Neuroprotection by adenosine receptors in aged rats – role of neuroinflammation

Neuroprotecção através dos receptores de adenosina em animais idosos – papel da neuroinflamação

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ABBREVIATIONS LIST

- **4-Br-A23187** - 4-bromo-calcium ionophore A23187
- **5'Ntase** - 5'-nucleotidase
- **8 Br-cAMP** - 8-bromo-adenosine-3',5'-cyclic monophosphate
- **A_1R** - Adenosine A_1 receptor
- **A_{2A}R** - Adenosine A_{2A} receptor
- **A_{2B}R** - Adenosine A_{2B} receptor
- **A_3R** - Adenosine A_3 receptor
- **AC** - Associational commissural pathway
- **ACB** - *Nucleus accumbens*
- **AD** - Alzheimer's disease
- **ADP** - Adenosine 5'-diphosphate
- **AK** - Adenosine kinase
- **AM** - Extended amygdala
- **AMP** - Adenosine monophosphate
- **AMPA** - α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid
- **AP** - Alkaline phospahatase
- **APP** - Amyloid precursor protein
- **APS** - Ammonium persulphate
- **AR** - Adenosine receptors
- **ARNO** - Nucleotide exchange factor
- **ATP** - Adenosine 5'-triphosphate
- **Aβ** - Beta-amyloid peptide
- **BBB** - Blood-brain barrier
- **BSA** - Bovine serum albumin
- **ca** - Commissural-associational zone
- **Ca^{2+}** - Calcium
- **cAMP** – Adenosine 3',5'-cyclic monophosphate
- **CB** - Cerebellum
- **CB_1R** - Cannabinoid 1 receptor
- **CC/VC** - Visual and cingulate cortex
- **CCPA** - 2-chloro-N^6-cyclopentyladenosine
- **CD73** - Ecto-5'-nucleotidase
- **cDNA** - Complementary deoxyribonucleic acid
- **CGS21680** - 4-[2-[[6-amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino] ethyl]benzenepropanoic acid
- **ChAT** - Choline acetyltransferase
- **Cl-IB-MECA** - 2-chloro-N6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide
- **CNS** - Central nervous system
- **CP** - Caudate putamen
- **CPA** - N^6-cyclopentyladenosine
- **CR3** - Complement type 3 receptor
- **CREB** - Cyclic-adenosine monophosphate responsive element-binding protein
- **D2R** - D2 dopamine receptor
- **DG** - Dentate gyrus

- **DIV** - Days *in vitro*
- **DMSO** - Dimethyl sulfoxide
- **DPCPX** - 1,3-Dipropyl-8-cyclopentylxanthine
- **D.P.X.** - Di-N-butylphthalate in xylene — mounting medium
- **DTT** - DL-Dithiothreitol
- **EAAT-1** - Excitatory amino acid transporter 1
- **EATT-2** - Excitatory amino acid transporter 2
- **EC** - Entorhinal cortex
- **ECF** - Enhanced chemifluorescence
- **ED1** - Macrosylianin
- **EDTA** - 2-[2-(bis(carboxylatomethyl)amino)ethyl-(carboxylatomethyl)amino]acetate
- **EFS** - Electric field stimulation
- **EGTA** - Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
- **ENT** - Equilibrative nucleoside transporters
- **E-NTPDases** - ectonucleoside triphosphate diphosphohydrolases
- **EU** - European Union
- **FAM** - Carboxyfluorescein
- **FCCP** - Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone
- **FJ-C** - FluoroJade C
- **GABA** - γ-Aminobutyric acid
- **GAPDH** - Glyceraldehyde 3-phosphate dehydrogenase
- **GFAP** - Glial fibrillary acidic protein
- **GIRK** - G-protein-dependent inwardly rectifying K^+ channels
- **GP** - *Globus pallidus*
- **GPCR** - G-protein-coupled-receptors
- **GRK-2** - G-protein coupled receptor kinase 2
- **H-89** - N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide
- **Halothane** - 1-bromo-1-chloro-2,2,2-trifluoroethane
- **HBM** - HEPES buffered medium
- **HBS** - HEPES buffered saline
- **HBSS** - Hank's balanced salt solution
- **Hcy** - Homocysteine
- **HEPES** - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **HEX** - Hexachloro-Fluorescein
- **HIP** - Hippocampus
- **HIV** - Human Immunodeficiency Virus
- **HPLC** - High performance liquid chromatography
- **HPRT1** - Hypoxanthine phosphoribosyltransferase 1
- **HYP** - Hypothalamus
- **icv** - Intracerebroventricular
- **IL-10** - Interlerukin-10
- **IL-1ra** - Interleukin-1 receptor antagonist
- **IL-1β** - Interleukin-1beta
- **INFγ** - Interferon-gama
- **ip** - Intraperitoneal
- **JNK/SAPK** - c-Jun N-terminal kinase/stress-activated protein kinase
- **KA** - Kainate
- **KD** - Equilibrium constant dissociation

- **kDa** - Kilodaltons
- **KO** - Knockout
- **KW6002** - ((E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6--dione)
- **LC** - *Locus coeruleus*
- **LEC** - Lateral enthorhinal cortex
- **LPS** - Lipopolysaccharide
- **LTP** - Long-term memory
- **LUF 5835** - 2-amino-4-(3-hydroxyphenyl)-6-(1H-imidazol-2-ylmethylsulfanyl)pyridine-3,5--dicarbonitrile
- **MAP-2** - Microtubule-associated protein 2
- **MAPK** - Mitogen-activated protein kinase
- **MDC** - Mature dendritic cells
- **MEC** - Medial enthorhinal cortex
- **MF** - Mossy fibers
- **mGluR5** - Metabotropic glutamate receptor 5
- **MHC class I or II** - Major histocompatibility class I or II
- **MK801** - [(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate)]
- **MPTP** - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
- **MRE2029-F20** - N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H--purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]acetamide
- **mRNA** - Messenger ribonucleic acid
- **MRS 1754** - N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8--yl)phenoxy]-acetamide
- **MTT** - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- **NC** - neocortex
- **NECA** - Adenosine-5′-N-ethylcarboxamide
- **NMDA** - N-methyl-D-aspartic acid
- **NO** - Nitric oxide
- **NTPDase 1** - CD39 - Ecto-nucleoside-triphosphate-diphosphohydrolase 1
- **OB** - Olfactory bulbs
- **OT** - Olfactory tubercle
- **PBS** - Phosphate buffer saline
- **PCR** - Polymerase Chain Reaction
- **PD** - Parkinson’s disease
- **PI** - Propidium iodide
- **PKA** - Protein kinase A
- **PLC** - Phospholipase C
- **PLD** - Phospholipase D
- **PMSF** - Phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride
- **PP** - Perforant pathway
- **PVDF** - Polyvinylidene fluoride
- **ROIs** - Regions of interest
- **SAH** - S-adenosylhomocysteine
- **SAHase** - S-adenosyl homocysteine hydrolase
- **Sb** - Subiculum
- **SB202190** - 4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol
- **SC** - Schaffer collateral

- **SCH58261**- 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2, 4]triazolo[1,5-c] pyrimidin-5-amine
- **SDS** - Sodium dodecyl sulfate
- **SDS-PAGE** - Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
- **SEM** - standard error of the mean
- **SNAP-25** - Synaptosome-associated protein of 25000 daltons
- **SYBR** - Green I nucleic acid gel stain
- **TBS-T** - Tris-Buffered Saline with Tween-20
- **TEMED** - N,N,N',N'-Tetramethylethylenediamine
- **TGF-β** - Transforming growth factor beta
- **TLE** - Temporal lobe epilepsy
- **TLR4** - Toll-like receptors 4
- **TMRM$^+$** - Tetramethyl rhodamine methyl ester
- **TNF-α** - Tumor necrosis factor alpha
- **TRIS** - Tris(hydroxymethyl)aminomethane
- **TTX** - tetrodoxin
- **vAChT** - Vesicular acetylcholine transporter
- **vGAT** - Vesicular GABA transporter
- **vGLUT1** - Vesicular glutamate transporter 1
- **vGLUT2** - Vesicular glutamate transporter 2
- **vGLUT3** - Vesicular glutamate transporter 3
- **vIAAT** - Vesicular inhibitory amino acid transporter
- **vMATs** - Vesicular monoamine transporters
- **VNTs** - Vesicular neurotransmitter transporters
- **WT** - Wild-type
- **ZM241385** - 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl)phenol
- **ΔΨm** - Modification of mitochondrial membrane potential

LIST OF PUBLICATIONS

The experimental work presented has been carried out by the author. In **sub-chapter 4.1**, tissue collecting, processing, and Western blot were made in collaboration with João Duarte, Ricardo Rodrigues and Attila Köfalvi (Center for Neuroscience of Coimbra – Purines at CNC); in **sub-chapter 4.2**, Aβ-injected model in rats was optimized in collaboration with Geanne Cunha (Department of Physiology and Pharmacology, Federal University of Ceará, Brazil), whereas viability assays and synaptotoxicity in hippocampal cultures was done in collaboration with Lisiane Porciúncula (Dept Biochemistry, ICBS, UFRGS, Porto Alegre, Brazil); in **sub-chapter 4.3**, Western blot and immunocytochemistry of synaptosomes was done in collaboration with Ricardo Rodrigues (Center for Neuroscience of Coimbra - Purines at CNC); in **sub-chapter 4.4**, electrical field stimulation and imaging of hippocampal cell culture was done in collaboration with Jorge Oliveira (REQUIMTE, Serviço de Farmacologia, Faculdade de Farmácia, Universidade do Porto); in **sub-chapter 4.5**, the results were obtained in collaboration with Geanne Cunha; in **sub-chapter 4.6**, perforant pathway lesion results were obtained in collaboration with Bente Finsen's group (Institute of Medical Biology, Anatomy and Neurobiology, University of Southern Denmark), whereas kainate-induced convulsions studies were carried out in collaboration with Lisiane Porciúncula and Inês Araújo (Center for Neuroscience of Coimbra). All the work presented in this thesis was supervised by Rodrigo Cunha, except the results presented in **sub-chapter 4.4**, which were co-supervised by Jorge Oliveira and Rodrigo Cunha and in **sub-chapter 4.6**, which were co-supervised by Bente Finsen and Rodrigo Cunha.

The following papers were published in peer-reviewed international scientific journals during the development of this thesis, some of them including data presented in this dissertation:

I- Rebola, N.; Canas, P.M.; Oliveira, C.R.; Cunha, R.A.; "Different subsynaptic distribution of adenosine A_{2A} receptors in the hippocampus and striatum of the rat." *Neuroscience* 132 (2005) 893–903.

II- Cunha, G.M.*; Canas, P.M.*; Oliveira, C.R.; Cunha, R.A.; "Increased density and synapto-protective effect of adenosine A_{2A} receptors upon sub-chronic restraint stress." *Neuroscience* 141 (2006) 1775-81.

III- Rodrigues, R.J.; Canas, P.M.; Lopes, L.V.; Oliveira, C.R.; Cunha, R.A.; "Modification of adenosine modulation of acetylcholine release in the hippocampus of aged rats." *Neurobiology of Aging* 29(10) (2008) 1597-601.

IV- Silva, C.G.*; Porciúncula, L.O.*; Canas, P.M.*; Oliveira, C.R.; Cunha, R.A.; "Blockade of adenosine A_{2A} receptors prevents staurosporine-induced apoptosis of rat hippocampal neurons." *Neurobiology of Disease* 27(2) (2007) 182-9.

V- Yu, L.; Shen, H.Y.; Coelho, J.E.; Araújo, I.M.; Huang, Q.Y.; Day, Y.J.; Rebola, N.; Canas, P.M.; Rapp, E.K.; Ferrara, J.; Taylor, D.; Müller, C.E.; Linden, J.; Cunha, R.A.; Chen, J.F.; "A_{2A} receptors modulate motor activity and MPTP neurotoxicity by distinct cellular mechanisms." *Annals in Neurology*, 63(3) (2008):338-46.

VI- Cunha, G.M.*; Canas, P.M.*; Melo, C.S.; Hockemeyer, J.; Müller, C.E.; Oliveira, C.R.; Cunha, R.A.; "Adenosine A_{2A} receptor blockade prevents memory dysfunction caused by β-amyloid peptides but not by scopolamine or MK-801." *Experimental Neurology*, 210(2) (2008) 776-81.

VII- Canas, P.M.*; Duarte, J.M.*; Rodrigues, R.J.*; Köfalvi, A.*; Cunha, R.A.; "Modification upon aging of the density of presynaptic modulation systems in the hippocampus." *Neurobiology of Aging* (2008), *in press*.

VIII- Shen, H.Y.; Coelho, J.E.; Ohtsuka, N.; Canas, P.M.; Day, Y.J.; Huang, Q.Y.; Rebola, N.; Yu, L.; Boison, D.; Cunha, R.A.; Linden, J.; Tsien, J.Z.; Chen, J.F.; "A critical role of the adenosine A_{2A} receptor in extrastriatal neurons in modulating psychomotor activity as revealed by opposite phenotypes of striatum and forebrain A_{2A} receptor knock-outs." *Journal of Neuroscience* 28(12) (2008) 2970-5.

IX- Gomes, C.A.*; Simões, P.F.*; Canas, P.M.*; Quiroz, C.; Sebastião, A.M.; Ferré, S.; Cunha R.A.; Ribeiro, J.A.; "GDNF control of the glutamatergic cortico-striatal pathway requires tonic activation of adenosine A_{2A} receptors." *Journal of Neurochemistry* 108 (5) (2009) 1208-19.

X - Canas P.M.*, Porciúncula L.O.*, Cunha G.M.A.*, Silva C.G., Oliveira J.M., Oliveira C.R., Cunha R.A., "Adenosine A_{2A} receptor blockade prevents synaptotoxicity and memory dysfunction caused by β-amyloid peptides via p38 MAPK kinase pathway.", *submitted for publication*.

XI -Quiroz-Molina C., Lujan R., Uchigashima M., Simões P., Lerner T., Borycz J., Kachroo A., Schwarzschild M., Canas P.M., Kreitzer A., Cunha R.A., Watanabe M., and Ferré S., "Key modulatory role of presynaptic adenosine A_{2A} receptors in cortical neurotransmission to the striatal direct pathway.", *submitted for publication*.

XII - Velloso N.A., Dalmolin G.D., Gomes G., Rubin M.A., Canas P.M., Cunha R.A., and Mello C.F., "Spermine improves recognition memory deficit in a rodent model of Huntington's disease." *Neurobiology of Learning and Memory* (2009), *in press*.

XIII - Rebola N., Canas P.M., Cunha G.M., Barry C., Oliveira C.R., Lynch M.A., Cunha R.A., "Blockade of adenosine A_{2A} receptors prevents the lipopolysaccharide-induced neuroinflammation and consequent neuronal dysfunction in the rat hippocampus.", *submitted for publication*.

*All these authors contributed equally to this study.

ABSTRACT

Neurodegenerative diseases are prevalent in the elderly but most studies exploiting the manipulation of presynaptic modulation systems to prevent these diseases were performed using young adult animals. It was now observed that aging causes an imbalance of excitatory *versus* inhibitory modulation systems; aging also decreased the density of presynaptic proteins and inhibitory A_1 (A_1R) and CB_1 receptors, whereas facilitatory mGluR5 and P_2Y_1 receptors density was roughly constant and the density of facilitatory A_{2A} adenosine receptor ($A_{2A}R$) increased at 18–24 months.

Adenosine is a neuromodulator that can either inhibit or facilitate synaptic transmission through A_1R or $A_{2A}R$ respectively. Both receptors are predominantly located in synapses in the limbic system and neocortex. Since noxious brain conditions enhance the extracellular levels of adenosine and the blockade of adenosine $A_{2A}R$ prevents synaptic dysfunction and confers neuroprotection, it was explored if $A_{2A}R$ blockade prevented synaptic dysfunction and memory impairment characteristic of Alzheimer's disease. Alzheimer's disease (AD) is characterized by memory impairment, neurochemically by accumulation of β-amyloid peptide (namely $A\beta_{1-42}$) and morphologically by an initial loss of nerve terminals. In a rodent model of Alzheimer's disease based on the intracerebral administration of soluble $A\beta_{1-42}$, the animals (rats or mice) presented, after two weeks, memory impairment and a loss of nerve terminal markers without overt neuronal loss, astrogliosis or microgliosis; this was prevented upon pharmacological blockade with SCH58261 ($A_{2A}R$ antagonist) in rats, and genetic inactivation of $A_{2A}R$ in mice. In the AD model there was a preferential loss of glutamatergic and cholinergic terminals, whereas there was an increase of GABAergic terminals. These results, which model the early events of AD, suggest that there is an asymmetric loss of nerve terminals in the hippocampus of amnesic mice. To further study the influence of $A_{2A}R$ in $A\beta_{1-42}$ induced synaptic loss, a nerve terminal preparation was used: SCH58261 prevented the $A\beta_{1-42}$ induced loss of viability and mitochondrial dysfunction; likewise, SCH58261 also prevented the initial synaptotoxicity and subsequent loss of viability of cultured hippocampal neurons exposed to $A\beta_{1-42}$.

