described by (Schmittgen and Livak, 2008). Briefly, after homogenization, samples were incubated for 5 min on ice followed by 10 min at room temperature. 0.2 ml of chloroform (per 1 ml of trizol) was added and shaked for 15 s followed by incubation for 10 min at room temperature. Samples were centrifuged at 14,000 r.p.m. for 15 min at 4 ºC and the clear aqueous phase was collected. 0.650 ml of 2-propanol was added (per 1 ml of trizol added during homogenization) followed by a

brief vortex and incubation for 10 min on ice. Samples were then centrifuged at 14,000 r.p.m. for 15 min at 4 ºC and the pellet washed with 75 % ethanol, followed by brief vortex and incubation on ice for 10 min. Samples were centrifuged at 14,000 r.p.m. for 15 min at 4 ºC, the supernatant discarded and the open tubes placed at -80 ºC for 30 min. RNase-free water was added and the quantification and purity of RNA was analyzed in a nanodrop. cDNA was synthetized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. The real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) using the $A_{2A}R$ primers 5'-TAGCCCTGTGACTGAGTGCATG and 5'-GCTGCTGACCTAGAAGTGG and the GAPDH primers 5' – TGGTCCAGGGTTTCTTACTCC and 5' - AGGTTGTCTCCTGCGACTTCA as the internal control in a realplex[4] thermocycler (Eppendorf). The data were analyzed as described previously (Schmittgen and Livak, 2008).

Accelerated rotarod

The mouse rotarod apparatus (Med associates inc.) consisted of a rubber roller with small grooves running along its turning axis. Thirty minutes after the acclimatization to the room, mice were tested for four consecutive trials. During each trial, animals were placed on the rod rotating at a constant speed (4 r.p.m.), then the rod started to accelerate continuously from 4 to 40 r.p.m. over 300 sec. The latency to fall off the rotarod was recorded. Animals that stayed on the rod for 300 sec were removed from the rotarod and recorded as 300 sec. Between each trial, mice were placed in their home cage for a 15–20 min interval.

Open-field test

The open-field test was used to evaluate anxiety-like behavior. The open-field arena consists of a white plastic box (41 cm × 41 cm × 25 cm) and was placed 50 cm above the floor. The arena was divided into a central field (center, 15 cm × 15 cm) and an outer field (periphery). Thirty minutes prior to the test the animals were acclimatized to the room. Individual mice were placed in the center of the open-field and the activity was recorded with a video camera during 8 min period. The ANY-maze software assessed the total distance traveled in the open-field, as well as the distance traveled, the number of entries and time spent in the center area.

Working memory

We first assessed working memory in a spontaneous alternation paradigm assessed in a *Y-maze*. Individual mice were placed at the end of one arm and allowed to freely explore the maze for 5 min. The sequence of entrance in each arm was recorded and the number of alternations (sequential entrance in the 3 different arms) was quantified. The percentage of spontaneous alternation consists in the percentage of alternations in the total possible alternations (total number of arms' changes minus 2).

We also assessed working memory in a more sensitive test using an *8 radial arm maze (RAM)* as previously described (Singer et al., 2012). To motivate performance in the RAM memory tasks, the animals were maintained on a food deprivation regime, which was gradually introduced with a progressive reduction of the daily available food, until the animals reached a stable weight of not less than 85 % of their *ad libitum* weight, at which time the food provided was stabilized. The RAM had 8 identical and equally spaced arms (56 cm long, 12 cm wide) radiating from a central octagonal platform (side-length = 12 cm). The mice were exposed to the maze for 5 min each day with a food reward at the end of each arm. The habituation was performed until the animals finished

the task within 5 min. In the *8 baited arms paradigm*, the 8 arms were set with a food reward and the animals allowed to freely explore the maze until they ate the 8 food rewards. Each time a mouse reentered in an arm where the reward was already ate, a working memory error (WME) was scored.

Associative learning: Pavlovian conditioned freezing

The apparatus (Gemini system; San Diego instruments) consisted of two conditioning chambers to provide two distinct contexts, as fully described before (Yee et al., 2007a). The operant chamber (context A) contained a grid floor to apply electric shocks (the unconditioned stimulus, US) and black walls. The second chamber (context B) had a brown plastic floor and white and pink striped walls. The conditioned stimulus (CS) was an 86 dB tone. The experiment consisted of three phases: conditioning, context test, and CS test. Conditioning was first conducted in context A and involved the presentation of three discrete paired CS-US trials. Each trial began with a 30 sec tone CS followed immediately by the delivery of a 1 sec foot-shock US set at 0.26 mA. Each trial was preceded and followed by a 180 sec intertrial interval. Mice were then returned to the home cage until the context freezing test on the next day when mice were again exposed to context A but left for a period of 8 min in the absence of any discrete stimulus. On the third day, conditioned freezing to the tone CS was assessed in the neutral context B. Following a 120 sec acclimatization period, the tone CS was turned on for 8 min.

Statistical analysis

Results are presented as mean ± SEM. Data with one condition and one variable (e.g. genotype) were analyzed with Student's *t* test. Data with more than one variable (e.g. genotype and time) and condition were analyzed with a two-way ANOVA followed

by Bonferroni post-hoc tests. Unless otherwise indicate the significance level was 95 %.

4. Results

4.1. $A_{2A}R$ mRNA is present in presynaptic nerve terminals and in a pure axonal fraction from the hippocampus

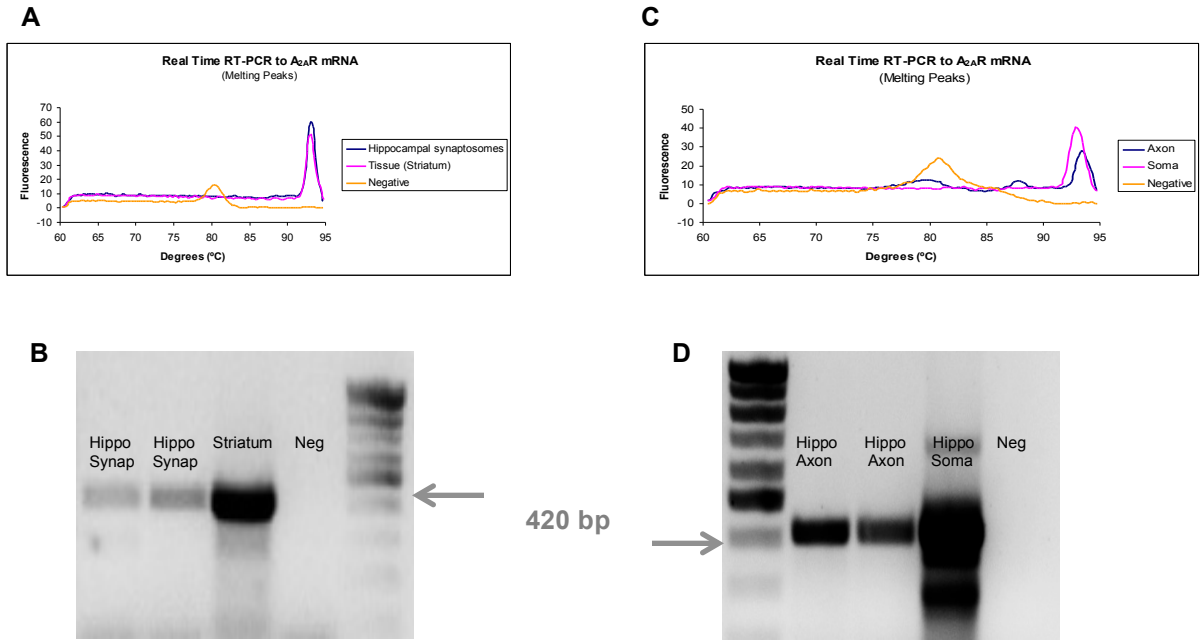

Figure 7.1. $A_{2A}R$ mRNA is present in presynaptic nerve terminals and in pure axonal fractions from the hippocampus. Hippocampal presynaptic nerve terminals previously incubated with ribonuclease showed the presence of $A_{2A}R$ mRNA (A, B). Panel A shows a representative melting curve of the $A_{2A}R$ mRNA real time RT-PCR (n = 4) for striatal RNA (pink), as a positive control, hippocampal synaptosomes RNA (blue) and negative control (yellow). Panel B is a representative image of the Southern blot showing the amplification and specificity of the real time RT-PCR, with a single amplicons (420 bp) from two hippocampal synaptosomes as well as from striatum, whereas no amplifications were detected in the negative control. Hippocampal axons from primary cultured neurons were shown to contain $A_{2A}R$ mRNA (C, D). Panel C shows a representative melting curve of the $A_{2A}R$ mRNA real time RT-PCR (n = 2) for soma fraction RNA (pink), as a positive control, axonal fraction RNA (blue) and negative control (yellow). Panel D is a representative image of the Southern blot showing the amplification and specificity of the real time RT-PCR, with single amplicons (420 bp) from two hippocampal axonal fractions, as well as from hippocampal somal fractions but no amplifications in the negative control.

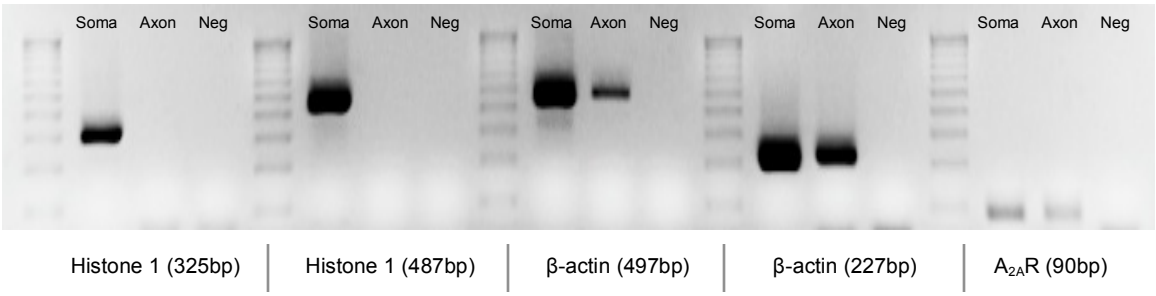

Figure 7.2. Hippocampal axonal fractions have β-actin mRNA, but not histone 1 mRNA. Real time RT-PCR followed by Southern blot in 2% agarose gel (n = 2) showed that the axonal fractions (axon) from hippocampal primary cultured neurons contain $A_{2A}R$ mRNA. This was demonstrated through the use of different sets of primers that produce an amplicon of 90 bp (last column); which were also positive for two different sets of primers for β-actin with amplicons of 497 and 227 bp (third and fourth columns respectively); moreover, the axonal fraction is negative for two different sets of primers for histone 1 with anplicons of 325 and 487 bp (first and second columns respectively). On the other hand, somal fraction (soma) of hippocampal primary cultured neurons was also shown to contain $A_{2A}R$, β-actin and histone 1 mRNAs.

Previous results obtained in the group (unpublished data from Attila Kövalvi) showed a very swift (≤ 30 min) gain of function of $A_{2A}R$ presynaptic nerve terminals ($A_{2A}R$-induced glutamate release after kainate (KA)) in the hippocampus. This prompted us to explore possible *de novo in loco* syntheses of $A_{2A}R$ protein. In agreement, we detected the presence of $A_{2A}R$ mRNA in hippocampal nerve terminals from mature brains, as well as in hippocampal pure axonal fractions from primary cultured neurons. Here we show that hippocampal nerve terminals previously incubated with ribonuclease, contain $A_{2A}R$ mRNA (Fig. 7.1A and 7.1B). Real time RT-PCR of RNA samples from hippocampal synaptosomes showed a specific amplification of a single amplicon (420bp), as well as from striatum (positive control) and no amplifications in the negative control (Fig. 7.1A and 7.1B). Hippocampal primary cultured neurons overlaid on microfluid plates were shown to contain $A_{2A}R$ mRNA in the somal fractions but also in the pure axonal fractions (Fig. 7.1 and 7.2). Real time RT-PCR with somal fractions showed the presence of all RNA investigated, i.e., $A_{2A}R$ mRNA, β-actin mRNA and histone 1 mRNA, tested with two different sets of primers for each condition (Fig. 7.1 and

7.2). Interestingly, real time RT-PCR with axonal fractions showed the presence of $A_{2A}R$ mRNA and β-actin mRNA but the absence of histone 1 mRNA, tested with two different sets of primers for each condition (Fig. 7.1 and 7.2).

4.2. *De novo* and *in loco* synthesis of $A_{2A}R$ in hippocampal presynaptic nerve terminals

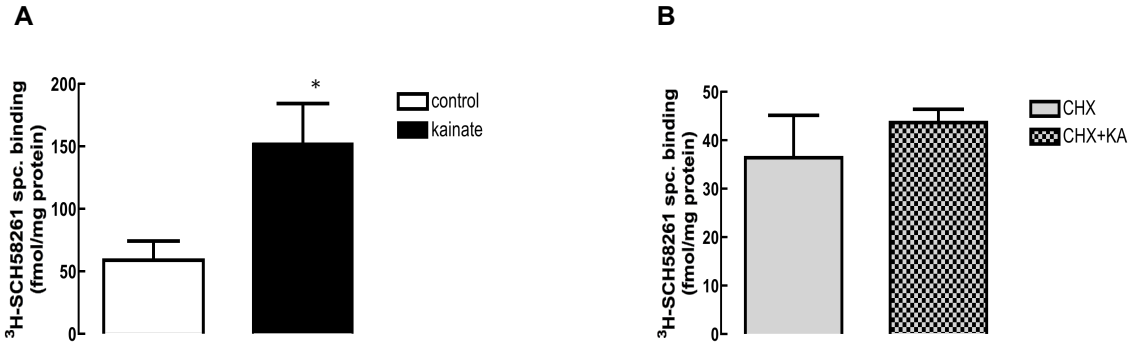

Figure 7.3. KA-induced increased $A_{2A}R$ in isolated hippocampal synaptosomes. Hippocampal synaptosomes incubated with kainate (5 μM) for 2 h followed by 2 h of incubation at 37 ºC without kainate showed increased [^{3}H]-SCH58261 specific binding (A); which was prevented by cyclohaximide (B).

7

In order to test the local protein synthesis in hippocampal synaptosomes we took advantage of KA stimulation that previously induced a very quick $A_{2A}R$-increased function. Accordingly, we incubated hippocampal synaptosomes with KA (5 μM) for 2 h at 37 °C in a 5 % CO_2 humidified atmosphere followed by 2 h of incubation without KA and next analyzed the $A_{2A}R$ density. Specific binding density for a specific $A_{2A}R$ antagonist ([^{3}H]-SCH58261) showed a significant increase after KA stimulation (Fig. 7.3A). In addition, KA-induced local synthesis was prevented by cycloheximide (an inhibitor of protein biosynthesis; Fig. 7.3B).