Additional investigation was engaged to discover which signaling pathways were associated with this $A_{2A}R$-mediated control of neurodegeneration upon exposure of rat

hippocampal cultured neurons to $A\beta_{1-42}$. It was observed that the neuroprotection afforded by $A_{2A}R$ blockade is independent from cAMP/PKA pathway, but involves p38 MAPK.

The ability of $A_{2A}R$ to control calcium deregulation and mitochondrial dysfunction associated with Aβ toxicity was further tested. It was found that the neurons surviving to $A\beta_{1-42}$ exposure displayed a calcium deregulation and loss of mitochondrial membrane potential, which were not altered by SCH58261, upon electrical field stimulation.

$A_{2A}R$ antagonists do not cause a generic prevention of memory impairment in rodents. In fact, SCH58261 or KW6002 (another $A_{2A}R$ antagonist) failed to modify scopolamine- or MK-801-induced amnesia, and were only effective against Aβ-induced memory impairment, where synaptotoxicity is known to occur.

Another mechanism proposed for $A_{2A}R$ blockade to confer a robust neuroprotection is through the control of neuroinflammation, a hypothesis based on the ability of $A_{2A}R$ to modulate inflammation in peripheral systems. In two *in vivo* models of neuroinflammation, SCH58261 prevented lipopolysaccharide-induced microglia activation and also prevented kainate induced neurodegeneration and microglia recruitment. However, in perforant pathway lesion, where a simultaneous increase of CD11b and $A_{2A}R$ mRNA was observed, neither the blockade of $A_{2A}R$ (SCH58261) nor inhibition of adenosine kinase prevented microgliosis. Further studies are required to understand the role of $A_{2A}R$ in the complex orchestration of neuroinflammation processes.

Overall this thesis provides evidence of the ability of $A_{2A}R$ to control Aβ-induced toxicity likely involving the control of p38 MAPK pathway rather than cAMP/PKA pathway, mitochondrial dysfunction or calcium deregulation. $A_{2A}Rs$ do not affect general processes of memory impairment, but instead play a crucial role restricted to neurodegenerative conditions involving an insidious synaptic deterioration leading to memory dysfunction. An alternative mechanism to achieve neuroprotection by $A_{2A}R$ in some chronic insults could be through control of neuroinflammation. The unraveled mechanisms may also explain the promising beneficial effects of caffeine consumption as a strategy to prevent neurodegenerative conditions albeit other hitherto unrecognized concurring mechanisms may also be involved.

RESUMO

As doenças neurodegenerativas são mais prevalentes durante o envelhecimento. Contudo a maioria dos estudos de neuroprotecção destas doenças foram efectuadas usando animais adultos. Ao levar a cabo um estudo de ontogenia a partir do animal jovem adulto até ao animal envelhecido, foi constatado que o envelhecimento causa um desequilíbrio dos sistemas moduladores excitatórios *versus* inibitórios. A densidade de proteínas pré-sinápticas e dos receptores A_1 e CB_1 inibitórios diminuiu com a idade, enquanto a densidade dos receptores $mGluR_5$ e P_2Y_1 facilitatórios se manteve constante e a densidade dos receptores A_{2A} ($A_{2A}R$) facilitatórios aumentou a partir dos 18-24 meses.

A adenosina é um neuromodulador que pode inibir ou facilitar a transmissão sináptica através dos receptores A_1 ou A_{2A} respectivamente; ambos estão predominantemente localizados nas sinapses no sistema límbico e no neocórtex. Em situações nocivas para o cérebro adulto ocorre um aumento de níveis extracelulares de adenosina e o bloqueio dos $A_{2A}R$ previne a disfunção sináptica, conferindo neuroprotecção. Foi por isso explorado se o bloqueio dos $A_{2A}R$ previne a disfunção sináptica e défice mnemónico, característicos da doença de Alzheimer. A doença de Alzheimer é caracterizada por um progressivo défice mnemónico, neuroquimicamente por uma acumulação do péptido β-amilóide (nomeadamente, $A\beta_{1-42}$) e morfologicamente por uma perda inicial de terminais nervosos. Num modelo em roedores da doença de Alzheimer baseado na administração intracerebral do péptido $A\beta_{1-42}$ solúvel, após 2 semanas os animais (ratos e murganhos) apresentam um défice mnemónico e uma perda de marcadores de terminais nervosos, sem ocorrer morte neuronal, microgliose ou astrogliose; estas alterações foram prevenidas por bloqueio dos $A_{2A}R$, com o antagonista SCH58261 em ratos, ou aquando da inactivação genética dos $A_{2A}R$ em murganhos. Neste modelo ocorre uma perda preferencial de terminais glutamatérgicos e colinérgicos, e um aumento de terminais GABAérgicos. Estes resultados sugerem uma perda assimétrica de terminais nervosos no hipocampo de murganhos amnésicos. Com o intuito de estudar a influência dos $A_{2A}R$ na perda sináptica induzida pelo péptido $A\beta_{1-42,}$ foi usada uma preparação enriquecida em terminais nervosos: o composto SCH58261 preveniu as alterações na viabilidade e disfunção mitocondrial induzidas pelo péptido, e de igual forma preveniu a sinaptotoxicidade inicial e subsequente perda de viabilidade de neurónios de hipocampo expostos a $A\beta_{1-42.}$

Adicionalmente testou-se quais as vias de sinalização associadas ao controlo de condições neurodegenerativas mediadas pelos $A_{2A}R$, após a exposição de neurónios em cultura ao $A\beta_{1–42}$. A neuroprotecção conferida pelo bloqueio $A_{2A}R$ é independente da via do AMP cíclico/PKA e envolve a p38/MAPK.

O papel dos $A_{2A}R$ na desregulação do cálcio e disfunção mitocondrial, também associada à toxicidade induzida pelo $A\beta_{1-42}$ foi igualmente testado. Os neurónios sujeitos a estimulação eléctrica de campo (EFS), em que as células tinham sido previamente incubadas com $A\beta_{1-42}$, apresentam uma desregulação de cálcio e uma perda do potencial membranar mitocondrial, que não foi alterado pelo SCH58261.

O bloqueio dos $A_{2A}R$ não confere protecção em todos os modelos de perca de memória. De facto, nem o SCH58261, nem o KW6002 (outro antagonista do $A_{2A}R$) preveniram os défices causados quer pela escopolamina ou pelo MK-801, tendo sido apenas efectivos na prevenção do défice mnemónico causado pelo péptido Aβ, em que está descrito a existência de sinaptotoxicidade.

Outro mecanismo proposto que parece estar envolvido na neuroprotecção através do bloqueio dos $A_{2A}R$ é o controlo da neuroinflamação, uma hipótese baseada no papel dos $A_{2A}R$ na modulação de respostas inflamatórias no sistema periférico. Em dois modelos *in vivo* em que a neuroinflamação está presente, observou-se que o SCH58261 evitou a activação de microglia por lipopolissacárido, além de prevenir neurodegeneração induzida por cainato e microgliose associada. No modelo de lesão da via perfurante ocorre um aumento ao longo do tempo do RNA mensageiro de CD11b e $A_{2A}R$, contudo nem o SCH58261 nem a inibição da adenosina cinase previnem a microglióse neste modelo. Estudos suplementares são necessários para perceber o papel dos $A_{2A}R$ nos processos complexos que estão envolvidos na neuroinflamação.

Esta dissertação apresenta evidências da capacidade dos $A_{2A}R$ de controlarem a toxicidade induzida pelo $A\beta_{1–42}$, envolvendo muito provavelmente o controlo da via p38 MAPK e excluindo a via do AMP ciclíco/PKA, da disfunção mitocondrial ou da desregulação de cálcio. Estes resultados indicam que os $A_{2A}R$ não afectam processos gerais que levam ao défice mnemónico, mas em vez disso têm um papel fundamental em condições neurodegenerativas em que existe uma deterioração sináptica subtil e que consequentemente leva a um défice mnemónico. Um mecanismo alternativo para obter a neuroprotecção conferida pelos $A_{2A}R$ em alguns estímulos crónicos, poderá ser através pelo controlo de neuroinflamação. Os mecanismos deslindados durante esta dissertação

poderão contribuir para compreender os efeitos benéficos do consumo da cafeína como estratégia de prevenção de condições neurodegenerativas, sem excluir outros possíveis mecanismos de acção ainda não identificados.

1. INTRODUCTION

1.1. HISTORICAL ASPECTS RELATED TO ADENOSINE

Adenosine is an ubiquitous metabolite involved in major pathways such as purinergic nucleic acid base synthesis, amino acid metabolism and modulation of cellular metabolic status (Stone, 1985). Adenosine can operate as a neuromodulator or a homeostatic regulator (Cunha, 2001), controlling neurotransmitters release and neuronal excitability in the nervous system.

The first suggestion that adenosine might affect physiological function was advanced 80 years ago by Drury and Szent-Györgyi, who reported that injecting adenosine into mammals causes a decrease in the arterial blood pressure, dilatation of the coronary arteries, sleep and relaxation of the small intestine (Drury and Szent-Györgyi, 1929). Forty years ago, it was reported that electrical stimulation increased the accumulation of 3',5'-cyclic adenosine monophosphate (cAMP) in brain slices (Kakiuchi et al., 1969), which could be blocked by methylxanthines (Sattin and Rall, 1970). In 1972 it was also shown that electrical stimulation could release adenosine from brain tissue (Pull and McIlwain, 1972) and that exogenous addition of adenosine could modulate neuromuscular transmission (Ginsborg and Hirst, 1972). This bolstered research on the neuromodulatory effects of adenosine. In the subsequent years it was found that adenosine was able to decrease the release of numerous neurotransmitters, such as acetylcholine, dopamine, serotonin and noradrenaline (Hedqvist and Fredholm, 1976; Vizi and Knoll, 1976; Harms et al., 1978, 1979; Fredholm and Hedqvist, 1980).

1.2. ADENOSINE METABOLISM

Adenosine can exist intra- or extracellularly and it is continuously produced under normal conditions (Fredholm et al., 2001). Its intracellular concentration in basal conditions is typically around 10-50 nM (Cunha, 2001). Intracellular adenosine is formed by the action of an AMP selective endo-5'-nucleotidase (E.C.3.1.3.5) and the rate of adenosine formation via this pathway is mainly controlled by the amount of AMP (Schubert et al., 1979; Zimmermann et al., 1998). Adenosine can also result from hydrolysis of S-adenosyl-homocysteine (Broch and Ueland, 1980) via a reversible reaction catalyzed by S-adenosyl-L-homocysteine hydrolase

(E.C. 3.3.1.1). This pathway is limited by the availability of L-homocysteine, which has very low concentration in brain tissue (Reddington and Pusch, 1983; Hack and Christie, 2003).

In the nervous system, extracellular adenosine can have three main sources (see Fig. 1): (1) adenosine release through bi-directional equilibrative nucleoside transporters (ENTs) after an increase in the intracellular levels of adenosine. These transport proteins have been cloned and were termed ENT1 and ENT2 (for the equilibrative transport proteins) (e.g. Williams and Jarvis, 1991; Anderson et al., 1996; Baldwin et al., 1999; Fredholm et al., 2001). Since all cell types so far investigated possess ENTs, it is expected that the intracellular levels of adenosine equilibrate with the extracellular levels of adenosine (Peng et al., 2005). (2) Extracellular conversion of released adenine nucleotides into adenosine by ecto-nucleotidases (White and MacDonald, 1990; Cunha et al., 1996a). Ecto-nucleotidases include ectonucleoside triphosphate diphosphohydrolases (E-NTPDases, which can hydrolyze ATP or ADP), ecto-nucleotide pyrophosphatase/phosphodiesterases and ecto-5'-nucleotidase (EC 3.1.3.5) (Zimmermann, 2000; Fredholm et al., 2001). These enzymes are essential for the nerve activity-dependent production of adenosine from released ATP under physiological conditions (Dunwiddie et al., 1997; Zimmermann et al., 1998). (3) Extracellular formation of adenosine after release of cAMP (Rosenberg and Li, 1995). However, this third pathway has been found to be of minor importance in more integrated neuronal preparations (Brundege et al., 1997).

At the synapse, extracellular adenosine can be formed upon catabolism of released ATP originated from synaptic vesicles (reviewed in Sperlágh and Vizi, 1996) whereas minor changes in intracellular ATP concentration lead to disproportional larger changes in the extracellular concentration of adenosine (Cunha, 2001) or from ATP released from astrocytes, as a gliotransmitter, setting a global inhibitory tonus in the brain rather than in a single synapse (Cunha, 2008b). Adenosine can also be released from the postsynaptic neuron as a consequence of the activation of ionotropic glutamate receptors (see Mitchell et al., 1993; Dunwiddie and Diao, 1994; Delaney and Geiger, 1995).

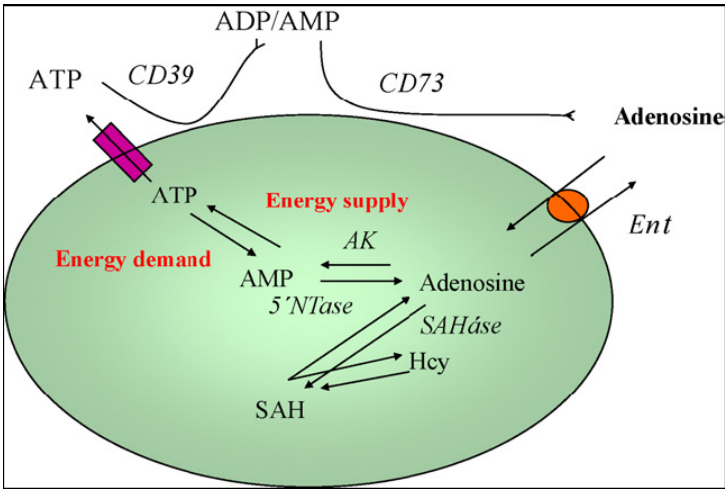


Figure 1: Mechanisms underlying formation of extracellular adenosine. Extracellular adenosine can be derived either from released adenine nucleotides sequentially broken down by ecto-enzymes including CD39 (NTPDase1) and CD73 (ecto 5'-nucleotidase) or from intracellular adenosine exported by equilibrative nucleoside transporters (ENT). A transporter for ATP is indicated, but its nature remains unclear. In addition, ATP can be exported from cells by vesicular exocytosis (complete or incomplete, so called ''kiss-and-run''). Adenosine is formed intracellularly from AMP by the action of an intracellular 5'-nucleotidase (5'NTase) or from S-adenosylhomocysteine (SAH) by means of S-adenosyl homocysteine hydrolase (SAHase) which also generates homocysteine (Hcy). The reaction between adenosine and AMP is reversible through the action of adenosine kinase (AK). The activity of S-adenosyl homocysteine hydrolase is reversible and the direction of the reaction is governed by the relative abundance of adenosine, Hcy and SAH. This figure was obtained from a review by Fredholm et al., 2007.

1.3. REGULATION OF ADENOSINE LEVELS

Regulation of intracellular adenosine concentration and clearance of extracellular adenosine occurs through the action of ENTs (Dunwiddie and Masino, 2001; Fredholm et al., 2005b). ENTs fulfill a dual role, because blockade of adenosine transport can inhibit either adenosine release or adenosine uptake in the hippocampus, depending upon its intra- and extracellular levels (Gu et al., 1995). The importance of ENT1 in regulating adenosine levels was also demonstrated in knockout mice lacking this protein (Choi et al., 2004). There are two enzymes that constitute the major pathways of adenosine removal: adenosine kinase (EC 2.7.1.20) and adenosine deaminase (EC 3.5.4.4). Intracellular adenosine can be converted to AMP by phosphorylation by adenosine kinase or degradation to inosine by adenosine deaminase (Arch and Newsholme, 1978; Lloyd and Fredholm, 1995; Svenningsson et al., 1999). Adenosine kinase is important at low levels of intracellular adenosine, with adenosine deaminase coming into play only when large amounts of adenosine have to be cleared (Fredholm et al., 2005b).

Adenosine kinase inhibitors are able to increase the extracellular levels of adenosine under physiological conditions, whereas adenosine deaminase inhibitors are able to increase the extracellular levels of adenosine during metabolic insults (Sciotti and Van Wylen, 1993; Lloyd and Fredholm, 1995).