4.3. Deletion of hippocampal $A_{2A}R$ that does not generate impairment on the rotarod task

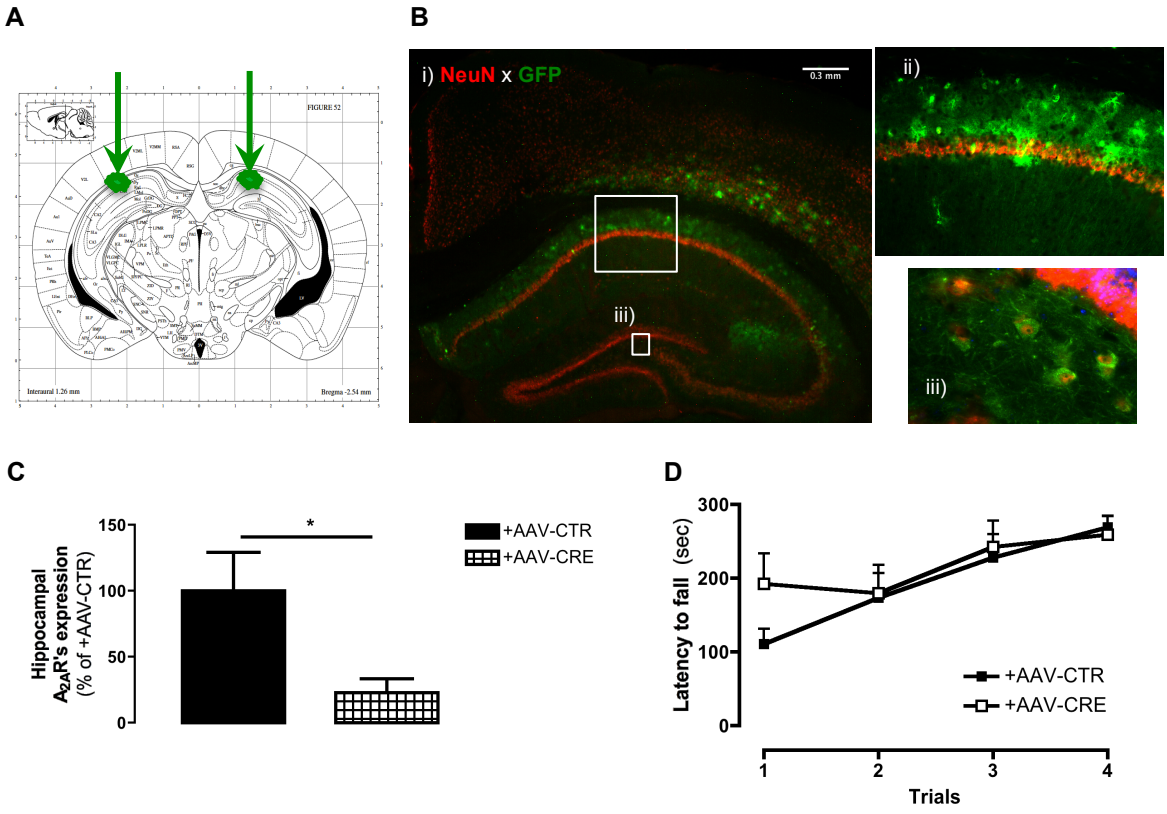

Figure 7.4. Deletion of hippocampal $A_{2A}R$ that does not generate impairment on the rotarod task. Deletion of hippocampal $A_{2A}R$ was obtained through local injection of adeno-associated virus (AAV)-Cre-GFP into the hippocampus of floxed-$A_{2A}R$ mice. Panel A is an illustration of the local of injection of AAV. Panel B shows a representative image (n = 4) of the immunohistochemistry showing NeuN (red) and GFP (green) expression in the hippocampus. Panel C depicts the qPCR analyses of hippocampal $A_{2A}R$ mRNA (n = 7) confirming the AAV-mediated $A_{2A}R$ deletion in hippocampus. Panel D shows the latency time during the trials in the accelerated rotarod of AAV-Cre-GFP (+AAV-CRE) and AAV-GFP (+AAV-CTR; n = 7). The data are mean ± SEM. **p* < 0.05, using a Student's *t* test in C or a two-way ANOVA followed by Bonferroni *post hoc* test in D.

Deletion of hippocampal $A_{2A}R$ was obtained through local injection of adeno-associated virus (AAV)-Cre-GFP into the hippocampus of floxed-$A_{2A}R$ mice. The expression of Cre was confirmed indirectly by the expression of a tag associated with Cre in the AAV, i.e. GFP (green) in the hippocampus (Fig. 7.4B). On the other hand, the knockdown of $A_{2A}R$ mediated by AAV-Cre on the hippocampus of floxed-$A_{2A}R$ mice was

confirmed by qPCR analyses of hippocampal $A_{2A}R$ mRNA, showing a reduced expression of $A_{2A}R$ in the AAV-CRE group of mice when compared with the control group (AAV-CTR; Fig. 7.4C).

It is well known that deletion of $A_{2A}R$ from medium spiny neurons in the dorsal striatum leads to impaired psychomotor coordination (Durieux et al., 2012). In order to discard a potential spread of AAV, we next tested if the mice knockdown on hippocampal $A_{2A}R$ were affected on the accelerated rotarod. We here showed that the latency time during the trials in the accelerated rotarod was similar between AAV-CRE and AAV-CTR (Fig. 7.4D), thus corroborating the accuracy of the hippocampal knockdown.

4.4. Anxiolytic phenotype observed in forebrain $A_{2A}R$ KO is not mimicked by hippocampal $A_{2A}R$ knockdown

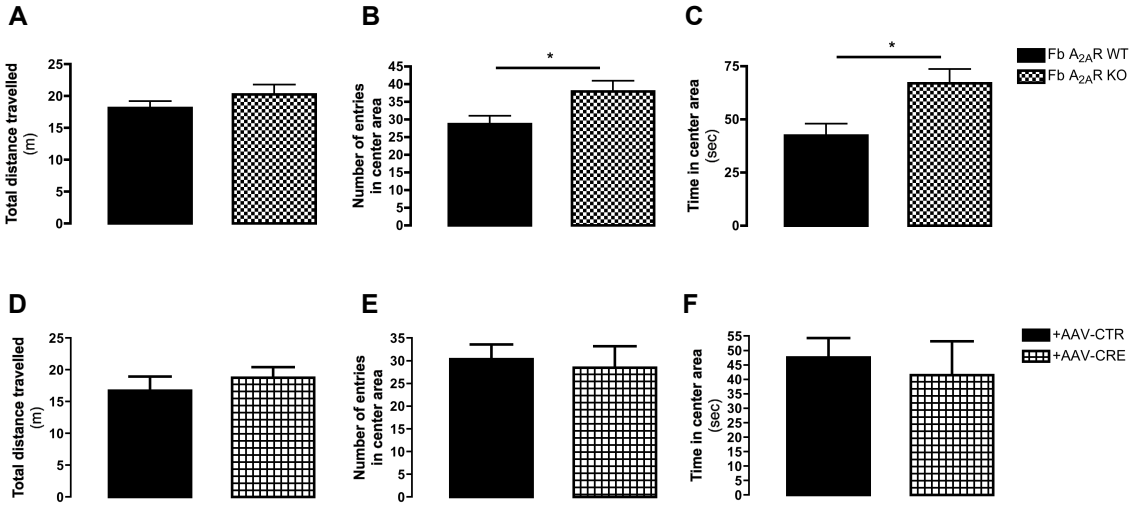

Figure 7.5. Anxiolytic phenotype observed in forebrain $A_{2A}R$ KO is not mimicked by hippocampal $A_{2A}R$ knockdown. The evaluation of spontaneous locomotion from forebrain $A_{2A}R$ (Fb $A_{2A}R$) KO mice or hippocampal $A_{2A}R$ knockdown (+AAV-CRE) was recorded in an open field. Panels A and D show that the total distance travelled by this two groups of animal lines was not altered when compared to their respective controls (Fb $A_{2A}R$ WT and +AAV-CTR). Panels B and C demonstrate that the number of entries (B) and time spent (C) in the center arena of open-field were increased in Fb $A_{2A}R$ KO mice when compared with the WT mice. On the other hand, panels E and F show that

the number of entries (E) and time spent (F) in the center arena of open-field were not changed in hippocampal $A_{2A}R$ knockdown (+AAV-CRE) when compared to the control group (+AAV-CTR). The data are mean ± SEM. *$p < 0.05$, using a Student's *t* test.

It was previously shown that deletion of $A_{2A}R$ in the entire forebrain (Fb $A_{2A}R$ KO) reduced anxiety-related behavior in the elevated plus maze but an indistinguishable behavior pattern was observed in striatal-selective $A_{2A}R$ deletion in the same paradigm (Catherine Wei PhD thesis). In order to know if hippocampal $A_{2A}R$ are the regional pool responsible for the anxiolytic profile observed in Fb $A_{2A}R$ KO, we tested both groups in the open field. In our paradigm, deletion of $A_{2A}R$ from the entire forebrain generated an anxiolytic profile, but an indistinguishable behavioral pattern in the hippocampal $A_{2A}R$ knockdown mice (+AAV-CRE; Fig. 7.5). In the open field Fb $A_{2A}R$ KO mice spent a greater proportion of time (Fig. 7.5C) and entered more frequently (Fig. 7.5B) into the center arena than did Fb $A_{2A}R$ WT mice. These findings are likely independent of the pattern of locomotion since the analysis of the distance traveled in the entire open field during the 8 min test failed to yield any genotypic difference between Fb $A_{2A}R$ KO mice and their respective WT controls (Fig. 7.5A). This anxiolytic-like behavior was not mimicked in hippocampal $A_{2A}R$ knockdown mice (+AAV-CRE), which presented no significant changes in the time (Fig. 7.5F) and number of entries (Fig. 7.5E) into the center arena than their respective controls, with no changes in the total locomotion in the open field (Fig. 7.5D). Together, these results suggest that the suppression of extra-striatal and extra-hippocampal $A_{2A}R$ are responsible to a reduced anxiety profile observed in Fb $A_{2A}R$ KO.

4.5. Working memory improvement observed in forebrain $A_{2A}R$ KO is mimicked by hippocampal $A_{2A}R$ knockdown

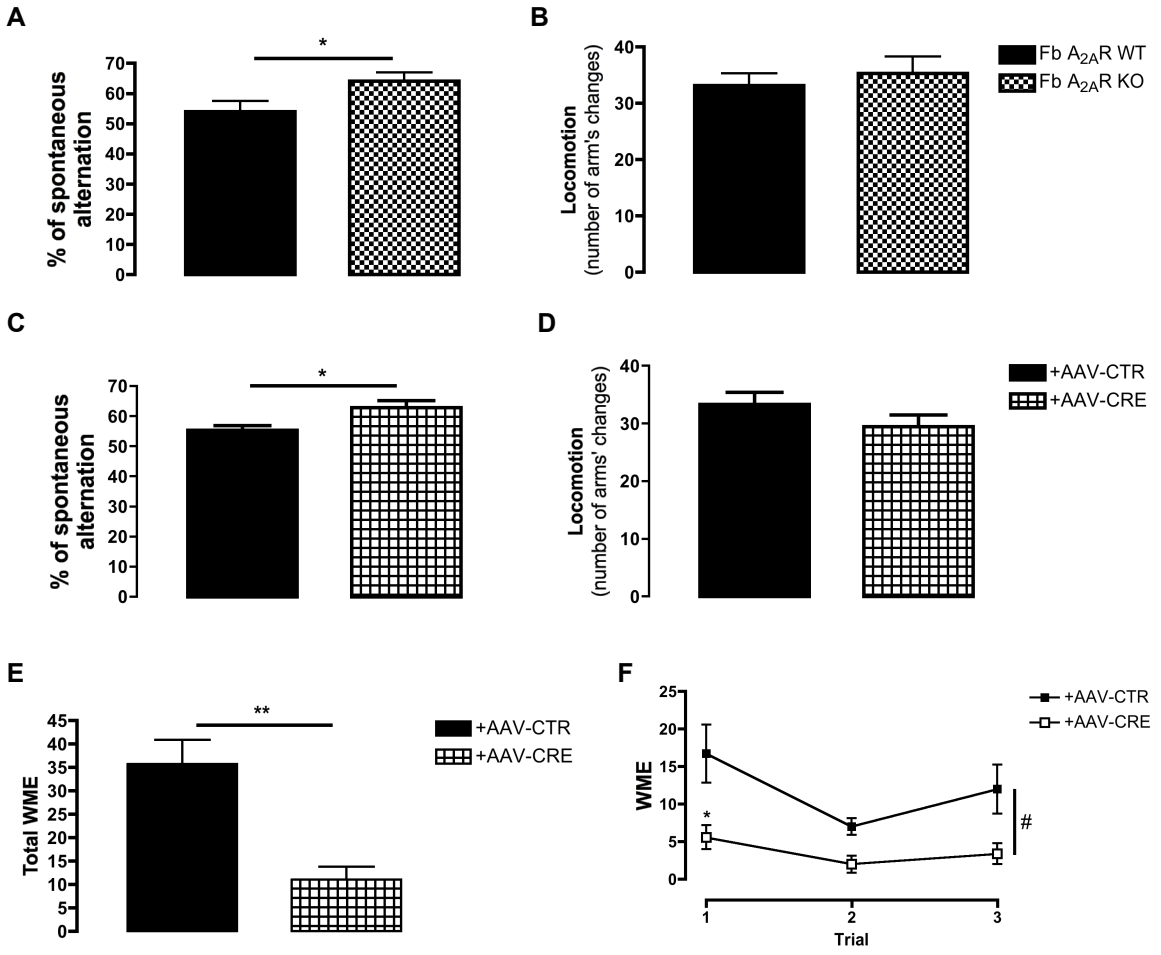

Figure 7.6. Working memory improvement observed in forebrain $A_{2A}R$ KO is mimicked by hippocampal $A_{2A}R$ knockdown. Forebrain $A_{2A}R$ KO mice (Fb $A_{2A}R$ KO) have an improved working memory when tested in a Y-maze paradigm analyzing their spontaneous alternation in comparison with the WT littermates (A), with no differences in locomotion (B), evaluated by number of arms' changes (n = 7-8). Hippocampal $A_{2A}R$ knockdown (+AAV-CRE) also have an improved working memory when tested in a Y-maze paradigm analyzing their spontaneous alternation in comparison with their control (C), with no differences in locomotion (D), evaluated by number of arms' changes (n = 7). In the 8 radial arm maze with 8 baited arms (E and F) +AAV-CRE displayed significantly less working memory errors (WME; scored each time an animal re-enter in a previous visited arm) in comparison with their control (n = 7). Panel E shows the total number of WME in the 3 trials, and panel F shows the total number of WME per trial. Data are mean ± SEM; *$p < 0.05$, **$p < 0.01$ using a Student's *t* test in A to E; #$p < 0.01$ in a two-way ANOVA followed by Bonferroni post-hoc test, *$p < 0.05$ in F.

It was previously shown that deletion of $A_{2A}R$ in the entire forebrain (Fb $A_{2A}R$ KO) improved spatial working memory in the water maze (Wei et al., 2011). In order to know if hippocampal $A_{2A}R$ also participate in the improvement of working memory observed in Fb $A_{2A}R$ KO, we tested both groups (Fb $A_{2A}R$ KO and hippocampal $A_{2A}R$ knockdown (+AAV-CRE)) in a spatial working memory paradigm where both showed improved working memory (Fig. 7.6). In the Y-maze Fb $A_{2A}R$ KO mice displayed more spontaneous alternations than the Fb $A_{2A}R$ WT mice (Fig. 7.6A). Likewise, in the same paradigm $A_{2A}R$ knockdown mice (+AAV-CRE) also exhibited more spontaneous alternations than their respective controls (Fig. 7.6C). These findings are likely independent of the pattern of locomotion since the analysis of the total arms' alternation profile during the 5 min test failed to yield any genotypic difference in both groups (Fig. 7.6B and 7.6D). In addition, in the 8 radial arm maze the hippocampal $A_{2A}R$ knockdown mice (+AAV-CRE) presented significant less working memory errors (WME) than the control littermates (Fig. 7.6E and 7.6F). Together, these results suggest that the suppression of hippocampal $A_{2A}R$ is conceivably responsible for the improved working memory observed in Fb $A_{2A}R$ KO.

4.6. Hippocampal $A_{2A}R$ knockdown selectively attenuate context (but not tone) fear conditioning

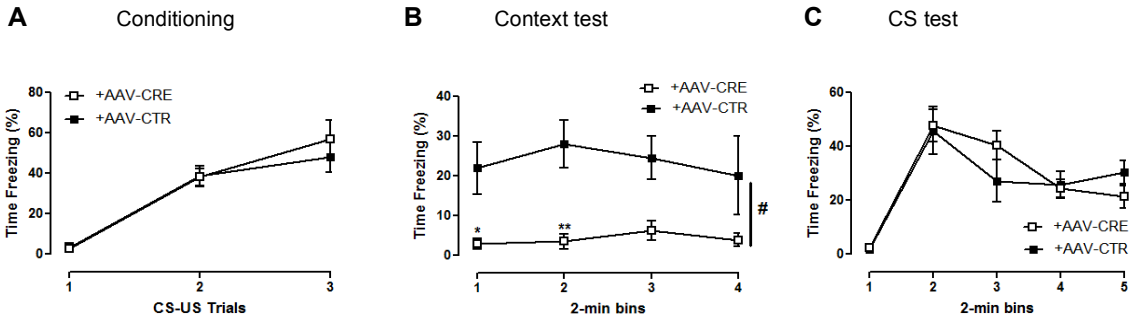

Figure 7.7. Deletion of hippocampal $A_{2A}R$ attenuates context (but not tone) fear conditioning. Panel A shows that acquisition of fear responses (conditioning) was similar between hippocampal $A_{2A}R$ knockdown mice (+AAV-CRE) and the control group (+AAV-CTR). Panel B shows that the context fear response (context test) was attenuated in the +AAV-CRE mice when compared with the +AAV-CTR mice. Panel C shows that the tone fear conditioning (CS test) was indistinguishable between groups. Data are mean ± SEM; #$p < 0.001$ using a two-way ANOVA followed by Bonferroni post-hoc test, *$p < 0.05$, **$p < 0.01$. CS, conditioned stimulus; US, unconditioned stimulus.