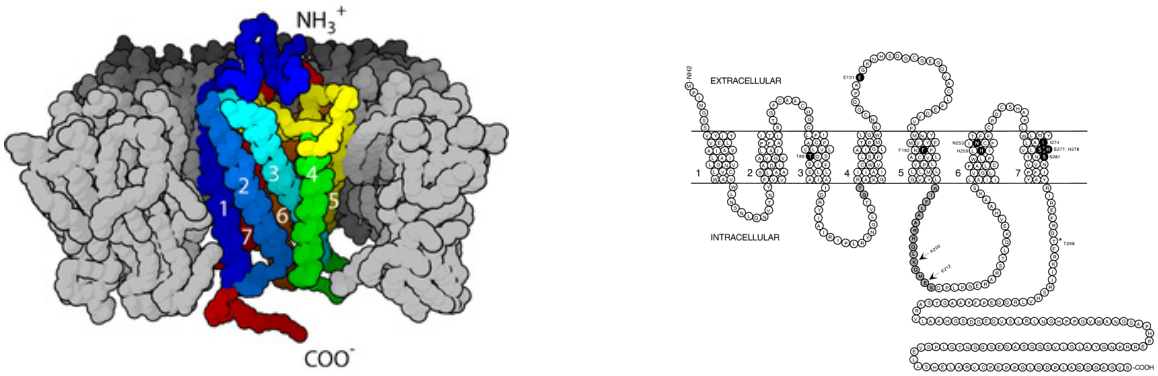
1.4. FORMATION OF EXTRACELLULAR ADENOSINE UPON STRESSFUL CONDITIONS

Several reports have documented an increase in the extracellular concentration of adenosine upon stressful challenges (Doolette, 1997; Stumpe and Schrader, 1997; Zhu and Krnjevic, 1997). Adenosine outflow can be triggered by hypoxia (Zetterström et al., 1982), ischemic insults (Berne and Rubio, 1974), electrical stimulation (Pull and McIlwain, 1972), metabolic poisoning (Zhu and Krnjevic, 1997), free radical induction (Masino et al., 1999), agonists of ionotropic glutamate receptors (Hoehn and White, 1990; Manzoni et al., 1994), NO in *in vivo* studies (Fischer et al., 1995; Delaney et al., 1998) and in *in vitro* studies (Fallahi et al., 1996) or arachidonic acid (Cunha et al., 2000). It has been already described that extracellular ATP catabolism provides a minor contribution for extracellular adenosine formation in stressful conditions (Lloyd et al., 1993; Pedata et al., 1993; Jurányi et al., 1999). Recent studies were left with the remaining hypothesis that adenosine is released as such (Pearson et al., 2001; Frenguelli et al., 2007; Martin et al., 2007) through mechanisms still to be resolved, which may involve carrier systems (Sperlágh et al., 2003), but independently of ENTs.

1.5. ADENOSINE RECEPTORS

The physiological effects of adenosine are transduced through four pharmacologically classified adenosine receptor types – A_1, A_{2A}, A_{2B} and A_3 receptors. Adenosine receptors have the following affinity for adenosine: A_1R – 70 nM; $A_{2A}R$ – 150 nM; $A_{2B}R$ – 5100 nM and A_3R – 6500 nM (Dunwiddie and Masino, 2001). These receptors were initially cloned from a canine thyroid library (Libert et al., 1989). All four subtypes are members of the superfamily of

G-protein-coupled-receptors (GPCRs) with seven transmembrane domains that signal through a variety of transduction mechanisms (see Fig. 2).



A **B**

Figure 2: A - The seven transmembrane α-helix structure of a G-protein-coupled receptor, from http://en.wikipedia.org/wiki/G_protein-coupled_receptor. **B - Schematic representation of the human A_{2A}R.** This picture was obtained from (Furlong et al., 1992).

1.6. SIGNALING PATHWAYS OF ADENOSINE RECEPTORS

Classically, the primary effector of all four adenosine receptors is adenyl cyclase (EC 4.6.1.1), whose activity is either stimulated or inhibited depending on the main receptor subtype present on a cell (van Calker et al., 1979; Londos et al., 1980). A_1R and A_3R mediate an inhibitory signal via Gi/o and Gi_3/G_q respectively, resulting in decreased levels of cAMP, whereas the A_{2A}R and A_{2B}R subtypes stimulate adenyl cyclase by activation of G_S/G_{olf} and G_S, respectively, with a consequent increase of cAMP (Abbracchio et al., 1995; Kull et al., 2000; Fredholm et al., 2001). However, it is now apparent that adenosine receptors also signal through other transducing pathways, namely phospholipase C (PLC), Ca^{2+} and mitogen-activated protein kinases (MAPKs) (for review see Schulte and Fredholm, 2003).

A_1R can activate G-protein-dependent inwardly rectifying K^+ channels (GIRKs), pertussis toxin-sensitive K^+ channels, as well as K_{ATP} channels and PLC, and inhibit Q-, P- and N-type Ca^{2+} channels through βγ G-protein subunits (Belardinelli et al., 1995; Fredholm et al., 2001; Rogel et al., 2005; Tawfik et al., 2005).

$A_{2A}R$ activate cAMP-dependent kinase/PKA, and phosphorylate cAMP responsive element-binding protein (CREB) (Josselyn and Nguyen, 2005). Several groups have documented the ability of neuronal $A_{2A}R$ to control neurotransmitter release im a manner independent of cAMP levels and mostly dependent on the control of protein kinase C activity (Cunha and Ribeiro, 2000 a, b; Gubitz et al., 1996; Norenberg et al., 1998; Queiroz et al., 2003; Rebola et al., 2003b). $A_{2A}R$ also activate MAPKs (De Cesare et al., 1999; Du et al., 2000; Cheng et al., 2002). This might result from converging signaling from CREB (De Cesare et al., 1999; Du et al., 2000) or from interaction and recruitment with the nucleotide exchange factor ARNO, which binds to the C terminus of the $A_{2A}R$ (Gsandtner et al., 2005). The $A_{2A}R$ can also recruit β-arrestin via a GRK-2 dependent mechanism (Khoa et al., 2006). Activation of β-arrestin can lead to novel types of signaling that are not directly related to G protein activation and which could have a different time course than classical signaling events (Lefkowitz, 2007).

$A_{2B}R$ can activate PLC that mediates many important functions of the receptors. Recently the arachidonic acid pathway was also demonstrated to be involved in the receptor activation (Daly et al., 1983; Brackett and Daly, 1994; Peakman and Hill, 1994; Feoktistov and Biaggioni, 1995; Donoso et al., 2005).

A_3R can activate PLC and PLD, and Ca^{2+} intracellular increase (Abbracchio et al., 1995; Englert et al., 2002; Fossetta et al., 2003; Shneyvays et al., 2004; Shneyvays et al., 2005).

1.7. LOCALIZATION OF ADENOSINE RECEPTORS IN THE BRAIN

A_1 adenosine receptor

A_1R is widely distributed in the brain, being highly expressed in cortex, cerebellum, hippocampus, dorsal horn of spinal cord, eye, adrenal gland and with intermediate levels in other brain regions (Reppert et al., 1991; Dixon et al., 1996; Ochiishi et al., 1999; Schindler et al., 2001; Rebola et al., 2003a; Fredholm et al., 2005b). At the cellular level, A_1R is found in neurons, astrocytes (Biber et al., 1997), microglia (Gebicke-Haerter et al., 1996) and oligodendrocytes (Othman et al., 2003). At the sub-cellular level, it is mostly found at nerve terminals both in the active zone and in the postsynaptic density in the rat hippocampus (Tetzlaff et al., 1987; Rebola et al., 2003a). This sub-localization may be related to receptor function. A_1R

efficiently inhibits intra-synaptossomal calcium transients and the evoked release of glutamate, acetylcholine, and serotonin from hippocampal neurons (Dunwiddie and Masino, 2001). A_1R immunoreactivity is evident in the postsynaptic density together with NMDA receptor subunits 1, 2A and 2B and with N- and P/Q-type calcium channel immunoreactivity (Rebola et al., 2003a). Post-synaptic A_1R is also known to control potassium channels, hyperpolarization of the resting membrane potential and control of NMDA function (Mogul et al., 1993; de Mendonça, 1995; Klishin, 1995; Rebola, 2003).

A_{2A} adenosine receptor

$A_{2A}R$ is highly expressed in striatopallidal GABAergic neurons (in caudate putamen and nucleus accumbens) and olfactory bulb, whereas in the rest of the brain $A_{2A}R$ is expressed at low levels in lateral septum, cerebellum, cortex and hippocampus (see Fig. 3) (Cunha et al., 1995b; Dixon et al., 1996; Svenningsson et al., 1997; Fredholm et al., 2005b). At the cellular level, it is expressed in neurons, in astrocytes (Li et al., 2001b; Nishizaki et al., 2002) and in microglia cells (Kust et al., 1999; Moreau and Huber, 1999). At the sub-cellular level, $A_{2A}R$ immunoreactivity is enriched in the active zone of presynaptic nerve terminals from hippocampus, whereas in the striatum it is predominantly located at the postsynaptic density, although a minority of striatal $A_{2A}R$ is located in the presynaptic active zone (Rebola et al., 2005a). The different localization in striatum and in hippocampus can explain different functions (Rebola et al., 2005a): in basal ganglia $A_{2A}R$ is recognized for the ability to postsynaptically control the signaling in striatopallidal neurons (reviewed in Fredholm and Svenningsson, 2003; Fuxe et al., 2003); in contrast, hippocampal $A_{2A}R$ mainly exert presynaptic action, controlling the release of neurotransmitters, either glutamate (e.g. Lopes et al., 2002), GABA (e.g. Cunha and Ribeiro, 2000a), acetylcholine (e.g. Jin and Fredholm, 1997; Rebola et al., 2002) or serotonin (e.g. Okada et al., 2001).

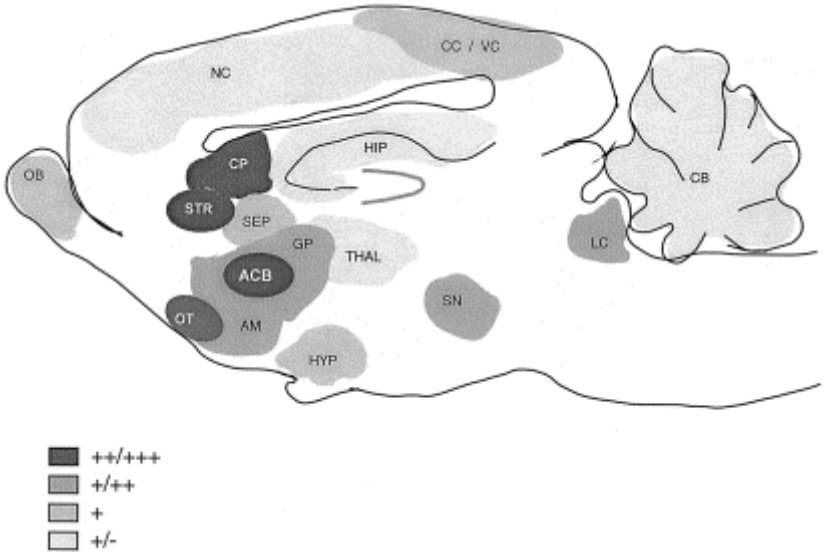


Figure 3: Expression and distribution of adenosine A_{2A}R in brain. A_{2A}Rs are found at the highest concentration in striatum, nucleus accumbens and olfactory tubercules, but also in other brain regions such as hippocampus, extended amygdala and cortex, as illustrated by differentially shaded areas (shading density correlates with receptor density). ACB: nucleus accumbens (++/+++); AM: extended amygdala (++); CC/VC: visual and cingulate cortex (+); CB: cerebellum (+/−); CP: caudate putamen (+++); GP: globus pallidus (+/++); HIP: hippocampus (+/−); HYP: hypothalamus (+); LC: locus coeruleus (+/++); NC: neocortex (+/−); OB: olfactory bulbs (+); OT :olfactory tubercle. This picture was obtained from Moreau and Huber, (1999).

A_{2B} adenosine receptor

A_{2B}R is expressed in intermediate levels in blood vessels, eye, median eminence, mast cells and in low levels in the adrenal and pituitary glands. At the cellular level, it has been demonstrated their biochemical existence in neurons and glial cells (Daly, 1977; Fredholm et al., 2005b).

A_3 adenosine receptor

A_3R is expressed at low levels in the rat or mouse brain, for example in cortex, amygdala, striatum, olfactory bulb, nucleus accumbens, hippocampus, hypothalamus, thalamus and cerebellum (Linden et al., 1993; Salvatore et al., 1993; Dixon et al., 1996). At the cellular level it is expressed in neurons (Lopes et al., 2003), astrocytes (Wittendorp et al., 2004) and microglial cells (Moreau and Huber, 1999; Hammarberg et al., 2003).

1.8. TRANSGENIC MICE WITH MODIFIED ADENOSINE RECEPTORS

The functions of the different adenosine receptors can be investigated using either genetic modifications of adenosine receptors or pharmacologic manipulations. Currently, there are transgenic mice with deletion of each of the 4 adenosine receptors, i.e. knockout (KO) mice. A_1R KO mice breed normally, but have reduced survival rates, anxiety, hyperalgia and decreased tolerance to hypoxia (Johansson et al., 2001; Gimenez-Llort et al., 2002; Fredholm et al., 2005a; Fredholm et al., 2005b). $A_{2A}R$ KO mice are viable and breed normally, but display anxiety, hypoalgesia, hypertension, increased tolerance to ischemia, altered sensitivity to motor stimulant drugs and decreased platelet aggregation (Ledent et al., 1997; Chen et al., 1999; Day et al., 2003; Fredholm et al., 2005a). $A_{2B}R$ KO mice were generated by Deltagen, Inc. and have a moderated inflamed phenotype baseline, including elevated adhesion molecule expression on vascular endothelial cells and elevated cytokine production (Yang et al., 2006). A_3R KO mice have lower intra-ocular pressure, altered inflammatory reactions, decreased edema and altered release of inflammatory mediators (Avila et al., 2002; Fredholm et al., 2005a). However, results from the use of knockouts (KO) can be influenced by the chosen genetic background, by developmental adaptations and by the lack of tissue specificity of global KO (Fredholm et al., 2005a). To overcome the developmental effects and tissue specificity, recently efforts were made to create tissue selective deletion of A_1R and $A_{2A}R$ in adult mice, using the Cre/loxP strategy (Boison et al., 2002; Shen et al., 2008).

1.9. PHARMACOLOGY OF ADENOSINE RECEPTORS

Ideally, for compounds to be useful for receptor classification, they should differ in potency by at least two orders of magnitude at different receptors. Few compounds used in classifying adenosine receptors fulfill this criterion (Fredholm et al., 2001). Generally, agonists for adenosine receptors are developed trough modifications of adenosine itself (Jacobson and Gao, 2006). The same strategy was applied searching for adenosine receptors antagonists, using xanthines as templates, such as caffeine and theophylline (Moro et al., 2006). Theophylline and

caffeine are classical non-selective xanthine antagonists of adenosine receptors, which display micromolar affinities for A_1R, $A_{2A}R$ and $A_{2B}R$ (Fredholm et al., 2001).

For instance, CPA is a selective agonist for A_1R but its half-life in the rat blood is 6 minutes (Mathôt et al., 1993). CCPA displays a greater affinity for A_1R when compared to CPA. DPCPX is a selective A_1R antagonist and it is the most frequently used (Fredholm et al., 2005b). NECA is a non-selective agonist for $A_{2A}R$ (Fredholm et al., 2001). CGS21680 (a drug developed using NECA as template, and more selective) is widely used but it is less potent and selective in humans than in rats (Hutchison et al., 1989; Kull et al., 1999). Watchfulness is necessary when using CGS21680, as it has been reported that it binds to other sites unrelated to $A_{2A}Rs$ in areas with low $A_{2A}R$ densities (Johansson et al., 1993; Cunha et al., 1996b; Lindström et al., 1996). SCH58261 is an antagonist for $A_{2A}Rs$ with high affinity for both human and rat receptors (Fredholm et al., 2005b; Yang et al., 2007). Presently, KW6002 is a suitable choice as an antagonist for $A_{2A}R$ *in vivo* animal studies, particularly for a CNS target because of its bioavailability, half life and brain penetration (Yang et al., 2007).

LUF5835 is the most potent activator of $A_{2B}R$ (Beukers et al., 2004); MRS1754 and MRE 2029-F20 are some examples of antagonists for $A_{2B}R$ (Ji et al., 2001; Gessi et al., 2005; Jacobson and Gao, 2006).

Cl-IB-MECA has been widely used has an A_3R selective agonist (Fredholm et al., 2001) although this compound can also bind (Lopes et al., 2003) and activate (Klotz et al., 1998) A_1Rs that largely out-number A_3Rs in the brain. In contrast to the other adenosine receptors, the A_3R is notably insensitive to several xanthines. Hence, most A_3R antagonists are dihydropyridines, pyridines or flavonoids, possessing a non-xanthine structure (Baraldi et al., 2000).

Table 1 presents a list with some examples of agonists and antagonists for the 4 adenosine receptors.