7

It was previously shown that deletion of $A_{2A}R$ in the entire forebrain produced an impaired on tone fear conditioning whereas striatal $A_{2A}R$ deletion generated an improved context and tone fear conditioning (Catherine Wei's PhD thesis). In order to determine if hippocampal $A_{2A}R$ is the pool responsible for any of this opposite behaviors observed between forebrain and striatal $A_{2A}R$ KO mice, we tested hippocampal $A_{2A}R$ knockdown mice in the same paradigm. During the conditioning phase, both the +AAV-CRE as well as their control (+AAV-CTR) showed similar acquired fear conditioning behavior over three training tone CS-US trials (Fig. 7.7A). On the following day, when these mice were exposed to the same environment (*i.e.* context test), the +AAV-CRE group displayed significantly weaker fear responses compared with the +AAV-CTR group (Fig. 7.7B). However, when the mice were tested for tone-induced conditioning (*i.e.* CS test), the fear response of +AAV-CRE and +AAV-CTR groups was identical (Fig. 7.7C). Thus,

deletion of $A_{2A}R$ restricted to the hippocampus by local AAV5-CMV-Cre-GFP infusion selectively reduced context (but not tone) fear conditioning.

5. Discussion

We here identified the presence of $A_{2A}R$ mRNA in two different types of preparations (*in vitro* and *ex vivo*) and revealed it to be local synthesized in hippocampal presynaptic nerve terminals. This important finding could be crucial for understanding the modulation of hippocampal functions, namely synaptic plasticity. In order to explore the direct participation of hippocampal $A_{2A}R$ in learning and memory behaviors, we developed a hippocampal $A_{2A}R$ knockdown model by local injection of a vector that expresses Cre into the dorsal hippocampus of $A_{2A}R$-floxed mice. These mice showed Cre expression throughout the dorsal hippocampus in addition to a decreased expression of $A_{2A}R$ in that brain area, with no contingent modifications in motor learning. Moreover, Fb $A_{2A}R$ KO mice displayed an anxiolytic phenotype that was not observed in the hippocampal $A_{2A}R$ knockdown mice. However, Fb $A_{2A}R$ KO mice displayed improved working memory, which was mimicked by hippocampal $A_{2A}R$ knockdown mice. Additionally, when hippocampal $A_{2A}R$ knockdown mice were tested in a fear-conditioning paradigm, they exhibited impaired context (but not tone) fear conditioning.

Synaptic plasticity is an important process for normal hippocampal activity. The presence of protein-synthesis machinery in axons and nerve terminals has created an entirely new perspective in the studies encompassing the cellular and molecular biology of neurons. The growth, differentiation, maintenance, plasticity and pathobiology of axons and nerve endings must now also be viewed in the context of local mechanisms that endow axons and nerve terminals with the capacity to respond in a fast and semiautonomous manner to local challenges (Alvarez et al., 2000; Alvarez, 2001). More recently, it was confirmed the *de novo* synthesis of ~80 different proteins in presynaptic

synaptosomes (Jiménez et al., 2002). In addition, the formation of long-term facilitation is crucially dependent on presynaptic protein synthesis (Martin et al., 1997; Casadio et al., 1999; Beaumont et al., 2001). Clearly, these observations indicate that synaptic plasticity is supported by local translational processes. In agreement, we here demonstrated the presence of $A_{2A}R$ mRNA in presynaptic nerve terminals, a receptor whose activation is involved in hippocampal synaptic plasticity, namely presynaptic release (Sebastião and Ribeiro, 1992; Cunha et al., 1994a; 1994b; 1994c; Latini et al., 1996; Ribeiro, 1999; Lopes et al., 2002). Despite, the fact that the available data for mammalian presynaptic mRNAs is still debatable (Alvarez et al., 2000), mRNA encoding for example, the presynaptic GAT-1 protein was recently found to be significantly enriched in rat synaptosomes (Crispino et al., 2001). It is known that growing neuritis and growth cones contain β-actin mRNA, (Bassell et al., 1994; Olink-Coux and Hollenbeck, 1996; Bassell et al., 1998), but not histone mRNA and we here showed that our preparation was also positive for β-actin mRNA (with two different sets of primers) and negative for histone mRNA (also with 2 different sets of primers). In addition, synaptosomal protein synthesis is strongly dependent on the ionic composition of the incubation medium, and on its osmolarity, namely Na^+ and K^+ concentrations (Autilio et al., 1968; Wedege et al., 1977; Weiler and Greenough, 1991; Crispino et al., 1993). In agreement, when we stimulated hippocampal presynaptic terminals with KA, which activates an ionotropic receptor permeable to Na^+ and K^+, the $A_{2A}R$ specific binding density significantly increased, which was prevented by cycloheximide. Perhaps one of the more intriguing questions concerning axonal and presynaptic protein synthesis centers on the mechanisms for local protein regulation. Such mechanisms might not be limited to the cognate cell body as an exclusive source of RNAs, but could also include the glial cell as a potential source, as indicated by evidence of local transcription of axonal RNAs (Edström et al., 1969; Alvarez et al., 2000). The possibility that periaxonal glial cells could transfer RNAs,

including mRNA, to the subjacent axon is clearly worthy of future investigation, as it might shed light on local modulatory mechanisms (Giuditta et al., 2008). However, our data do not support this latter mechanism, since $A_{2A}R$ mRNA was detected in axonal fractions of primary cultured neurons.

The hippocampus plays pivotal roles in different cognitive functions, namely memory that is critical for brain function and allows the encoding, storage and retrieval of information from the outside environment. Memory formation requires a well-organized orchestration of different mechanisms, and can be divided in short and long-term memory. The short-term phase does not require protein synthesis but the long-term phase does (Bacskai et al., 1993). The synaptic changes that occur during short-term memory are expressed even when protein synthesis is inhibited and seem to be mediated by a second messenger system such as cyclic AMP (cAMP) (Schwartz et al., 1971). Increased presynaptic cAMP, induces a Ca^{2+} influx, through PKA, contributing to presynaptic facilitation (Brunelli et al., 1976; Kandel et al., 1976; Klein and Kandel, 1980). Actually, it is known that $A_{2A}R$ activation increases cAMP and activates PKA (Chern et al., 1993; Gubitz et al., 1996; Chang et al., 1997; Huang et al., 2001; Chen et al., 2013) which in turn facilitates neurotransmitters' release (Gubitz et al., 1996; Cunha, 2008b; Ferré, 2010). Working memory is a type of short-term memory, which is known to have an important contribution from the hippocampus. Working memory captures important elements of cognitive flexibility, notably the capacity to maintain or update information held online and select appropriate behavioral responses in accordance to shifting positive and negative stimulus-response contingencies (Goldman-Rakic, 1995; Marié and Defer, 2003; Dalley et al., 2004). In addition, it is well established that the hippocampus contains a cellular representation of extrapersonal space - a cognitive map of space - and consequently hippocampal function is able to interfere with spatial tasks (Grant et al., 1992). Additionally, it was shown that global deletion of $A_{2A}R$ in mice

selectively enhanced working memory (Zhou et al., 2009). Conversely, overexpression of $A_{2A}R$ in the brain of transgenic rats impaired working memory performance, in transgenic rats (Giménez-Llort et al., 2007). Furthermore, it was shown that Fb $A_{2A}R$ KO and striatal $A_{2A}R$ KO revealed largely similar phenotypes with enhanced working memory (Wei et al., 2011), leading the authors to conclude that targeting striatal $A_{2A}R$ alone may be sufficient to facilitate working memory (Wei et al., 2011). The novel finding of our study is that selective inactivation of hippocampal $A_{2A}R$ alone was sufficient to reproduce the pro-cognitive phenotypes resulting from $A_{2A}R$ deletion extending to the entire forebrain (Wei et al., 2011). Our study is in agreement with an earlier transgenic study that suggested that $A_{2A}R$ in the cortex and in the hippocampus are able to modulate working memory (Giménez-Llort et al., 2007). However, the $A_{2A}R$'s well documented functional effects on neuronal plasticity at the hippocampus (Sebastião and Ribeiro, 1992; Cunha et al., 1994a; 1994b; 1994c; Latini et al., 1996; Ribeiro, 1999; Lopes et al., 2002) should be further explored in the $A_{2A}R$ knockdown mice.