Table 1: Adenosine receptors pharmacology, from Jacobson and Gao (2006)

Adenosine receptor subtype	Compound	K_i value for AR (nM)			
		A_1AR*	A_{2A}AR*	A_{2B}AR*	A_3AR*
Agonists					
A_1	CPA	2.3	794	18,600¶	72
	CCPA	0.83	2,270	18,800¶	38
	S(–)-ENBA	0.38	>10,000	>10,000¶	915
	ADAC	0.85‡	210‡	N.D.	13.3
	AMP579	5.1‡	56‡	N.D.	N.D.
	NNC-21-0136	10‡	630‡	N.D.	N.D.
	GR79236	3.1‡	1,300‡	N.D.	N.D.
	CVT-510 (Tecadenoson)	6.5§	2,315§	N.D.	N.D.
	SDZ WAG 994	23§	25,000§	>10,000§,¶	N.D.
	Selodenoson	1.1‡	306‡	N.D.	N.D.
A_{2A}	NECA	14	20	140¶	25
	CGS21680	289	27	>10,000¶	67
	DPMA	168	153	>10,000¶	106
	Binodenoson	48,000	270	430,000¶	903
	ATL-146e	77	0.5	N.D.	45
	CV-3146	>10,000	290	>10,000¶	>10,000
A_{2B}	LUF5835	4.4	21	10¶	104
A_3	IB-MECA	51	2,900	11,000¶	1.8
	Cl-IB-MECA	220	5,360	>100,000¶	1.4
	LJ568	193	223	N.D.	0.38
	CP-608039	7,200	N.D.	N.D.	5.8
	MRS3558	260	2,300	>10,000¶	0.29
	MRS1898	136	784	N.D.	1.5
Antagonists					
A_1	DPCPX	3.9	129	56	3,980
	WRC-0571	1.7	105	N.D.	7,940
	BG 9719	0.43	1,051	172	3,870
	BG 9928	29	4,720	690	42,110
	FK453	18	1300	980	>10,000
	FR194921	2.9	>10,000	N.D.	>10,000
	KW3902	1.3‡	380‡	N.D.	N.D.
A_{2A}	KW6002	2,830	36	1,800	>3,000
	CSC	28,000‡	54‡	N.D.	N.D.
	SCH 58261	725	5.0	1,110	1,200
	SCH 442416	1,110	0.048	>10,000	>10,000
	ZM241,385	774	1.6	75	743
	VER 6947	17	1.1	112	1,470
	VER 7835	170	1.7	141	1,931
	'Schering compound'	82	0.8	N.D.	N.D.
A_{2B}	MRS1754	403	503	2.0	570
	MRE 2029-F20	245	>1,000	3.0	>1,000
	OSIP-339391	N.D.	N.D.	0.5	N.D.
A_3	OT-7999	>10,000	>10,000	>10,000	0.61
	MRS1292	12,100‡	29,800‡	N.D.	29.3
	PSB-11	1640	1,280	2,100¶	3.5
	MRS3777	>10,000	>10,000	>10,000¶	47
	MRS1334	>100,000	>100,000	N.D.	2.7
	MRE 3008-F20	1,200	141	2100	0.82
	MRS1220	305‡	52.0‡	N.D.	0.65
	MRS1523	15,600‡	2,050‡	N.D.	18.9
	'Novartis compound'	197	1,670	3.0¶	10.0

*Binding experiments at recombinant human A_1R, A_{2A}R, A_{2B}R and A_3R (ARs), unless noted. ‡Binding experiments at rat ARs. §Binding or functional experiments at porcine ARs. ¶Data are from a cyclic AMP functional assay. N.D., not determined or not disclosed.

1.10. ROLE OF ADENOSINE IN THE CENTRAL NERVOUS SYSTEM

At a cellular level, adenosine has a neuromodulatory role on nerve activity, by modulating the release of neurotransmitters, the post-synaptic responsiveness and the action of other receptor systems (Cunha, 2001).

A_1R inhibits synaptic transmission, acting both pre and post-synaptically in brain regions with a high concentration of these receptors, such as the hippocampus (Dunwiddie and Masino, 2001). A_1R inhibits the release of most classical neurotransmitters: glutamate, acetylcholine, norepinephrine, 5-hydroxytryptmanine, dopamine and other transmitters (Dunwiddie and Haas, 1985; Schubert et al., 1986; Proctor and Dunwiddie, 1987; Barrie and Nicholls, 1993; Ambrósio et al., 1997).

$A_{2A}R$ mediates facilitation of the release of neurotransmitters such as glutamate (Lopes et al., 2002; Cunha and Ribeiro, 2000a; Shindou et al., 2002), acetylcholine (Rebola et al., 2002) and serotonin (Okada et al., 2001), among others. In many regions this effect is only seen if A_1R is present (Lopes et al., 2002), but $A_{2A}R$ can also facilitate the release of neurotransmitters independently of A_1R, as typified by the control of the evoked release of GABA (Gubitz et al., 1996; Cunha and Ribeiro, 2000a; Brooke et al., 2004). The final target of $A_{2A}R$ modulation in nerve terminals seems to be P-type calcium channels (Mogul et al., 1993; Umemiya and Berger, 1994; Gubitz et al., 1996; Gonçalves et al., 1997).

The overall neuromodulatory role of adenosine in the CNS is a balance between A_1R and $A_{2A}R$ functions, because they are the two mainly expressed adenosine receptors in the brain (compared to $A_{2B}R$ and A_3R) and they can be located at the same synapse (Rebola et al., 2005d). In the hippocampus, the percentage of nerve terminals with $A_{2A}R$ that are simultaneously endowed with A_1R is 80% (Rebola et al., 2005d). $A_{2A}R$ has a major role in controlling A_1R through intracellular transducing systems (Dixon et al., 1997; Lopes et al., 1999) or through receptor dimerization (Ciruela et al., 2006).

Adenosine can possess other functions such as controlling the rate of metabolism of neurons and astrocytes (Håberg et al., 2000; Hammer et al., 2001), axonal growth (Rivkees et al., 2001) or axonal guidance (Corset et al., 2000; Stein et al., 2001). Adenosine receptors can also control astrogliosis, the release of neuroactive substances (Hindley et al., 1994; Ciccarelli et al., 2001), inflammation (Ohta and Sitkovsky, 2001) and control vascular resistance (Olsson and Pearson, 1990).

1.11. ADENOSINE AND NEUROPROTECTION

Adenosine is released upon stressful situations (Fredholm et al., 2005b), as discussed in sub-section 1.4. Consequently, a possible neuroprotective strategy is the control of the levels of adenosine by manipulation of adenosine kinase activity (Gouder et al., 2004). This enzyme acts as key sensor and regulator of ambient adenosine and can play a pivotal role in fine-tuning glutamatergic and dopaminergic neurotransmission, based on adenosine's activation of its receptors with opposing activities (A_1R *versus* $A_{2A}R$) (Boison, 2008). Gouder and collaborators reported that inhibition of adenosine kinase (thus increasing extracellular endogenous adenosine) effectively decreased chronic convulsive behavior, in an animal model of epilepsy (Gouder et al., 2004). Increasing extracellular adenosine could be considered a suitable therapeutic target to obtain neuroprotection in other brain conditions besides epilepsy, such as ischemia (Pignataro et al., 2007), stroke (Kowaluk et al., 1998; Boison, 2006), chronic pain (McGaraughty and Jarvis, 2006) or schizophrenia (Lara et al., 2006). However, pharmacological manipulation of adenosine kinase activity can lead to the appearance of severe side effects (Ugarkar et al., 2000; Gouder et al., 2004). A possible alternative is adenosine-releasing cell transplants (Boison, 2007b, a).

Adenosine receptors can have a role in pathological situations. A_1Rs can play a role in neuroprotection since their activation decreases metabolic rate, as an attempt to hamper the detrimental effects caused by a noxious stimulus (Cunha, 2001), and they can also decrease glutamate release and hyperpolarize neurons (Gerber and Gahwiler, 1994; Cunha, 2005). It has been described that A_1R agonists and antagonists consistently attenuate and potentiate brain damage, respectively (de Mendonça et al., 2000). Thus, the activation of A_1R protects against ischemic brain injury in adult animals in global or transient focal ischemia (Rudolphi et al., 1992; Von Lubitz et al., 1994; Von Lubitz et al., 1995) and against other brain noxious stimulus such as excitotoxicity induced by kainate and quinolinic acid (MacGregor et al., 1993; MacGregor et al., 1997) or dopaminergic neurotoxicity (Delle Donne and Sonsalla, 1994). Therefore, tonic activation of A_1R can be considered an endogenous neuroprotective system in stressful brain situations (Cunha, 2005). However, the use of A_1R agonists as a neuroprotective strategy has several disadvantages, namely the occurrence of prominent cardiovascular effects (Olsson and Pearson, 1990; Shryock and Belardinelli, 1997), the poor brain permeability of A_1R agonist and their short "window of opportunity" (Cunha, 2005). Neuroprotection achieved by A_1R is limited in time, because the A_1R system is prone to desensitization (Lee et al., 1986;

Coelho et al., 2006) that occurs in time frames of 12-24h. Accordingly, the effects operated by A_1R undergo desensitization upon chronic noxious brain conditions (Cunha, 2005).

Given that chronic noxious stimuli cause a down-regulation of A_1R and an up-regulation of $A_{2A}R$, there is a trend to emphasise the interest of $A_{2A}R$ compared to A_1R in neuroprotection (Cunha, 2005). In fact, chronic stressful stimuli cause an increased expression and density of $A_{2A}R$ in animal models of Parkinson's disease (Pinna et al., 2002; Tomiyama et al., 2004), of epilepsy (Rebola et al., 2005c), diabetes (Duarte et al., 2006) or restraint stress (Cunha et al., 2006). Most notably, $A_{2A}R$ antagonists confer neuroprotection in several pathological conditions in adult animals such as upon ischemia (Gao and Phillis, 1994; Phillis, 1995; Von Lubitz et al., 1995; Monopoli et al., 1998; Chen et al., 1999), or excitotoxicity (Jones et al., 1998; Behan and Stone, 2002). In humans there is an inverse association between caffeine consumption and Parkinson's (PD) disease (Ross et al., 2000). $A_{2A}R$ antagonists are currently being developed as anti-Parkinsonian drugs, since they are claimed to provide a double benefit: 1) symptomatically they prevent motor dysfunction; 2) they also provide neuroprotection (Chen et al., 2007). In fact, caffeine and other $A_{2A}R$ antagonists provide functional protection against dopaminergic neurotoxicity and also reduce degeneration of the dopaminergic system in the MPTP model of PD (Chen et al., 2001; Xu et al., 2002). Chen and colleagues reported that $A_{2A}R$-mediated control of psychomotor function and neuroprotection involves distinct cellular mechanisms, using forebrain neuronal-specific $A_{2A}R$ knockout mice (Yu et al., 2008). $A_{2A}R$ activity in forebrain neurons is critical for control of psychomotor activity, but not for neuroprotection against brain injury, which might indicate a role of $A_{2A}R$ in glial cells (Yu et al., 2008).

At this moment there is no consensus about the mechanisms by which $A_{2A}R$ blockade confer a robust neuroprotection in noxious situations. Two leading hypotheses are currently being explored to explain the neuroprotection afforded by $A_{2A}R$ blockade: control of glutamate excitotoxicity and control of neuroinflammation (Cunha, 2005).

1.12. ADENOSINE AND GLUTAMATE EXCITOTOXICITY

Glutamate has several roles in the brain, namely as a protein constituent, as a neurotransmitter and in the intermediary metabolism (for a review, see Shank and Campbell, 1983).When acting as a neurotransmitter, released glutamate activates a family of ligand gated

ion channels that were originally named according to the exogenous agonists that are selective for each subtype and include α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) (for reviews, see e.g. Monaghan et al., 1989; Hollmann and Heinemann, 1994; Wollmuth and Sobolevsky, 2004).

Glutamate can also play a role in brain pathology. It can be highly toxic to neurons, a phenomenon known as "excitotoxicity", the process by which the over-activation of excitatory neurotransmitter receptors leads to neuronal cell death (Olney, 1969); this results in either rapid necrosis or delayed apoptosis of the neuron, depending on the severity of the insult (Bonfoco et al., 1995). In neurons, NMDA receptors, which are highly permeable to calcium and distributed widely on CNS neurons, are the major initiators of excitotoxicity (Matute et al., 2006). Glutamate excitotoxicity has been implicated in acute injury to the CNS and in chronic neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's diseases (Choi, 1988; Lipton and Rosenberg, 1994; Lee et al., 1999). In Parkinson's disease, depletion of nigrostriatal dopamine results in disinhibition of striatal neurons which initiate glutamatergic overactivity (Reichmann et al., 2005). This regulation classifies Parkinson's disease as a "secondary glutamate overactivity syndrome" (Reichmann et al., 2005). In Alzheimer's disease, glutamate may be one of the major executors of neuronal damage, through its relation with Aβ and tau protein, hallmarks of AD (Koutsilieri and Riederer, 2007). Huntington's disease can be initiated by a massive release of glutamate from the corticostriatal terminals (cortical dysfunction) leading to striatal excitotoxicity (e.g. Gil and Rego, 2008). In other noxious conditions, such as stroke or brain trauma, glutamate release occurs from the neurons residing in the damaged core region (Ikonomidou et al., 2000; Palmer, 2001). After being released, glutamate overactivates excitatory pathways, a phenomenon that is also observed in other disorders, including epilepsy and neuropathic pain (e.g. Villmann and Becker, 2007).

$A_{2A}R$ can control glutamate release and clearance from astrocytes (Marcoli et al., 2003; Cunha, 2005). Several studies identified $A_{2A}R$ as being responsible for the release of glutamate in noxious situations (O'Regan et al., 1992; Popoli et al., 2002; Melani et al., 2003). Thus, the blockade of $A_{2A}R$ can be a neuroprotective strategy, by preventing glutamate excitotoxicity that is present in several neurodegenerative diseases.

1.13. ADENOSINE AND NEUROINFLAMMATION

While neuroinflammation is present in different conditions of brain damage, it should be made clear that it is a double-edged sword, possibly contributing for brain damage, but also for the repair and regeneration of brain tissue (Schwartz and Moalem, 2001; Weiner and Selkoe, 2002; Elward and Gasque, 2003; Kerschensteiner et al., 2003; Schwartz, 2003; Marchetti and Abbracchio, 2005).

Microglia cells are one type of resident cells in the CNS, having a main role in the onset and progression of central inflammatory responses (Kreutzberg, 1996; Streit et al., 1999). Microglia cells are rapidly up-regulated in response to infection or tissue injury (Kreutzberg, 1996; Streit et al., 1999). Upon response to pathological conditions, activated microglia cells release cytokines, which later on will mediate inflammation (Rothwell and Luheshi, 2000; Chavarria and Alcocer-Varela, 2004). Cytokines can contribute to initiation, propagation and regulation of inflammatory reactions in CNS (Benveniste, 1998). The major pro-inflammatory cytokines are Il-1β and TNF-α, while IL-10, TGF-β and IL-1ra are anti-inflammatory, their classification being dependent on the final balance of their effects in the immune system (Vilček, 2003).

Adenosine is a metabolic switch to sense inflammation and tissue damage reflecting and triggering responses (Sitkovsky, 2003). There is a paradoxical modulation of peripheral inflammation and neuroinflammation by $A_{2A}R$ (Sitkovsky, 2003). Therefore, activation of $A_{2A}R$ prevents peripheral inflammation; in contrast, in the CNS, it is the blockade of $A_{2A}R$ that prevents neuroinflammation (Cunha et al., 2007). This contradictory modulation by $A_{2A}R$ can reflect the complexity of $A_{2A}R$ actions on neuronal, glial and vascular components, which may have distinct effects in brain injury (Chen et al., 2007; Cunha et al., 2007).

In the periphery, extracellular adenosine binds to $A_{2A}R$ and acts as a 'STOP' signal of immune responses, constituting one mechanism of immediate tissue protection (Sitkovsky, 2003; Sitkovsky et al., 2004; Sitkovsky and Ohta, 2005). $A_{2A}R$ activation has been shown to confer protection against tissue damage from ischemia-reperfusion injury in different organs such as heart (e.g. Maddock et al., 2001; Platts et al., 2003), blood vessels (McPherson et al., 2001), kidney (Okusa et al., 2000; Day et al., 2003), liver (Harada et al., 2000; Day et al., 2004; Odashima et al., 2005a), lung (Khimenko et al., 1995; Ross et al., 1999), intestine (Odashima et al., 2005c) or stomach (Odashima et al., 2005b). Moreover, $A_{2A}R$ blockade actually exacerbates

tissue damage involving inflammatory reactions in the periphery (reviewed in Hasko and Cronstein, 2004; Sitkovsky et al., 2004).

In the central nervous system the opposite occurs: the blockade of $A_{2A}R$ mediates neuroprotection in chronic stimulus, by controlling neuroinflammation, for example in Parkinson's disease models (Pierri et al., 2005; Yu et al., 2008), in a rodent model of temporal lobe epilepsy (Lee et al., 2004), and in ischemia models (Yu et al., 2004; Melani et al., 2006).

1.14. ADENOSINE AND EPILEPSY

Epilepsy is a neurological disorder, characterized by the recurrent appearance of spontaneous seizures due to neuronal hyperactivity in the brain and has a number of subtypes (CCTILAE, 1981, 1989; Engel, 2001). Temporal lobe epilepsy (TLE) is the most prevalent type of refractory epilepsy and is characterized by complex partial seizures (e.g. Sperk et al., 2007). The limbic system, including the hippocampus, is thought to play a pivotal role in the initiation and propagation of TLE seizures (Wieser et al., 1993). TLE, at the cellular level, is characterized by both neuronal and glial dysfunction (Barone and Feuerstein, 1999; del Zoppo et al., 2000).

Seizures are the outcome of recurrent firing of excitatory neurons resulting from an imbalance towards hyperexcitability (Coutinho-Netto et al., 1981; Reynolds, 1995). This imbalance can be caused by an abnormality in the transmitter systems of the brain such as impaired inhibitory transmission (through GABA) or excessive excitatory transmission (through glutamate) (Dichter and Ayala, 1987; Meldrum et al., 1999; Coulter, 2001). Increased glutamate levels can mediate: 1) hyperexcitability of neuronal circuits (Coutinho-Netto et al., 1981; Chapman, 1998; Löscher, 1998); 2) synaptic plasticity phenomena involving both functional and structural changes in neuronal circuits (Cain et al., 1989); 3) excitotoxicity and neuronal cell death (Lipton, 1999).

Adenosine is an endogenous anticonvulsant in the brain (Dunwiddie, 1980; Lee et al., 1984; Dragunow et al., 1985). It exerts antiepileptic and neuroprotective effects (Fredholm et al., 1996; Ribeiro et al., 2003). A_1Rs, which have high expression levels in the hippocampus, one of the main regions affected in epilepsy (Fredholm et al., 2001), prevent the spread of epileptogenic activity (Fedele et al., 2006; Kochanek et al., 2006). However, synaptic activation of $A_{2A}Rs$ can subsequently downregulate A_1Rs or its responses (Lopes et al., 1999; Ciruela et al., 2006). In

fact, in chronic epilepsy, there is an up-regulation of adenosine kinase that is paralleled by changes in G-protein coupled receptor density such as down-regulation of A_1Rs and up-regulation of A_{2A}Rs (Ekonomou et al., 2000; Rebola et al., 2003c; Rebola et al., 2005c). The local delivery of adenosine to an epileptic focus by means of implanted cells appears to be a promising strategy for the long term control of seizures, by disruption of their adenosine kinase gene (Huber et al., 2001; Boison et al., 2002; Güttinger et al., 2005a; Güttinger et al., 2005b). A_1R agonists are effective in seizure suppression (Boison, 2007b). A_1Rs are believed to provide beneficial extra-synaptic effects, which are based on a decrease in brain metabolism (Håberg et al., 2000) and the control of astrocyte function (van Calker and Biber, 2005). However, as described previously, A_1R agonists have severe side effects (Olsson and Pearson, 1990; Shryock and Belardinelli, 1997; Cunha, 2005). Currently the role of A_{2A}R in epilepsy is unclear, since caffeine given acutely can aggravate seizures (Ault et al., 1987; Whitcomb et al., 1990; De Sarro et al., 1999; Luszczki et al., 2006), but chronic ingestion of caffeine or genetic inactivation of A_{2A}R is able to protect against acute pentylenetetrazol induced seizures (El Yacoubi et al., 2008).

1.15. ADENOSINE AND MEMORY

Memories for events, individuals, places, foods, motor behaviors and emotions are extremely important for the survival, well-being and adaptation of complex organisms (Tronson and Taylor, 2007). The hippocampus is one of the main regions involved in memory formation (O'Keefe and Conway, 1978). It integrates information from multiple brain regions with inputs from neocortex arriving via entorhinal cortex (EC) and the perforant pathway (PP) (Witter et al., 2000; van Groen et al., 2003). The involvement of the hippocampus in memory processes was discovered in 1957 when the patient H.M. presented severe memory impairment after the bilateral removal of his hippocampi for the treatment of epilepsy (Scoville and Milner, 1957). Hippocampal lesioned rodents confirmed this conclusion, as the animals presented memory impairment for space and context, which reinforced the key role of hippocampus in memory (O'Keefe and Conway, 1978).

Dynamic changes in synaptic efficacy provide a cellular basis for information storage in the nervous system (Martin and Morris, 2002). Current theories suggest the importance of activity-dependent synaptic plasticity including long-term potentiation (LTP) as a form of

synaptic plasticity widely regarded as a substrate of memory encoding (Martin and Shapiro, 2000). NMDA receptors are required for induction of LTP (Morris, 1989). Moreover it was reported that a tonic activation of $A_{2A}R$ seems required for the implementation of long-term potentiation, both in striatal and hippocampal synapses (Fujii et al., 1999; d'Alcantara et al., 2001; Fontinha et al., 2008; Rebola et al., 2008). Recently, it was reported that LTP of NMDA receptor mediated synaptic transmission depends on an elevation of intracellular calcium and requires the coactivation of NMDA receptors and mGluR5 in addition to $A_{2A}R$ (Rebola et al., 2008).

Adenosine can have an essential role controlling neurons, since administration of $A_{2A}R$ agonists has a hindering effect on memory performance (Normile et al., 1994; Hooper et al., 1996; Ohno and Watanabe, 1996; Pereira et al., 2005; Prediger and Takahashi, 2005). Similarly, in a transgenic model overexpressing $A_{2A}R$, memory deficits were correlated with increased $A_{2A}R$ mRNA levels, $A_{2A}R$ protein levels and increased receptor binding (Gimenez-Llort et al., 2007). On the contrary, caffeine (or theophylline, another non-selective antagonist of adenosine receptors present in different beverages) consumption in moderate doses by rodents indicate that there is a memory performance improvement (Cestari and Castellano, 1996; Angelucci et al., 1999; Hauber and Bareiss, 2001; Angelucci et al., 2002; Prediger and Takahashi, 2005; Costa et al., 2008b).

1.16. AGING AND MEMORY

Memory function often declines with age (Craik, 1977). Aged animals show impairment in cognitive (learning and memory), emotional (motivation) and motor functions (Jolles, 1986). These alterations are the consequence of changes that occur in the brain with aging. In agreement with memory loss, LTP impairment has also been reported in aged rats (Murray and Lynch, 1998). In fact, in the hippocampus of aged rats there is an increase in basal extracellular concentrations of glutamate (Freeman and Gibson, 1987) and a decrease of NMDA receptor density (reviewed in Segovia et al., 2001). However, there is an increase in the affinity of the NMDA receptor for glutamate (Cohen and Müller, 1992). Additionally, there is a loss of GABAergic input or decreased GABA release (Vela et al., 2003). A loss of inhibitory inputs during aging might be reflected in some of the properties of hippocampal neuronal network

(integrative properties, firing rate and general excitability), influencing memory processes (Jolles, 1986). Aged animals also display modifications of the densities of A_1R and $A_{2A}R$ in the brain, favoring facilitatory $A_{2A}R$ (Lopes et al., 1999; Rebola et al., 2003b) and a modification of the extracellular metabolism of adenosine (Cunha et al., 2001). This apparent loss of an adenosinergic inhibitory tonus and the appearance of a stimulatory one in aged individuals may be important to the enhancement of synaptic efficacy and might be a consequence of the decreased number of functional synapses (Takahashi et al., 2008). At the same time, a physiological cost may be represented by an increased vulnerability of senescent neurons to excitatory amino acid toxicity (Meldrum and Garthwaite, 1990; Halle et al., 1997; Johansson et al., 2001).

Coffee (whose main active substance is caffeine) is probably the most widespread consumed psychoactive drug worldwide (see Fredholm et al., 1999). Caffeine consumption can afford a series of psychoactive effects such as diminishing fatigue and improving alertness and mood (Rogers and Dernoncourt, 1998; Fredholm et al., 1999; Lorist and Tops, 2003). Caffeine has significantly greater beneficial effects on cognition in aged individuals (Jarvis, 1993; Rees et al., 1999; Johnson-Kozlow et al., 2002; but see Rogers and Dernoncourt, 1998; Hameleers et al., 2000) and is able to attenuate the age-associated cognitive decline (Ritchie et al., 2007; but see van Boxtel et al., 2003). Caffeine abrogates the age-dependent decline in memory performance in rodents (Prediger et al., 2005; Costa et al., 2008a) an effect mimicked by antagonists of adenosine A_{2A} but not A_1 receptors (Prediger et al., 2005). In aging, caffeine acts as a memory normaliser (Cunha, 2008a).

These results highlight the importance of the blockade of $A_{2A}R$ as a possible neuroprotective strategy in neurodegenerative disorders, such as Alzheimer's disease, which is more prevalent in elderly.

1.17. ALZHEIMER'S DISEASE

Alzheimer's disease is characterized by progressive cognitive impairment in which the early affected areas in the brain are the cortex and hippocampus (Khachaturian, 1985; Gomez-Isla et al., 1997; Rosenblum, 1999; Uylings and de Brabander, 2002). Histologically, Alzheimer's disease can be described by synaptic loss, neuronal loss, amyloid plaques,

neurofribrillary tangles and microglial activation (McGeer and McGeer, 1995; Sisodia and Price, 1995; Selkoe, 2002). At present there are 3 hypotheses to explain Alzheimer's disease: cholinergic hypothesis, amyloid hypothesis and tau hypothesis.

Cholinergic hypothesis was the first to be developed, and it essentially states that a loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and AD (reviewed by Bartus, 2000). Forty years ago, it was discovered that scopolamine (muscarinic cholinergic receptor antagonist) produces amnesic syndromes (Longo, 1966). Later on, it was reported a loss of choline acetyltransferase (ChAT) activity (Davies and Maloney, 1976), loss of basal forebrain cholinergic neurons (Whitehouse et al., 1982) and that cholinergic projections are severely impaired (Reinikainen et al., 1990; Bierer et al., 1995) in AD. It was also observed a decline in cortical cholinergic presynaptic bouton number in transgenic animal models of AD (Wong et al., 1999).

Amyloid hypothesis states that the gradual cerebral accumulation of soluble and insoluble assemblies of the β-amyloid (Aβ) in limbic and association cortices triggers a cascade of biochemical and cellular alterations that produce the clinical phenotype of Alzheimer's disease (AD) (Selkoe, 2000; Hardy and Selkoe, 2002). Aβ is formed from the proteolytic cleavage of amyloid precursor protein (APP) by secretases (Haass et al., 1992; Shoji et al., 1992; Busciglio et al., 1993; Haass and Selkoe, 1993). Synapses are the initial target in AD, and it was shown that Aβ induces changes in synaptic efficacy *in vivo* (Selkoe, 2002). Synaptic alterations are the morphological parameter that correlates better with cognitive dysfunction in AD (Coleman and Yao, 2003). To explain Aβ toxicity two mechanisms have been proposed: 1) inflammatory mechanism in which Aβ promotes damaging inflammation reaction; there are some evidences that nonsteroidal anti-inflammatory drugs afford protection (McGeer et al., 2006); 2) the ability of Aβ to promote mitochondrial dysfunction and apoptosis, which play a key role in the later stages of AD (Caspersen et al., 2005; Manczak et al., 2006; Reddy and Beal, 2008).

Tau hypothesis supports the idea that tau protein abnormalities initiate the disease cascade (Mudher and Lovestone, 2002). Tau protein becomes hyperphosphorylated in response to an imbalance of physiological kinase/phosphatase activities (Alonso et al., 2001). Once hyperphosphorylated, tau affinity for microtubules is reduced and tau can detach, leading to changes in microtubule dynamics and cell cycle abnormalities in various animal models that potentially result in toxicity (Zhu et al., 2007; Sorrentino et al., 2008).

However, none of these hypotheses by itself is sufficient to clarify the diversity of biochemical and pathological changes in AD, but instead an integration of hypotheses may be required to allow devising of a model providing a better explanation of AD.

1.18. ADENOSINE AND ALZHEIMER'S DISEASE

A retrospective epidemiological study showed that the incidence of Alzheimer's disease was inversely associated with the consumption of coffee in the previous two decades of life (Maia and de Mendonça, 2002). Likewise, in other neurodegenerative diseases, $A_{2A}R$ were found to be up-regulated in cortical regions both in animals models (Arendash et al., 2006) as well as in cortical tissue from patients with Alzheimer's disease (Angulo et al., 2003; Albasanz et al., 2008). *In vitro* studies reported that Aβ-induced toxicity in cultured cerebellar neurons was prevented by caffeine and ZM241385 (an $A_{2A}R$ antagonist) (Dall'Igna et al., 2003). *In vivo* studies, using AD animal models revealed that: 1) administration of propentofylline – mixed blocker of nucleoside transporters and adenosine receptors (Parkinson et al., 1994) – to a transgenic animal model with the Sweddish mutation (transgenic mice overexpressing mutated human amyloid precursor protein, presenting several features of AD) attenuated pathological changes (Chauhan et al., 2005); 2) a beneficial role of chronic ingestion of caffeine in multiple cognitive tasks, in the same transgenic animals (Arendash et al., 2006); 3) administration of caffeine or SCH58261 to a model of intracerebral administration of β-amyloid peptide fragments (model of AD) prevented cognitive impairment (Dall'Igna et al., 2007); 4) administration of caffeine ameliorates high cholesterol diet-induced increases in disruptions of the BBB, in a rabbit model of AD (Chen et al., 2008).

In neuronal cell cultures from transgenic animal model with the Sweddish mutation, caffeine also reduced the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides (Arendash et al., 2006), whereas propentofylline attenuated tau phosphorylation in these same cultured cells (Chauhan et al., 2005). However, the mechanism by which $A_{2A}R$ antagonists confer protection in different AD animal models is still unclear.

2. AIM

- **Analyse how the density of adenosine receptors changes with aging in rat hippocampal synaptosomes.**

- **Test if A_{2A} adenosine receptor blockade prevents $A\beta_{1-42}$-induced synaptotoxicity and memory dysfunction and explore the underlying signaling pathways.**

- **Define if there is any particular type of nerve terminal that is more susceptible to $A\beta_{1-42}$.**

- **Test if A_{2A} adenosine receptors antagonists influence mitochondrial dysfunction and calcium deregulation induced by $A\beta_{1-42}$.**

- **Evaluate if A_{2A} adenosine receptor blockade affords a general beneficial effect in different experimental paradigms disturbing memory performance in rodents.**

- **Study the ability of A_{2A} adenosine receptor to control neuroinflammation.**

3. ANIMAL MODELS AND METHODS

3.1. ANIMALS

Male Wistar rats or C57BL6 mice with 8 to 10 weeks were obtained from Charles River (Barcelona, Spain). Male $A_{2A}R$ knockout (KO) mice and their littermates, male C57BL6 mice, with 8 to 10 weeks (genetic background of $A_{2A}R$ KO) were generously provided by Jiang-Fan Chen (Univ. Boston, USA). The animals were maintained under controlled environment (23±2 °C, 12 h-light/dark cycle, free access to food and water) and were handled according to the EU guidelines for use of experimental animals (86/609/EEC). All behavioral experiments were conducted between 10:00 a.m. and 4:00 p.m.

3.2. ANIMAL MODEL OF ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common chronic neurodegenerative disease characterized clinically by an atrophy of hippocampal regions and a progressive cognitive impairment. The parameters that correlate better with memory dysfunction in AD are the levels of soluble Aβ, mainly $Aβ_{1-42}$, and a decreased density of nerve terminals in cortical areas (Selkoe, 2001; Coleman et al., 2004). Thus, a major lead for the development of novel therapeutic strategies for AD might be to explore mechanisms able to prevent this early synaptotoxicity caused by $Aβ_{1-42}$. Unlike non-human primates and a few other animals (Van Dam and De Deyn, 2006), aging rodents do not spontaneously develop the characteristic hallmarks of AD, so inducible models of AD comprise the central injection of Aβ peptides into healthy adult animals. A great advantage of this model is that AD can be mimicked in rodents by a single intracerebroventricular (icv) injection of Aβ peptides, inducing stable long-term deficits. Depending on where they are infused, Aβ can lead to AD-like behavioral alterations, deficits in spatial and nonspatial learning and memory (Nag et al., 1999; Harkany et al., 2000; Nakamura et al., 2001; Medeiros et al., 2007), disruption of cholinergic functions (Harkany et al., 2000) and loss of functional synapses (Medeiros et al., 2007; Prediger et al., 2007). These models based on intracerebral Aβ injection support the hypothesis that these peptides play a central role in AD pathogenesis and allow preclinical tests of drugs.

Experimental procedure:

The β-amyloid (1-42) peptide fragment ($A\beta_{1-42}$) or the non-amyloidogenic reverse peptide $A\beta_{42-1}$ ($A\beta_{42-1}$) were dissolved in bidistilled water at a concentration of 2.257 mg/ml and stored at -20°C until use. This led to the formation of soluble oligomers (Resende et al., 2008) and 2 nmol in 4 μl were intracerebroventricularly (icv) administrated, as previously described (Dall'Igna et al., 2007). Control animals were icv infused with a similar volume of bidistilled water. The behavioral performance was evaluated 15 days after the administration of $A\beta_{1-42}$ or $A\beta_{42-1}$. The rats were treated with the selective A_{2A} receptor antagonists SCH58261 (generously provided by Scott Weiss, Vernalis, UK) or KW6002 (synthesized as described previously – see Hockemeyer et al., 2004), each of these drugs was injected ip (0.05 mg/kg of SCH58261 dissolved in 10% dimethylsulphoxide in saline; 3 mg/kg of KW6002 dissolved in 5% Tween 80 in saline) 30 min before the administration of Aβ and thereafter once daily. It was chosen to administer SCH58261 and KW6002 ip since this route of administration of these particular doses of SCH58261 and KW6002 afford effective brain concentrations of these A_{2A} receptor antagonists within 30 min and have previously been shown to afford neuroprotection against different types of neuronal injuries without peripheral effects (reviewed in Cunha, 2005). The control rats were injected intra-peritoneally with saline with 10% dimethylsulphoxide or 5% Tween 80.