On the other hand, the long-term memory requires the synthesis of new proteins. Long-term memory depends on the growth of new synaptic connections, which produce a structural changes paralleling the duration of the behavioral memory (Bailey and Chen, 1988; 1989; Bailey et al., 1992; Bailey and Kandel, 1993). In addition, it is well known that in the CA1 region of the hippocampus, LTP is induced postsynaptically by activation of NMDA receptors to glutamate, interfering with memory storage (Bliss and Lomo, 1973; Morris et al., 1986). Additionally, it is known that repeated trains of electrical stimuli generate the late phase of LTP, which requires the activation of PKA, MAPK, and CREB signaling pathways, which in turn induce new protein synthesis that appears to lead to the growth of new synaptic connections (Frey et al., 1993; Bourtchuladze et al., 1994; Nguyen et al., 1994; Yin and Tully, 1996; Bolshakov et al., 1997; Muller, 1997; Impey et al., 1998; Engert and Bonhoeffer, 1999; Ma et al., 1999; Nicoll and Malenka,

1999). Furthermore, the late phase of LTP associated with PKA mediated *de novo* protein synthesis is essential for the stabilization of the long term spatial map / place fields (Kentros et al., 2004). In agreement, PKA activation and protein synthesis in the hippocampus have a selective effect in long-term contextual memory, namely eliciting contextual fear conditioning, without changes in short-term memory (Abel et al., 1997). The production of newly synthesized proteins was additionally shown following training for memory consolidation (Davis and Squire, 1984; Matthies, 1989; Sutton and Schuman, 2006). Protein synthesis following training is composed of phases, beginning immediately after training and lasting for a few hours (Matthies, 1989; Belelovsky et al., 2009) and in some instances days (Bekinschtein et al., 2010). Many proteins synthesized after learning are products of new mRNAs that are also *de novo* transcribed. However, it was shown that protein synthesis during memory formation and its molecular correlates, LTP or LTD, can be independent of new RNA synthesis (Sutton and Schuman, 2006; Costa-Mattioli et al., 2009). It was also demonstrated that, within the dendrites of the hippocampus, new protein synthesis occurred too quickly (within minutes) to be explained by distant synthesis in the cell body and subsequent transport into the dendrites (Feig and Lipton, 1993), being preferably explained by a immediate local protein synthesis in dendrites (Kang and Schuman, 1996).

In agreement with our results, it is known that the activation of $A_{2A}R$ can activate MAPK (Cheng et al., 2002; Chen et al., 2013) and is crucial for LTP in the hippocampus (Rebola et al., 2008; Fontinha et al., 2009). Both processes are required for the generation of long-term contextual memory. In addition, it was shown that $A_{2A}R$ deletion in striatal neurons enhanced context and tone fear conditioning without affecting anxiety-like behavior. On the other hand, deleting $A_{2A}R$ in the entire forebrain normalized context and impaired tone fear conditioning, while also producing an anxiolytic phenotype in elevated plus maze (Wei et al., 2013). This led the authors to conclude that striatal and

extrastriatal $A_{2A}R$ may exert opposite control over fear conditioning, prompting consideration whether forebrain $A_{2A}R$ could be considered novel therapeutic targets to manage maladaptive fear responses. In accordance, our data showed that $A_{2A}R$ deletion in the hippocampus attenuated context (but not tone CS) fear conditioning, without modifications in the anxiety profile. These data reveal that hippocampal $A_{2A}R$ have an opposite phenotype to the striatal $A_{2A}R$, in respect to contextual fear conditioning, contributing to the normalized behavior observed when $A_{2A}R$ was deleted in the entire forebrain. The weaker conditioned freezing response in hippocampal $A_{2A}R$ knockdown mice may reflect an impaired learned fear response involving impaired long-term mnemonic processes, such as weaker memory traces of the learned stimulus- and context-shock associations. This notion is consistent with the impaired LTP effects seen after pharmacologic blockade of $A_{2A}R$ in the hippocampus (Rebola et al., 2008; Fontinha et al., 2009). Regarding the tone fear conditioning, we can conclude that the opposite behavior observed between striatal and forebrain $A_{2A}R$ deletion cannot be explained by a reduction of hippocampal $A_{2A}R$. In particular, the experiments with hippocampal $A_{2A}R$ knockdown revealed the role of this pool of $A_{2A}R$ in the control of context (but not tone) fear conditioning. Conversely, cortical $A_{2A}R$ may enhance tone fear conditioning since inactivating perirhinal, parietal, or cingulate cortical activity selectively impairs fear tone conditioning (Sacchetti et al., 2002; Bissière et al., 2008; Biedenkapp and Rudy, 2009). $A_{2A}R$ in the amygdala also might contribute to this result, given the role of this brain region in fear responses. Additional analyses with focal deletion of prefrontal cortical and amygdala $A_{2A}R$ by AAV-Cre local injection may identify the forebrain structure(s), where the loss of $A_{2A}R$ overrides the opposite impact of striatal $A_{2A}R$ deletion.

One behavioral aspect that could interfere with fear conditioning is the locomotion and anxiety behaviors. When $A_{2A}R$ deletion was restricted to the striatum, no change in anxiety-like behavior was observed, but when the deletion was extended to the entire

forebrain, anxiolytic behavior in the elevated plus maze was observed (Wei et al., 2013). This finding is consistent with the studies showing that the ventral hippocampus and cortex are relevant regions controlling anxiety (Bannerman et al., 2004; Adhikari et al., 2010; McHugh et al., 2011). However, the anxiolytic behavior observed after forebrain $A_{2A}R$ inactivation contrasts with the anxiogenic effect induced by acute caffeine (a nonselective adenosine receptor antagonist) (Cunha et al., 2008) and with genetic association studies between the $A_{2A}R$ gene and panic disorders (Deckert, 1998; Hamilton et al., 2004; Hohoff et al., 2010). Therefore, $A_{2A}R$ in brain regions beyond the forebrain need to be examined to better clarify this discrepancy. In agreement with the previous report (Catherine Wei's PhD thesis), we here showed an anxiolytic phenotype in the Fb $A_{2A}R$ KO in the open field, without changes in the locomotion. This phenotype is not attributable to dorsal hippocampal $A_{2A}R$ function since reduction of $A_{2A}R$ in that area did not show modifications in the open field.

In conclusion, we identified the presence of $A_{2A}R$ mRNA and the local synthesis of $A_{2A}R$ in the nerve terminals from mice adult brain. This is an important finding demonstrating that $A_{2A}R$ synthesis is prompt to a rapid and local regulation in order to rapidly respond to shifting synaptic conditions. In addition, to the best of our knowledge, this is the first study, to directly demonstrate that hippocampal $A_{2A}R$ activation impairs working memory and improves contextual fear conditioning. Importantly, these responses of hippocampal $A_{2A}R$ knockdown mice cannot be attributed to general emotional or hyperactivity behaviors.

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CHAPTER 8

GENERAL CONCLUSIONS

Conclusions

Adenosine is a neuromodulator that plays important roles in many physiological and pathological processes within the mammalian CNS, fine-tuning brain neurotransmission mainly acting through inhibitory A_1R and facilitatory $A_{2A}R$ (Fredholm et al., 2005a). However, the adenosinergic field is particularly challenging due to the difficulty in discriminating between the different sources of endogenous extracellular adenosine in the different pathways, with the involvement of the different adenosine receptors, frequently with opposite functions. Thus, the precise mechanisms of how the concentration of extracellular adenosine changes after the different stimuli remains controversial. This uncertainty stems from the potential complexity of adenosine origin, with a variety of release mechanisms, which may be different depending on the brain region, cellular type and on the properties of the stimulus (Latini and Pedata, 2001; Wall and Dale, 2008). Actually, the source of endogenous extracellular adenosine, namely during physiological conditions of neuronal firing has been one of the less studied aspects of adenosine neuromodulation until recently (Cunha, 2008a).