3.3. SCOPOLAMINE AND MK–801 MODELS OF MEMORY IMPAIRMENT

Scopolamine is a commonly used drug in the study of memory (e.g. Mihara et al., 2007). It blocks muscarinic cholinergic receptors, which are involved in learning and memory (Bartus and Johnson, 1976; Sitaram et al., 1978; Watts et al., 1981). In animal studies, scopolamine administration has been used as a simple method to induce cholinergic deficits, which produce impairments of a wide range of learning and memory tasks (Bartus and Johnson, 1976; Sitaram et al., 1978; Watts et al., 1981). Scopolamine also induces horizontal locomotor activity and impairs spontaneous alternation, an indication of memory impairment in the Y-maze in mice (Rubaj et al., 2003).

MK-801 is another pharmacological tool that causes an acute disruption of memory performance by inhibiting NMDA receptors, which play a crucial role in the implementation of several forms of synaptic plasticity in the mammalian central nervous system (Coan et al., 1987). The effects of MK-801 on memory may be explained by: (1) blockade of hippocampal NMDA receptors located on the pyramidal neurons (Wedzony et al., 1997); (2) an excessive release of serotonin (Löscher et al., 1991; Whitton et al., 1992); (3) or enhancement of dopamine release in the prefrontal cortex that could also contribute to hyperactivity (Wedzony and Golembiowska, 1993; Adams and Moghaddam, 1998).

Experimental procedure:

Scopolamine and MK-801 were dissolved in saline (0.9% NaCl) and were injected intra-peritoneally (ip) at doses of 2 mg/kg and 0.25 mg/kg, respectively. The rats were analyzed behaviorally 30 min after the administration of each drug. Control rats were injected ip with saline. When rats were treated with the selective A_{2A} receptor antagonists SCH58261 (generously provided by Scott Weiss, Vernalis, UK) or KW6002 (synthesized as described previously – see Hockemeyer et al., 2004), each of these drugs was injected ip (0.05 mg/kg of SCH58261 dissolved in 10% dimethylsulphoxide in saline; 3 mg/kg of KW6002 dissolved in 5% Tween 80 in saline) 30 min before the administration of memory-disturbing drugs.

3.4. LPS-INDUCED NEUROINFLAMMATION MODEL

Lipopolysaccharide (LPS) is a part of Gram negative bacteria wall and is a classical activator of the immune system including microglia cells (e.g. Kim et al., 2000; Kloss et al., 2001; Lee and Lee, 2002). Experimental administration of LPS has been used as a model of inflammatory response to brain infections (e.g. Kim et al., 2000; Kloss et al., 2001). LPS is the major inducer of microglia production of pro- and anti-inflammatory cytokines, chemokines, prostaglandins and nitric oxide (Bernardino and Malva, 2007). LPS systemic injection can orchestrate a correlation of responses in liver, serum and brain at 1 hour after, that are likely due to liver and serum monocyte-macrophage like cells secreting cytokines into serum that are transported into brain (Qin et al., 2008). *In vivo*, normal liver secretes little or no TNFα; however, in response to LPS, liver Kupffer cells and resident hepatic macrophages secrete TNFα

and other cytokines (Iwai et al., 2001; Xia et al., 2007; Qin et al., 2008). Alternatively, LPS may passively diffuse across the BBB, especially under severe endotoxemia (Rivest, 2003), allowing the direct interaction of LPS with its receptors in parenchyma microglia. Lehnard and colleagues described microglia cells as the major LPS-responsive cell type in the brain parenchyma due to the presence of TLR4 (Lehnardt et al., 2003; Bernardino and Malva, 2007). Peripheral inflammation can stimulate central inflammatory cytokine mRNA and protein synthesis (Laye et al., 1994; Pitossi et al., 1997); thus cytokines such as TNFα and IL-1β coordinate a group of adaptive behavioral changes collectively known as sickness behavior (Konsman et al., 2002). These two master pro-inflammatory cytokines possess pleiotropic and largely overlapping functions. They are produced by microglia and blood-derived macrophages during CNS inflammation and severe LPS-induced endotoxemia (Bernardino and Malva, 2007).

The LPS model is based on previous work done by Lynch's group (Dublin, Ireland), where it has been described that systemic LPS injection inhibits LTP in perforant path-granule cell synapses, through the increase of INFγ and IL-1β concentration in the hippocampus and induces microglia activation (e.g. Hauss-Wegrzyniak et al., 2002; Kelly et al., 2003; Barry et al., 2005; Maher et al., 2005). IL-1β can also activate JNK and p38 MAPK, which in turn leads to LTP deficit, since selective inhibitors of the MAPK prevent LTP impairment (Kelly et al., 2003; Minogue et al., 2003).

Experimental procedure:

The rats were divided in four groups: Control – vehicle icv and vehicle ip; SCH – SCH58261 icv and vehicle ip; LPS – vehicle icv and LPS ip; SCH+LPS – SCH58261 icv and LPS ip. Wistar male rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and then injected with the selective $A_{2A}R$ antagonist SCH58261 (50 nM) dissolved in 10% dimethilsulfoxide (DMSO in 0.9% NaCl) or vehicle administrated in the third ventricle (coordinates calculated using atlas of Paxinos and Watson, 0.8 mm posterior to the bregma on the midsagittal line and implanted 6.5 mm below the outer surface of the skull). After 30 min animals were injected with LPS (200 μg/kg) or vehicle (0.9% NaCl). The animals were sacrified by decapitation, under severe anesthesia with halothane, 4 hours after ip injection.

3.5. PERFORANT PATHWAY LESION

This mechanical lesioning model provides excellent features for studying the activation and proliferation of microglial cells to neural injury the perforant pathway (PP) lesion model (Ladeby et al., 2005). It is based on the highly organized anatomical structure of the PP projection, which originates in the entorhinal cortex and terminates in the hippocampal dentate gyrus (Amaral and Witter, 1995).

The model is based on the highly organized anatomical structure of the hippocampal formation and its connective integration with the entorhinal cortex. Transection of the entorhino-hippocampal perforant path (PP) axonal projection leads to anterograde axonal and terminal degeneration of presynaptic elements in the area of termination distal to the site of the axonal transection (Hjorth-Simonsen and Jeune, 1972; Fifkova, 1975; Matthews et al., 1976; Amaral and Witter, 1995). This degeneration comprises approximately 85% of the presynaptic elements in the outer two thirds of the dentate molecular layer (Matthews et al., 1976). This leads to activation of microglial and astroglial cells (Rose et al., 1976; Gall et al., 1979; Fagan and Gage, 1994; Jensen et al., 1994; Jensen et al., 1997), as well as activation of oligodendrocytes and profound changes in myelin (Bechmann and Nitsch, 1997; Jensen et al., 1999), concurrent to reactive sprouting and synaptogenesis of other afferent fiber systems terminating in the denervated areas (Lynch et al., 1972; Fagan and Gage, 1994; Deller et al., 1995; Deller et al., 1996).

Experimental procedure:

Transection of the PP projection was made using a stereotaxic frame (Stoelting, Wood Dale, IL, or David Kopf Instruments, Tujunga, CA) fitted with an adjustable wire knife (David Kopf Instruments). The knife was angled 10 degrees lateral and rotated 15 degrees rostral. The nosebar was set at -3 mm. Lesions were made with the mice under anesthesia by an intraperitoneal injection with a 1:1:1 mixture of xylazine, ketamine and sterile water (SAD, Denmark) at a dosage of 0.006 ml/g. A lesion was made by inserting the closed wireknife into the brain 2.0 mm lateral to the lambda and 0.4 mm caudal to the lambdoid suture through a drilled trepanation. The knife was unfolded 2.5 mm at a distance of 3.6 mm ventral to the meninges, and a 3.3 mm long cut was made. The knife was folded back into the needle, and retracted completely (Jensen et al., 1999). The lesioned mice were supplied with subcutaneous

sterile saline and buprenorphine injections for postoperative analgesia. After the surgical procedure, the animals were placed in a warm environment for recovery from anesthesia (Wirenfeldt et al., 2005).

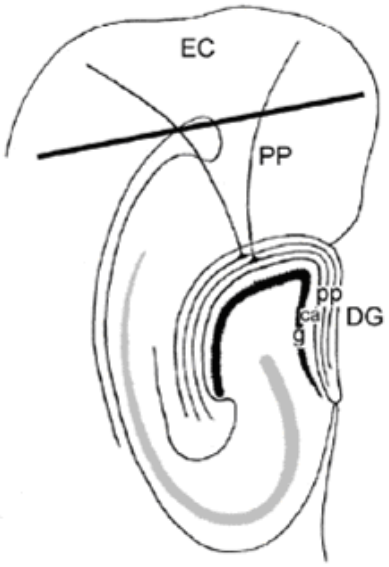


Figure 4: Perforant Pathway lesion – schematic illustration - The lesion-induced degeneration leads to a region-specific microglial reaction in the PP termination zone of the dentate gyrus. ca, Commissural-associational zone; DG, dentate gyrus; EC, entorhinal cortex; g, granule cell layer; pp, perforant pathway termination zone. Figure adapted from Dissing-Olesen et al., 2007.

The animals treated with the selective $A_{2A}R$ antagonist SCH58261 (generously provided by Scott Weiss, Vernalis, UK) were injected intraperitoneally (ip), 30 min before the perforant pathway lesion and daily with an effective dose (0.05 mg/kg) of SCH58261 (see Cunha et al., 2006; Dall'Igna et al., 2007) dissolved in 10% dimethylsulfoxide in saline (0.9% sodium chloride). The vehicle (10% dimethylsulfoxide in saline - 0.9% sodium chloride) was injected daily in non-treated control animals submitted to perforant pathway lesion.

Some of the animals were treated with an adenosine kinase inhibitor, 5-iodotubercidin, ip daily at an effective dose (3.1 mg/kg, see Gouder et al., 2004) dissolved in 20% dimethylsulfoxide in saline (0.9% sodium chloride). The vehicle (20% dimethylsulfoxide in saline) was injected daily in non-treated animals submitted to perforant pathway lesion. The animals were sacrified by ip injection of sodium pentobarbital (200 mg/kg), 3 days after the mechanical lesion.

3.6. KAINATE-INDUCED CONVULSION MODEL

Excitotoxic cell death is most often induced experimentally by the administration of kainate (KA), a potent agonist of AMPA/kainate receptors (Choi, 1988; Ohno et al., 1997; Doble, 1999; Wang et al., 2005). Thus, KA administration has been widely used as a model to study glutamate-induced excitotoxicity and seizure related neurologic diseases (Schauwecker and Steward, 1997). In rodents, peripheral injections of KA result in recurrent seizures (Ben-Ari et al., 1980; Sperk, 1994) and the subsequent degeneration of select populations of neurons in specific brain regions, such as the hippocampus, piriform cortex, thalamus and amygdala (Coyle et al., 1983; Sperk et al., 1985). In the hippocampus, the CA3 pyramidal cells and the interneurons in the hilus of the dentate gyrus are the most vulnerable, followed by CA1 pyramidal cells (Coyle et al., 1983; Sperk et al., 1985; Tauck and Nadler, 1985). KA administration induces neuronal death, astrogliosis, microgliosis and the consequent increase in the expression of genes implicated in the production of nitric oxide and cytokines, which might lead to expansion of brain injury and the delayed loss of neurons (Barone and Feuerstein, 1999; del Zoppo et al., 2000).

Experimental procedure:

In experiments carried out in mice, wild type and $A_{2A}R$ knockout mice were evaluated in parallel and were either injected with saline or with kainate (25-35 mg/kg – it is necessary to adjust kainate doses to obtain the same behavioral profile – accordingly with the different batches of kainate) injected subcutaneously (sc). After the injections the animals were placed in individual cages kept at room temperature and observed for 3 hours 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).

Table 2: Six-point seizure scale for mice, (from Schauwecker and Steward, 1997).

Level	Characteristic behaviors
1	Unmoving and crouched in a corner, staring
2	Stretches body out, tail becomes straight and rigid, ears laid back, bulging eyes
3	Repetitive head bobbing, rears into a sitting position with forepaws resting on belly
4	Rearing and falling tonic clonic seizures broken by periods of total stillness, jumping clonus, running clonus
5	Continuous level 4 seizures
6	Body in clonus, no longer using limbs to maintain posture, usually precursor to death

3.7. HIPPOCAMPUS

The hippocampus is perhaps the most studied structure in the brain. The hippocampus is a brain structure located inside the medial temporal lobe of the cerebral cortex, and therefore is part of the telencephalon (forebrain). It belongs to the limbic system. The hippocampus and its associated medial temporal lobe structures are required for the formation, consolidation, and retrieval of episodic memories (Morris et al., 1982; Squire and Zola, 1997; Eichenbaum, 2000; Scoville and Milner, 2000). Studies creating lesions in the rodent hippocampus or adjacent regions (septum, fimbria/fornix or parahippocampus) usually lead to impaired spatial and working memory (O'Keefe and Conway, 1978).

The hippocampal formation is a bi-lateral limbic structure, which in overall shape resembles two “Cs” leaning together at the top and spread apart at the base. The top portion of the formation is known as the “dorsal hippocampus” and because of its proximity to the septum, a structure at the midline of the brain, the dorsal tip of the hippocampus is called the “septal pole”. Cross-section taken perpendicular to the long axis (septal-temporal) will reveal the internal structure as two interlocking “Cs”, one reversed in relation to the other, each with its own principal cell layer. One “C” makes up the Ammon's Horn or *Cornu Ammonis* (CA1-CA3), also known as the “Hippocampus proper”. The principal cell layer of Ammon's Horn is the *stratum pyramidale*, or the pyramidal cell layer. The other “C” is made up of the dentate gyrus,

of which the *stratum granulosum*, or granule cell layer is the principal cell layer. The hippocampus, when cut transverse to its longitudinal (septal-temporal) axis, exibits a strong afferent set of three connected pathways known as the **"trisynaptic" circuit or loop** (Andersen et al., 1966; Swanson et al., 1978; Swanson, 1982; Witter et al., 1989), which is essentially represented by the following three subdivisions of the hippocampus according to Ramon and Cajal: CA1, CA2 and CA3 areas (see Fig. 5). **First**, layers II and III or the "surface layers" of the entorhinal cortex project to the granule cells of the dentate-gyrus, via the perforant-path. **Second**, the granule cells of the dentate gyrus project to the large pyramidal cells of *Cornu Amonnis*, subfield 3 (CA3), via the mossy fibers system. **Third** and finally, the CA3 pyramidal cells project to the pyramidal cells of the CA1 subfield, via the Schaffer collateral system (Blackstad, 1956, 1958; Amaral, 1978; Bayer, 1985; Amaral and Witter, 1989; Witter et al., 1989).

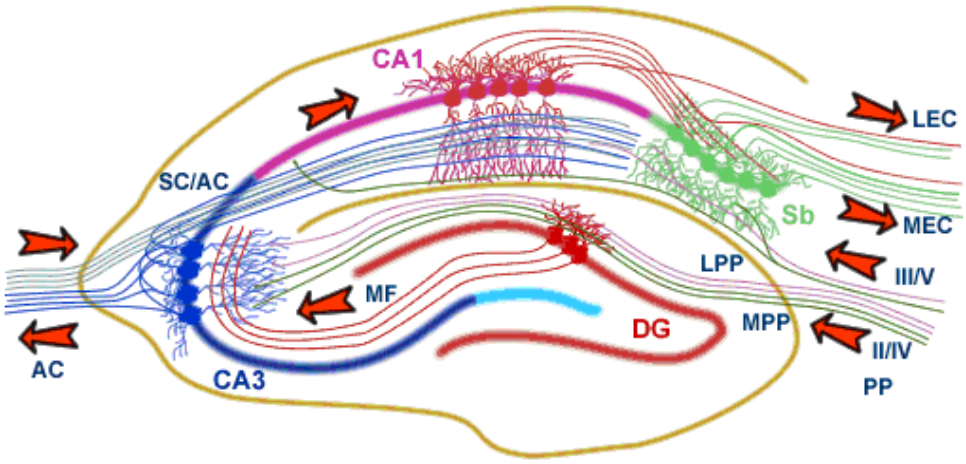


Figure 5: Hippocampal network. Dentate gyrus (DG); perforant path (PP); mossy fibers (MF); Schaffer collateral pathway (SC); associational commissural pathway (AC); subiculum (Sb); lateral enthorhinal cortex (LEC); medial enthorhinal cortex (MEC) adapted from http://www.bristol.ac.uk/synaptic/info/pathway/hippocampal.htm

The "trisynaptic" circuit has been considered to be the fundamental network of the hippocampus and is thought to be involved in neuronal information processing (Amaral and Witter, 1989). Hippocampal neuronal network mainly results from physiologic balance between inhibitory GABAergic and excitatory glutamatergic neurotransmissions; however, there are other neurotransmitters that can have a minor contribution to neurotransmission and a major contribution to fine-tune transmission (Lopes da Silva et al., 1990).