Adenosine can be directly released by active transport through bidirectional non-concentrative (equilibrative) nucleoside transporters (ENT) (Jonzon and Fredholm, 1985; White and MacDonald, 1990; Gu et al., 1995; Lovatt et al., 2012) or through direct exocytotic release by neurons (Klyuch et al., 2012). Adenosine can also be indirectly released following rapid extracellular ATP metabolism (Dunwiddie et al., 1997), with ATP released by exocytosis from neurons (White and MacDonald, 1990; Jo and Schlichter, 1999; Pankratov et al., 2007) or glial cells (Zhang et al., 2003; Pascual et al., 2005; Serrano et al., 2006), or even via gap junction hemi channels (Pearson et al., 2005; Huckstepp et al., 2010). Extracellular ATP can then be extracellularly metabolized into adenosine and activates adenosine receptors (Koizumi et al., 2003; Newman, 2003; Zhang et al., 2003; Pascual et al., 2005; Serrano et al., 2006; Panatier et al., 2011).

Adenosine can also be reuptaked by ENT, deaminated by adenosine deaminase (ADA) under normal metabolic conditions (Latini and Pedata, 2001) or phosphorylated by adenosine kinase (ADK) back into ATP (Boison, 2006). In addition, ATP has also been shown to be reuptaked and stored in synaptic vesicles on nerve terminals (Sperlágh et al., 2003) and astrocytes (Coco et al., 2003). Most of these adenosine release mechanisms have been described for some time, however the difficulties to discriminate the contribution of the different components were made more complex by the limitations of contemporaneous techniques (see chapter 1, section 4.4). In addition, the release characteristics might vary between different preparations, brain area, cell type and type of stimulus.

Adenosine can either inhibit or facilitate neurotransmission, and either be neuroprotective or promote neurodegeneration during pathological states depending on the brain region affected and the subtype of receptor activated (Cunha, 2008a; Boison, 2009; Dale and Frenguelli, 2009). A_1R are abundantly expressed throughout the brain, inhibiting synaptic transmission (Dunwiddie and Masino, 2001). A_1R activation may depend on the tissue workload (Cunha, 2001a) and therefore ADK activity has been established as a key regulator of endogenous adenosine when activating A_1R (Boison, 2011). By contrast to A_1R, the source of the adenosine activating $A_{2A}R$ is poorly characterized. In order to explore a functional association between ecto-5'-nucleotidase (CD73), the only enzyme able to dephosphorylate extracellular AMP into adenosine in the brain (Lovatt et al., 2012), and $A_{2A}R$, we here explored the macroscopic location of CD73, showing that it is highly expressed and co-localized with $A_{2A}R$ in different nuclei of the basal ganglia, with a preferential postsynaptic location, where $A_{2A}R$ is prominently located (Schiffmann et al., 1991b; Rebola et al., 2005a). This association was further corroborated by a high-resolution proximity ligation assay, showing a close proximity between CD73 with $A_{2A}R$. These data were additionally confirmed by recent findings

obtained by Ena et al., 2013, showing that CD73 is specifically expressed in the striatopallidal neurons of the indirect pathway, specifically where $A_{2A}R$ are also most abundantly located (Schiffmann et al., 1991a). In addition we showed that CD73 is responsible for the formation of the adenosine that activates striatal $A_{2A}R$, since the release of inorganic phosphate from AMP was almost abolished in striatal synaptosomes from CD73 KO mice, as well as the formation of cAMP after incubation with a new prodrug that requires dephosphorylation from CD73 to become an adenosine agonist. These data were also corroborated by Ena et al., 2013, who showed that CD73 is responsible for the generation of most of the extracellular ATP-derived adenosine in the striatum, since the formation of adenosine from ATP was almost abolished in the striatal slices from CD73 KO mice.

Interestingly, the concentration of evoked adenosine varies between different brain regions, and it was showed that the highest concentration of adenosine is evoked in the nucleus accumbens and dorsal caudate-putamen (Pajski and Venton, 2013), where high levels of CD73 and $A_{2A}R$ are present (Schiffmann et al., 1991b). On the other hand, A_1R are expressed in every brain region and do not correlate as well with high levels of stimulated adenosine release (Pajski and Venton, 2013). In agreement, it was shown that $A_{2A}R$ are engaged to assist the implementation of synaptic plasticity changes in excitatory synapses (Cunha, 2008a), by facilitating NMDA receptor-mediated responses (Rebola et al., 2008), by increasing glutamate release (Rodrigues et al., 2005) and by desensitizing presynaptic inhibitory modulation of systems like A_1R (Lopes et al., 2002; Ciruela et al., 2006a) or cannabinoid CB_1R (Martire et al., 2010). Therefore, $A_{2A}R$ play a key role in modulating the plasticity of neuronal circuits, such as upon learning and memory (Zhou et al., 2009; Wei et al., 2011) or drug addiction (Chen et al., 2003). Notably, neurodegenerative conditions are accompanied by an up-regulation of $A_{2A}R$ (Cunha, 2005), justifying that $A_{2A}R$ blockade controls the burden of Parkinson's (Chen et

al., 2001b) or Alzheimer's diseases (Canas et al., 2009b). In agreement, our studies showed that CD73 activity is responsible for the formation of the adenosine involved in different basal ganglia functions that have the participation of $A_{2A}R$, like the hypolocomotion, motor coordination or working memory, but also in different disorders like drug sensitization, MK-801-induced psychomotor activity and MPTP-induced neurodegeneration.

Notably, this proposed activation of $A_{2A}R$ selectively by CD73-mediated formation of adenosine seems to be present not only in the striatum, but also in other tissues and cell types. Indeed, most of the collected data were obtained in hippocampal preparations, where it was previously shown that different sources of adenosine activate A_1R or $A_{2A}R$ (Cunha et al., 1996a) and that $A_{2A}R$ are selectively activated upon extracellular catabolism by ecto-nucleotidases of ATP (Cunha et al., 1996a; Rebola et al., 2008). In agreement, it was shown that the ATP-derived formation of adenosine by ecto-nucleotidases is limited and controlled by CD73 activity in the hippocampus (Cunha, 2001b). Additionally, it was shown that the inhibition of CD73 blunts the ability of $A_{2A}R$ to control synaptic plasticity in hippocampal slices (Rebola et al., 2008). In agreement, several groups concluded that the inhibition or genetic deletion of CD73 failed to affect the modulation of synaptic transmission by A_1R either in physiological or pathological conditions (Brundege and Dunwiddie, 1996; Cunha et al., 1996a; Lovatt et al., 2012; Zhang et al., 2012), in contrast to the conclusions derived from a transgenic mouse with hampered release of gliotransmitters (Pascual et al., 2005). However, presently it is still difficult to establish if the close proximity between CD73 activity and $A_{2A}R$ activation is a widespread and general phenomenon or if it occurs only in specific pathways or only plays a role under particular conditions, e.g., basal stimulation, high frequency stimulation or different pathological conditions.

Regarding the different sources of adenosine in the hippocampus, most of the

studies explored A_1R functions and it is known that astrocytes play an important role, starting with the finding of an astrocytic source of released ATP and ensuing adenosine (Fields and Burnstock, 2006; Haydon and Carmignoto, 2006). In agreement, it was shown that inducible transgenic mice with reduced astrocytic release of gliotransmitters, showed reduced ATP release in hippocampal CA1 region, which in turn led to a lower accumulation of adenosine and the disappearance of the tonic A_1R-mediated inhibition on synaptic transmission in hippocampal slices (Pascual et al., 2005). This implies that the endogenous extracellular adenosine responsible for the tonic A_1R-mediated inhibition of excitatory synaptic transmission is largely derived from ATP released from astrocytes, excluding the role of ENT, which was shown to participate in the uptake rather than release of adenosine (Pascual et al., 2005). Another elegant study also showed that astrocytes in the hippocampal CA1 region were able to detect synaptic activity induced by single-synaptic stimulation, increasing basal synaptic transmission through ATP-derived adenosine release, which led to the activation of presynaptic $A_{2A}R$, a mechanism that was shown to be dependent of astrocytic mGlu5 receptor activation (Panatier et al., 2011). In contrast, the study of Lovatt et al., (2012) showed that selective activation of postsynaptic CA1 neurons leads to direct release of adenosine and following synaptic depression mediated by A_1R activation, supporting the idea that adenosine-mediated synaptic depression is not a consequence of astrocytic ATP release, but is instead a direct neuronal adenosine release (Lovatt et al., 2012). In contrast, Zhang et al. (2012) showed that the inhibitory effects of A_1R activation in hippocampal slices were enhanced by an ENT inhibitor, showing that ENT capture but do not release adenosine. Further adding to the complexity and controversy of the topic was the finding that the formation of adenosine that activates A_1R in the CA1 area of the hippocampus during basal, hypoxic or ischemic conditions is not CD73-dependent (Zhang et al., 2012). In addition, using cerebellum slices, it was described an additional

mechanism of adenosine release that occurs in parallel to the release of ATP, that represents the direct release of adenosine, and that strongly supports an exocytotically mechanism of adenosine release (Klyuch et al., 2012). More recently Wall et al. (2013) provided evidence for the occurrence of these two parallel distinct mechanisms of adenosine release – ATP exocytosis and adenosine transporter-mediated – in the CA1 area of the hippocampus, both contributing to A_1R activation. The fast, direct release via neuronal ENT, while the slower exocytotic release via the catabolism of extracellular ATP released from glial cells. In addition, Lee et al. (2013) found that mice with decreased ATP release from astrocytes have lower thresholds to induce LTP, demonstrating that a low concentration of adenosine acting through A_1R in GABAergic neurons selectively attenuated inhibitory neuronal activity.