The hippocampus is an excellent choice to focus the study on the role of $A_{2A}R$ in neurodegenerative diseases and neuroinflammation, because it is one of the mainly affected

regions in Alzheimer's disease and epilepsy (Khachaturian, 1985; Wieser et al., 1993; Gomez-Isla et al., 1997; Rosenblum, 1999; Uylings and de Brabander, 2002). Furthermore, it is possible to perform behavioral animal studies dependent on this region, electrophysiological studies and biochemical studies.

3.8. CULTURED HIPPOCAMPAL NEURONS

There is a widespread use of cortical or hippocampal cultures neurons to study Aβ neurotoxicity (e.g Allen et al., 1999; Canu and Calissano, 2003; Plant et al., 2006) because the cortex and hippocampus are two of the main regions affected in AD.

Synaptic connections between hippocampal neurons in culture do not form until after 4 days *in vitro* (Bito et al., 1996) and functional synapses do not appear until 6-8 days *in vitro* (Deisseroth et al., 1996).

Experimental procedure:

Hippocampal neurons were cultured from embryonic day 18 Wistar rats fetuses in accordance with the protocol approved by the National Ethical Requirements for Animal Research and the European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purposes. Briefly, hippocampi were dissected out in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (GIBCO BRL, Paisley, Scotland, UK) with 10 mM of HEPES and digested with 1 mg/ml of trypsin (Sigma-Aldrich, Portugal) for 10 min. Cells were plated on 16 mm diameter coverslips placed in 12-well dishes, or plated on 6 wells dishes (both previously coated with 10 mg/ml of poly-D-lysine). For viability and immunocytochemistry assays, cells were plated at the density of 5×10^4 per coverslip and 0.75×10^6/well for Western blot analysis. Hippocampal neurons were grown at 37º C in a 5% CO_2 humidified atmosphere in Neurobasal medium supplemented with B-27 supplement (Gibco-BRL, Paisley, Scotland, UK), glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml- GIBCO BRL, Paisley, Scotland, UK).

3.9. CULTURED MICROGLIA

Cultured microglia are a simple model that allows to unravel the mechanisms of action of compounds that initiate or are involved in neuroinflammation processes. LPS is commonly used to mimic what happens upon inflammation, and alternatively, ATP or glutamate are used since they are released in large quantities in situations of cell death (Fields and Stevens, 2000; Honda et al., 2001).

Experimental procedure:

The preparation of microglia cultures was adapted from a method described by Kingham and Pocock (Kingham and Pocock, 2000). Briefly, 8 brains of 7-day-old rat pups were removed into 20 to 25 ml ice-cold phosphate buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and homogenised. The homogenate was centrifuged at 80x *g* for 1 min (short-spin). The pellet was discarded and the supernatant was centrifuged at 500x *g* for 10 minutes. The pellet was ressuspended in 45 ml of 70% isotonic Percoll (GE Healthcare) diluted with PBS. This was overlaid with 35% isotonic Percoll made up in PBS and finally PBS. The Percoll gradient was then centrifuged at 1300x *g* for 45 minutes at 20ºC. The cells at the 35/70% interface were removed and washed once in PBS. The mixture was centrifuged at 500x *g* for 10 minutes and the pelleted was plated on 10 mm coverslips at a density of 35000 cells per coverslip. Cells were maintained at 37 ºC in 5% CO_2 humidified atmosphere in filtered astrocyte conditioned medium (sterile filters with 0.22 μm diameter pore). The cells were stimulated after 3 days *in vitro*.

3.10. SYNAPTOSOMAL PREPARATIONS

Neural tissue is composed of neurons and their support cells, the glia. Neurons do not survive homogenization intact and the cell bodies are sheared from their processes, which break up into discrete fragments. The plasma membrane of the cell fragments may reseal to form osmotically active particles and when such particles contain the organelles of the synapse they are known as **synaptosomes** (see Fig. 6). Subcellular fractions enriched in synaptosomes are

sufficiently pure to permit the study of certain physiological and pharmacological aspects of synaptic function (Turner and Backelard, 1987).

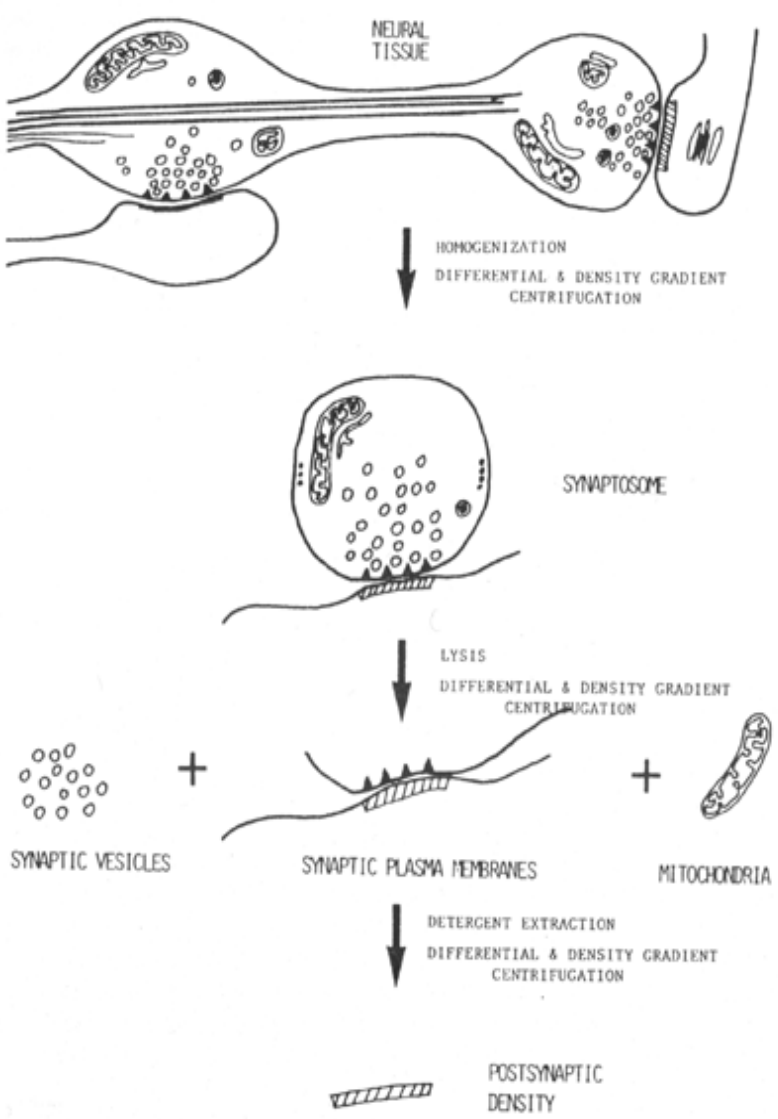


Figure 6: Diagram illustrating the derivation of synaptosomes and their subcellular fractions from a homogenate of neural tissue, adapted from (Turner and Backelard, 1987).

The isolation of synaptosomes can be achieved with two different isolation methods:

Rapid isolation of mice synaptosomes using a Percoll gradient

The animals were anesthetized under halothane atmosphere before being sacrificed by decapitation. Membranes from Percoll-purified hippocampal synaptosomes were prepared as previously described (Rebola et al., 2005c). Briefly, the two hippocampi from one animal were

homogenized at 4°C in sucrose solution (0.32 M) containing 1 mM EDTA, 10 mM HEPES, albumin bovine serum (BSA) 1 mg/ml pH 7.4, centrifuged at 3000x *g* for 10 min at 4°C, the supernatants collected, centrifuged at 14000x *g* for 12 min at 4°C and the pellet was resuspended in 1 ml of a 45% (v/v) Percoll solution made up in a Krebs solution (composition 140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose, pH 7.4). After centrifugation at 14000x *g* for 2 min at 4ºC, the top layer was removed (synaptosomal fraction), washed in 1 ml Krebs solution and resuspended in Krebs-HEPES medium (140 mM NaCl, 5 mM KCl, 10 mM HEPES, 5 mM glucose, pH 7.4).

Isolation of synaptosomes with a discontinuous Percoll gradient

This protocol was adapted from Dunkley and colleagues (Dunkley et al., 1986). Hippocampi were homogenized in a medium containing 0.25 M sucrose 10 mM HEPES (pH 7.4). The homogenate was spun for 3 min at 2000x *g* at 4ºC and the supernatant spun again at 9500x *g* for 13 min. Then, the pellets were re-suspended in 2 ml of 0.25 M sucrose and 10 mM HEPES (pH 7.4) and were placed onto 3 ml of Percoll discontinuous gradients containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM dithiothreitol and 3, 10, or 23 % Percoll, pH 7.4. The gradients were centrifuged at 25000x g for 11 min at 4ºC. Synaptosomes were collected between the 10 and 23 % Percoll bands and diluted in 15 ml of HEPES buffered medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). After centrifugation at 22000x *g* for 11 min at 4ºC, the synaptosomal pellet was removed. This procedure for separation of synaptosomes (in the absence of calcium) is crucial to reduce the amount of post-synaptic density material.

3.11. WESTERN BLOT

Cultured hippocampal neurons were washed twice with PBS and gently scraped with ice-cold lysis buffer composed of 25 mM HEPES-Na, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and supplemented with 2 mM DTT (Sigma-Aldrich, Portugal), 100 µM phenylmethanesulfonylfluoride (PMSF, from Sigma-Aldrich, Portugal), 2 mM orthovanadate (Sigma-Aldrich, Portugal), 50 mM sodium fluoride (Sigma-Aldrich, Portugal), and a protease inhibitor cocktail containing leupeptin, pepstatin A, chymostatin and aprotinin (1 mg/ml, all from

Sigma-Aldrich, Portugal). The synaptossomal extract from rat or mice was solubilized in 5% sodium dodecyl sulfate (SDS – Bio-Rad, Portugal) supplemented with 2 mM DTT and 100 μM PMSF and rapidly sonicated. After determining the amount of protein using the bicinchoninic acid method (Pierce, Dagma, Portugal), 1/6 volume of 6x SDS-PAGE sample buffer was added before storage at -20º C. Electrophoresis was carried out using a 10% or 7.5% SDS-PAGE gel after loading of different amounts of each sample.

Table 3: Gel formulation

GEL FORMULATION (1 GEL)	4% (Stacking gel)	7.5%	10%
Tris-buffer, 1.5 M, pH 8.8 (Resolving gel)	----------	3.022 ml	2.5 ml
Tris-buffer, 0.5 M, pH 6.8 (Stacking gel)	2.5 ml	------	----------
Acrylamide 30 % (Bio-Rad)	1.3 ml	2.25 ml	3.3 ml
Water	6.1 ml	3.45 ml	4.1 ml
SDS 10 % (Sigma-Aldrich, Portugal)	100 μl	195 μl	100 μl
TEMED (Sigma-Aldrich, Portugal)	10 μl	6 μl	5 μl
APS 10 % (freshly prepared, dilute in water)	50 μl	90 μl	50 μl

Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckingamshire, UK). Membranes were blocked for 1 h at room temperature with 5% low-fat milk in Tris-buffered saline or 3% bovine serum albumin (depending on the antibodies used), pH 7.6, and containing 0.1% Tween 20 (TBS–T). Membranes were then incubated overnight at 4 °C with primary antibodies (see Table 4). After washing with TBS-T, membranes were incubated IgG secondary antibodies (see Table 4). After washing, membranes were revealed by an enhanced chemifluorescence (ECF) kit (GE Healthcare, Buckingamshire, UK) and visualized in a VersaDoc 3000 (Bio-Rad, Portugal).

To determine phosphorylation *ratio* of p38 and JNK, the membranes were re-probed with rabbit anti-total JNK/SAPK or rabbit anti-p38 MAPK total.

Table 4: Primary and secondary antibodies for Western blot

Antibodies	Supplier	Host	Type	Dilution
Synaptophysin	Sigma	Mouse	IgG1	1:20000
SNAP-25	Sigma	Mouse	IgG1	1:20000
α-Tubulin	Sigma	Mouse	IgG1	1:20000
vGAT	Calbiochem	guinea-pig	polyclonal	1:1000
vGLUT1	Millipore	guinea-pig	polyclonal	1:10000
vGLUT2	Millipore	guinea-pig	polyclonal	1:10000
vAChT	Millipore	guinea-pig	polyclonal	1:500
GAPDH	Sigma	Rabbit	IgM	1:10000
A_{2A}	Santa Cruz	Goat	polyclonal	1:500
mGluR5	(Chemicon) Millipore	Rabbit	polyclonal	1:1000
CB1	Gift from Ken Mackie	Rabbit	polyclonal	1:750
A_1	Affinity Bioreagents	Rabbit	polyclonal	1:500
P_2Y_1	Santa Cruz	Goat	polyclonal	1:500
Phospho-p38 MAPK	Cell Signaling	Mouse	IgG1	1:1000
p38 MAPK total	Cell Signaling	Rabbit	IgG	1:1000
Pospho- SAPK/JNK	Cell Signaling	Mouse	IgG1	1:1000
SAPK/JNK total	Cell Signaling	Mabbit	IgG	1:1000
Rabbit- alkaline phosphatase conjugate (AP)	Amersham Biosciences	Goat	IgG (H+L)	1:20000
Mouse- AP	Amersham Biosciences	Goat	IgG + IgM (H+L),	1:20000
Goat-AP	Santa Cruz	Rabbit	IgG	1:2500
Guinea-pig-AP	Sigma	Goat	IgG	1:5000

3.12. BEHAVIORAL ANALYSIS

Each behavioral test was performed in sound-isolated room with lighting conditions and environmental cues held constant throughout testing. During the tests the experimenter stayed in the room adjacent to the one in which the experiments were performed. To remove the smell traces left by mice, the floor and walls of the equipment were carefully cleaned before testing the next animal. To avoid influences of circadian rhythms on performance of the animals each behavioral test was carried out between 10:00 and 16:00.

Open field

The open field test is designed to measure behavioral responses such as locomotor activity, hyperactivity, and exploratory behaviors. The open field is also used as a measure of anxiety and/or impulsivity. Rats and mice tend to avoid brightly illuminated, novel, open spaces, so the open field environment acts as an anxiogenic stimulus and allows for measurement of anxiety-induced locomotor activity and exploratory behaviors (Walsh and Cummins, 1976; Belzung and Griebel, 2001). Like the elevated plus maze, open field testing is a one trial test with little or no impact on the animal's subsequent behavior. Locomotor activity was monitored in an open field arena (50 x 50 cm, divided in 4 squares of 25 cm for rats and 30 x 30 cm, divided in 9 squares for mice) and the exploratory behavior of the animals was evaluated by counting the total number of line crossings (horizontal explorations) and the number of rearings (vertical explorations) over a period of 5 min.

Y-maze

Y-maze test is a hippocampal-dependent memory performance test that is assessed by measuring spontaneous alternation performance (reviewed in Hughes, 2004). Spontaneous alternation is a measure of exploratory behavior, most often evaluated in rodents (Dember and Fowler, 1958, 1959). Rats and mice normally alternate at levels significantly above chance, indicating their willingness to explore novel environmental stimuli (Dember and Fowler, 1958, 1959). Spontaneous alternation is also dependent on spatial memory capacity (Lalonde, 2002). In the free-trial procedure, the alternation rate is dependent on whether the animal possesses a bias of turning either to the left or to the right side (Lalonde, 2002). However, the free-trial

procedure, when compared to forced-trial, has been the most prevalent method of evaluating the effects of lesions on spontaneous alternation (Lalonde, 2002).

The Y-maze apparatus is constituted by 3 equal arms with an angle of 120 degrees between them. The spontaneous alternation is measured by the visual record of the series of arm entries done by the rodents (rats or mice) during 8 minutes. An alternation was defined as entries in all three arms on consecutive occasions. The percentage of alternation was calculated as total number of alternations divided by (total arm entries - 2), as previously described (e.g. Dall'Igna et al., 2007). Evaluation was performed under blind conditions to different treatments and the same animals were used for evaluation of locomotion and memory performance.

3.13. HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Perfusion and fixation of the brain was performed through trans-cardiac perfusion with 4% paraformaldehyde (in 0.9% sodium chloride and 4% sucrose from Sigma-Aldrich, Portugal), as previously described (e.g. Cunha et al., 2006). Frozen brains were sectioned (20 μm coronal sections) using a cryostat Leica CM1850 (Leica Microsistemas, Lisboa, Portugal) and the sections were mounted on slides coated with 2% gelatin plus 0.08 % chromalin (chromium and potassium sulfate, from Sigma-Aldrich, Portugal), allowed to dry at room temperature and stored at -20ºC until use.