It is noticeable that the field of adenosine release has been characterized by considerable multiplicity of mechanisms and lack of clarity, and even conflict, as to which mechanisms may be physiologically important. For a long time direct adenosine release was seen as occurring only under pathological conditions. A widely held consensus is that, except under pathological conditions, extracellular adenosine arises only from ecto-nucleotidases-mediated metabolism of previously released ATP. Actually, the adenosine can theoretically, be released through ENT (Kong et al., 2004). However, the effect of pharmacologically manipulating of the ENT in most of the studies is an increase of the extracellular levels of adenosine implying that their role is to uptake rather than release adenosine (Fredholm et al., 2005a). Thus, in integrated brain preparations under physiological conditions, most of the studies do not support the argument that adenosine is released as such through ENT. However, unlike extracellular adenosine accumulation detected in response to physiological stimuli, the source of adenosine released by metabolic distress is mainly intracellular (Frenguelli et al., 2007; Martín et al., 2007; Dale and Frenguelli, 2009), which may involve also carrier systems (Sperlágh et al., 2003).

This phenomenon is probably related with ADK expression and activity under those conditions (Boison, 2013). Thus, it is also important to take into account the age of the mice used in the different studies, since the expression of ADK could change, producing changes in intracellular adenosine concentration in the different cellular compartments, which in turn changes the adenosine efflux (Studer et al., 2006).

Interestingly, most of the studies in the hippocampus explored astrocytic ATP release and A_1R function. In order to clarify if astrocytic ATP release participates in $A_{2A}R$ activation, further studies like the one performed by Panatier et al. (2011) are required. In addition, CD73 activity could be correlated with neuronal release of ATP. Actually, it is possible that some gliotransmitters may indirectly cause the release of ATP and/or adenosine from neurons. In agreement, adenosine could be formed extracellularly upon catabolism of released ATP originated from synaptic vesicles (Sperlágh and Vizi, 1992), or in addition, could be released from the postsynaptic neurons as a consequence of the activation of ionotropic glutamate receptors (Vizi et al., 1992; Dunwiddie and Diao, 1994). In fact, it was shown that stimulated nerve terminals can directly release ATP, which is stored in synaptic vesicles (Pankratov et al., 2006). However, this stimulation-evoked release of ATP from nerve terminals seems to differ from the release of classical neurotransmitters (Rabasseda et al., 1987; Fariñas et al., 1992; Santos et al., 1999; Coco et al., 2003; Magalhaes-Cardoso et al., 2003). In particular, this release of ATP is disproportionally larger at higher frequencies of nerve stimulation (Wieraszko et al., 1989; Cunha et al., 1996b).

Recently, a vesicular nucleotide transporter (VNUT) capable of transporting ATP into vesicles was identified in the brain (Sawada et al., 2008). Interestingly, VNUT colocalized with synaptic vesicles in excitatory and inhibitory terminals of hippocampal formations and was enriched in preterminal axons and present in postsynaptic dendritic spines (Larsson et al., 2012), mimicking the distribution of hippocampal $A_{2A}R$ (Rebola et

al., 2005a). It was also shown the presence of VNUT in a subset of vesicular glutamate transporter 1 (VGLUT1)-containing vesicles, showing that VNUT mediates transport of ATP into synaptic vesicles of hippocampal glutamatergic neurons, thereby conferring a purinergic phenotype to these cells (Larsson et al., 2012). This supports the role of facilitation of excitatory transmission operated by $A_{2A}R$, which are activated by adenosine formed from neuronally released ATP (Cunha, 2001a). In order to known if the association observed between CD73 activity and $A_{2A}R$ activation could be correlated with neuronal release of ATP, further studies with conditional KO mice for VNUT in astrocytes and different pools of neurons should be performed.

Our study also explored the role of CD73 and $A_{2A}R$ in the hippocampus. We here showed that CD73 does not generate the anti-convulsive adenosine that activates A_1R in epilepsy. Instead CD73 generates adenosine that produces neuronal loss, a role that is usually associated to $A_{2A}R$ activation. This suggests an important unappreciated role of CD73-derived adenosine on $A_{2A}R$ activation on epilepsy. In addition, our results suggest that astrocytic $A_{2A}R$ activation is anticonvulsive and neuroprotective, in opposition to neuronal $A_{2A}R$, whose activation appears to be proconvulsive and induces neuronal loss. These data imply a differential and delicate coordinated action of astrocytic $A_{2A}R$ and neuronal $A_{2A}R$ to fine-tune glutamatergic transmission. Overall, this work implies the necessity to investigate if pharmacological inhibition of CD73 can be therapeutically relevant in epilepsy and points to the necessity in discriminating between the two cellular compartments (neuron or astrocytic) during the development of adenosinergic-based therapeutic strategies for epilepsy and other disorders of the central nervous system. In addition, we showed the presence and local synthesis of $A_{2A}R$ mRNA at the presynaptic nerve terminals from the hippocampus. This is an important finding, showing that $A_{2A}R$ synthesis is fast and locally regulated in order to promptly modulate the synaptic environment. In agreement we showed that hippocampal $A_{2A}R$ participates in cognitive

behaviors; impairs working memory and improves contextual fear conditioning. This suggests that the participation of hippocampal CD73-derived adenosine and hippocampal $A_{2A}R$ is far more complex than previously thought and therefore advocates the need of further studies in order to better clarify these two cellular sources of adenosine.

In conclusion, it is still debatable if the close proximity between CD73 activity and $A_{2A}R$ activation is a specific and general mechanism. Additionally, it is still difficult to understand the spatial cellular and subcellular specificity of this system. Addressing such questions may require the further refinement and development of new techniques in order to monitor the CD73-dependent adenosine and the subcellular $A_{2A}R$ activation. However, due to the preferencial localization of CD73 at the postsynaptic density in all brain areas studied, it is tempting to suggest that most of this specific association may occur postsynaptically. This is also supported by our data obtained in the striatum and hippocampus, namely the data showing that inhibition of CD73 blunts $A_{2A}R$ function postsynaptically (Rebola et al., 2008). Additionally, this may suggest that the trigger of this specific system is the neuronal release of ATP, which would corroborate the view that the facilitation of $A_{2A}R$ is associated to tetanized synapses where extracellular ATP is generated in sufficient amounts to activate $A_{2A}R$ (Almeida et al., 2003); this would provide a biological meaning to the feed-forwardly inhibition of CD73 by ATP, which results in a delayed, 'burst-like' adenosine production (Cunha, 2001b). This might only occur upon high-frequency stimulation, characteristic of long-term potentiation (Rebola et al., 2008), representing a local mechanism to facilitate the implementation of synaptic plasticity, with the participation of VNUT, CD73 and postsynaptic $A_{2A}R$. The previous association of ATP and A_1R activation is mainly associated with astrocytic ATP release, despite some recent studies that do not correlate CD73-dependent adenosine and A_1R activation (Lovatt et al., 2012; Zhang et al., 2012). However, this could be also explained

by the recent report that AMP is an A_1R agonist (Rittiner et al., 2012), showing that A_1R activation could be ATP-dependent and CD73-independent. Despite the complexity regarding the extracellular endogenous adenosine, the data here presented suggest that CD73 is a possible therapeutic target to fine-tune $A_{2A}R$ activation in the brain. However, the use of CD73 inhibitors will avoid the side effect of $A_{2A}R$ blockade as vasoconstrictor (Nair et al., 2011), but it could have a proinflammatory side effect (Koszalka et al., 2004; Takedachi et al., 2008; Reutershan et al., 2009).

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