Cresyl violet

Neuronal morphology in hippocampal sections was evaluated by cresyl violet staining of Nissl bodies, as previously described (Lopes et al., 2003). Briefly, sections were incubated for 10 min with cresyl violet (Sigma-Aldrich, Sintra, Portugal) solution (0.5% in acetate buffer). Sections were then washed twice with acetate buffer, twice in 100% ethanol, cleared with xylene and mounted with vector medium (Vector Laboratories, Batista Marques, Lisbon, Portugal).

Fluoro-Jade C

Degenerating neurons were detected using Fluoro-Jade C, which fluorescently labels them independently of the mechanism of cell death (Schmued et al., 2005).

Brain sections in slides were immersed in 0.1% NaOH in 80% ethanol for 5 min. After rinsing for 2 min in 70% ethanol and for 2 min in distilled water, the slides were then transferred to a solution of 0.06% potassium permanganate for 10 min and were gently shaken on a rotating platform. This solution when kept in a sealed glass container remains usable for a period of about 1 week. The slides were rinsed for 1 min in distilled water and then transferred to the Fluoro-Jade C (from Histo-Chem Inc. – Jefferson, AR, USA) staining solution (0.0001% working solution of Fluoro-Jade C – the proper dilution was accomplished by first making a 0.01% stock solution of the dye in distilled water and then adding 1 ml of the stock solution to 99 ml of 0.1% acetic acid vehicle) where they are gently agitated for 10 min. After staining, the sections were rinsed three times for 1 min with distilled water. Excess water was drained off, and the slides were rapidly air dried on a slide warmer or with a hot air gun. When dried, the slides were immersed in xylene and then coversliped with D.P.X (Sigma-Aldrich, Portugal). Sections were examined under a transmission and fluorescence Zeiss Axiovert 200 microscope, with Axiovision software 4.6 (PG-Hitec, Lisbon, Portugal).

Immunohistochemistry for synaptophysin, CD11b and glial fibrillary acidic protein

Detection of nerve terminals was carried out as previously described (Cunha et al., 2006), using immunohistochemical detection of synaptophysin, a synaptic protein (Masliah and Terry, 1993). Immunohistochemistry detection of CD11b, a marker of microglia (Jensen et al., 1997), and of glial fibrillary acidic protein (GFAP), a marker of astrocytes (Pekny and Nilsson, 2005), was carried out to evaluate microgliosis and astrogliosis, respectively. The sections were first rinsed for 5 min with phosphate buffered saline PBS and then three times for 5 min with Trizma base buffer (TBS - 0.05 M containing 150 mM of NaCl, pH 7.2) at room temperature. Sections were then permeabilized and blocked with TBS containing 0.2% Triton X-100 and 10% goat serum during 45 min, incubated in the presence of the mouse anti-synaptophysin antibody (1:500 dilution in TBS containing 0.2% Triton X-100 and 10% normal goat serum), or rat anti-mouse-CD11b (for mouse sections), or mouse anti-rat-CD11b (for rat sections) (1:600, Serotec, Lisboa, Portugal) or anti-GFAP-cy3 (1:500, Sigma-Aldrich) for 72 h at 4ºC. Then the sections were rinsed three times for 10 min in TBS and subsequently incubated with goat anti-mouse or goat anti-rat secondary antibody conjugated with a fluorophore (Alexa Fluor 488, Invitrogen, Lisbon, Portugal) (1:100 dilution in 0.1 M TBS containing 0.2% Triton X-100 and 10% normal serum) for 2 hours at room temperature. After rinsing twice for 10 min in TBS and

once for 10 min in distilled water, the sections were dehydrated and passed through xylene before mounting on slides, using Vectashield mounting medium (Vector Laboratories).
All sections were examined under a transmission and fluorescence Zeiss Axiovert 200 microscope, with Axiovision software 4.6 (PG-Hitec, Lisbon, Portugal).

Immunohistochemistry co-localization of CD11b and A_{2A} adenosine receptors

In mice previously injected with kainate 25-35 mg/kg, the perfused brains were sectioned coronally (30 μm). The sections were rinsed three times for 5 min PBS at room temperature. Sections were then blocked with PBS containing 5% goat serum during 45 min, and then incubated in 0.25% Triton X-100 + 5% goat serum in PBS, in the presence of the anti-mouse $A_{2A}R$ antibody (1:500, monoclonal, Upstate) in combination with rat anti-mouse-CD11b antibody (1:100, IgG1, Serotec) for 48 h at 4ºC. The slices were rinsed three times for 10 min in PBS + 0.25% Triton X-100 and subsequently incubated with an goat anti-mouse IgG conjugated with AlexaFluor 488 and an goat anti-rat IgG conjugated with AlexaFluor 594 (1:200 dilution in 0.25% Triton X-100 + 5% goat serum in PBS, Molecular Probes), for 2 hours at room temperature. After rinsing once for 10 min in PBS, nuclei were stained with Hoechst 33342 (2 μg/ml – from Molecular Probes, Leiden, The Netherlands) for 10 minutes. Slices were rinsed three times in PBS and were mounted in a glass slide with Dako fluorescent mounting medium (Dako, USA). The brain sections were analyzed in a laser scanning confocal microscope (LSM 510 META, Zeiss).

Immunocytochemistry of hippocampal neurons or microglia cells

After fixation with paraformaldehyde, cells were permeabilized with PBS plus 0.2 % Triton X-100 for 2 minutes and incubated with 3 % of BSA in PBS for 30 min. The cells were incubated with primary antibodies (Table 5) for 1 hour at room temperature or overnight at 4ºC. After three washes with PBS, cells were incubated with secondary antibody conjugated with a fluorophore (Table 5). The cells were visualized by a transmission and fluorescence Zeiss Axiovert 200 microscope, with Axiovision software 4.6 (PG-Hitec, Lisbon, Portugal) or with confocal microscopy MRC 600, Bio-Rad (Hercules, CA, USA) or with a laser scanning confocal microscope (LSM 510 META, Zeiss).

Table 5: Primary and secondary antibodies for immunocytochemistry

Antibodies	Supplier	Host	Type	Dilution
Synaptophysin	Sigma	mouse	IgG1	1:200
SNAP-25	Sigma	mouse	IgG1	1:200
MAP-2	Santa Cruz	rabbit	IgG1	1:400
Phospho-p38	Cell signaling	mouse	IgG1	1:100
Synaptophysin	Sigma	rabbit	IgG1	1:200
A_{2A}	Santa Cruz	goat	polyclonal	1:500
CD11b	Serotec	mouse	IgG1	1:100
ED1	Serotec	mouse	IgG1	1:750
Alexa Fluor 488 anti-mouse	Invitrogen	donkey	IgG (H+L)	1:200
Alexa Fluor 488 anti-goat				
Alexa Fluor 594 anti-rabbit				
Alexa Fluor 488 anti-rabbit				

Immunocytochemistry in synaptosomes

Nerve terminals from the mice hippocampus were purified through a discontinuous Percoll gradient and placed onto coverslips previously coated with poly-D-lysine (from Sigma-Aldrich, Portugal), fixed with 4% paraformaldehyde (4% paraformaldehyde and 4% sucrose in 0.9% NaCl) for 15 min and washed twice with PBS medium. The synaptosomes were permeabilized in PBS with 0.2% Triton X-100 for 10 min and then blocked for 1 h in PBS with 3% bovine serum albumin (BSA) and 5% normal bovine serum. The synaptosomes were then washed twice with PBS and incubated with primary antibodies: markers for the different vesicular transporters and synaptophysin (see Table 6) for 1 h at room temperature. The synaptosomes were then washed three times with PBS with 3% BSA and incubated for 1 h at room temperature with secondary antibodies labeled with a fluorescent dye (see Table 6). It was confirmed that none of the secondary antibodies produced any signal in preparations to which the addition of the corresponding primary antibody was omitted. Most importantly, it was confirmed that the individual signals in double-labeled fields are not enhanced over the signals under single-labeling conditions. After washing and mounting on slides with Prolong Antifade

(Molecular Probes, Leiden, The Netherlands), the preparations were visualized in a transmission and fluorescence Zeiss Axiovert 200 microscope, with Axiovision software 4.6 (PG-Hitec, Lisbon, Portugal). Each coverslip (three to four per experiment) was analyzed by counting three to four different fields from each coverslip and in each field a minimum of 900 individualized elements. The values are presented as the percentage of the total number of glutamatergic, GABAergic or cholinergic terminals that were labeled with different vesicular markers in total population terminals stained with synaptophysin, as mean±S.E.M. of *n* experiments (i.e. in preparation obtained from different mice).

Table 6: Primary and secondary antibodies for immunocytochemistry of synaptosomes

Antibodies	Supplier	Host	Type	Dilution
Synaptophysin	Sigma	Mouse	IgG1	1:200
vGAT	Calbiochem	guinea-pig	polyclonal	1:1000
vGLUT1	Millipore	guinea-pig	polyclonal	1:1000
vGLUT2	Millipore	guinea-pig	polyclonal	1:1000
vAChT	Millipore	guinea-pig	polyclonal	1:500
Alexa Fluor 488 anti-mouse	Invitrogen	Donkey	IgG (H+L)	1:200
Alexa Fluor 594 anti-guinea pig				

3.14. VIABILITY OF SYNAPTOSOMES – MTT ASSAY

The redox status of the synaptosomes is affected by exposure to β-amyloid peptides (Mattson et al., 1998). The alteration in the redox status by Aβ is related with biochemical alterations characteristic of apoptosis, which consequently lead to synaptic dysfunction (Mattson et al., 1998). The redox status was measured by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, from Sigma-Aldrich, Portugal), as previously described (Silva et al., 2007). Synaptosomes were incubated for 2 h at 37 ºC in Krebs buffer in the absence or presence of $A\beta_{1-42}$ (500 nM) and/or SCH58261 (50 nM). MTT (0.5 mg/ml) was then added and incubated for 1 h at 37 ºC in the dark. As MTT is converted to a water-insoluble blue product (formazan) by viable terminals, the precipitated dye

can be spectrophotometrically (570 nm) quantified after exposing the synaptosomes to isopropanol containing 0.04 M HCl. Values were expressed as the percentage of optical density of control synaptosomes, in the absence of added drugs.

3.15. MITOCHONDRIAL MEMBRANE POTENTIAL OF SYNAPTOSOMES – FUNCTIONAL ASSAY

Mitochondrial membrane potential ($\Delta\Psi_m$) is a key marker of mitochondria function, generated by the pumping of protons across the inner mitochondrial membrane in association with electron transport (Buckman and Reynolds, 2001). In turn, $\Delta\Psi_m$ drives many key mitochondrial functions, including ATP synthesis, calcium accumulation and maintenance of ion gradients that permit the influx of substrates and the eflux of metabolic products (Buckman and Reynolds, 2001).

Tetramethyl rhodamine methyl ester ($TMRM^+$) is a fluorescent probe to monitor $\Delta\Psi_m$. $TMRM^+$ is a derivative of rhodamine 1,2,3, that exhibits a low binding to mitochondria. This indicator dye is a lipophilic cation, which accumulates within mitochondria in proportion to $\Delta\Psi_m$, in accordance to the Nernst equation, and the fuorescent properties of this dye are altered upon mitochondrial accumulation (Scaduto and Grotyohann, 1999). These major changes in the fluorescent properties of this indicator upon mitochondrial accumulation correspond to a fluorescent quenching and a red shift in the wavelength of maximum excitation and emission energy (Scaduto and Grotyohann, 1999). This probe is innocuous since it does not cause loss of mithocondrial function and/or depletion of $\Delta\Psi_m$ (Scaduto and Grotyohann, 1999).

The mitochondrial membrane potential of synaptosomes was measured by a fluorimetric assay adapted and optimized for synaptosomes from a fluorimetric protocol used in isolated brain mitochondria (Oliveira et al., 2007). Synaptosomes were incubated for 2 h at 37 ºC in Krebs buffer in the absence or presence of $A\beta_{1-42}$ (500 nM) and/or SCH58261 (50 nM), followed by 1 h incubation with 2 nM $TMRM^+$ (dissolved in methanol from Molecular Probes, Leiden, The Netherlands) and a short-spin centrifugation. The pellet was resuspended in 150 µl Krebs-HEPES with 2 nM $TMRM^+$. The functional assay was performed in a fluorescence spectrometer (Spectra Max Gemini EM, Molecular Devices, USA), using 540 nm excitation and 590 nm emission, with a cut off of 570 nm, and analyzed with SoftMax Pro V5 (Molecular

Devices, Sunnyvale, CA, USA). The experiment is initiated by measuring a baseline (370±8 fluorescent arbitrary units, n=8) for 10 min, followed by the simultaneous addition of FCCP (2 μM) and oligomycin (1 μg/ml) and measurement of fluorescence for 10 minutes to establish the new baseline, yielding a change of relative fluorescence of 607±28 fluorescent arbitrary units (control, n=8). The effect of the tested drugs was measured as changes in this difference between final and initial baseline and are expressed as the percentage of the difference observed in control conditions.

3.16. VIABILITY OF CULTURED CELLS – SYTO-13/PI ASSAY

Viability assays were performed by double-labeling (3 min incubation) with the fluorescent probes Syto-13 (4 μM) and propidium iodide (PI, 4 μg/ml) (from Molecular Probes, Leiden, The Netherlands) followed by fluorescence microscopy cell counting. As previously described (Rebola et al., 2005b), viable neurons present nuclei homogenously labeled with Syto-13 (green fluorescent nuclei), whereas apoptotic neurons show condensed and fragmented nuclei labeled with Syto-13 (primary apoptosis) or with Syto-13 plus PI (secondary apoptosis) and necrotic neurons present intact nuclei labeled with PI (red fluorescent nuclei). In parallel, the nuclear morphology of hippocampal neurons was analyzed by fluorescence microscopy using Hoechst 33342 (2 μg/ml for 10 min; from Molecular Probes), as previously described (Almeida et al., 2004). Each experiment was repeated using different cell cultures in duplicate, and cell counting was carried out in at least six fields per coverslip, with a total of approximately 300 cells.

3.17. HPLC QUANTIFICATION OF ADENOSINE LEVELS

After addition at time zero of $A\beta_{1-42}$ (500 nM) to Neurobasal medium bathing hippocampal neuronal cultures maintained at 37ºC in a 5% CO_2 humidified atmosphere, samples (125 μl) were collected from the incubation medium after 0, 3, 12, 24 and 48 hours. Each sample was filtered through 0.22 μm filters (Millex-GV from Millipore, Interface, Lisbon, Portugal) and

then stored at -20 °C until high performance liquid chromatography (HPLC) analysis (Cunha and Sebastião, 1993). Separation of adenine nucleotides was performed at room temperature using a reverse-phase column [LiChroCART 125x4 mm LiChrospher 100 RP-18 (5 μm) cartridge fitted into a ManuCART holder (Merck Darmstadt, Germany)], using a GOLD^{TM} system (Beckman, UK) equipped with a UV detector set at 254 nm. The eluent was a 100 mM KH_2PO_4 solution with 15% methanol (pH 6.5) with a flow rate of 1.75 ml/min. The identification of the peak corresponding to adenosine was performed by comparison of relative retention times with adenosine standards and confirmed by complete disappearance of the peak after incubation of samples with 4 U/ml adenosine deaminase (Cunha and Sebastião, 1993). The quantification of adenosine was achieved by calculating the peak area and then converting to concentration values (correcting the change of incubation volume over time) by calibration with known standards (0.03–3 μM).

3.18. MONITORING OF DYNAMIC CHANGES IN INTRACELLULAR FREE CALCIUM CONCENTRATION AND MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\Psi_M$) IN A MODEL OF ELECTRICAL FIELD STIMULATION (EFS) IN HIPPOCAMPAL NEURONS

FURA-2 ($C_{44}H_{47}N_3O_{24}$) is a calcium (Ca^{2+}) indicator, a dye-molecule that binds Ca^{2+} since its structure is derived from EGTA [ethylene glycol-bis(2-aminoethylether)--N,N,N',N'-tetraacetic acid] with 4 extra carboxyl groups to bind calcium (Grynkiewicz et al., 1985). FURA-2 molecule has a dissociation constant (K_D) for calcium near the basal $[Ca^{2+}]_i$ of a mammalian cell (100 nM) (values provided by the supplier). FURA-2 is a ratiometric dye and Ca^{2+} binding shifts rightward the excitation spectrum (Grynkiewicz et al., 1985; Hirst et al., 1999). In the presence of calcium, maximum FURA-2 fluorescence (at 510 nm emission) is observed at wavelength of 340 nm and in Ca^{2+}-free conditions at 380 nm (Grynkiewicz et al., 1985; Hirst et al., 1999). Therefore, it follows that the concentration of free intracellular Ca^{2+} is proportional to the ratio of fluorescence at 340/380 (Hirst et al., 1999). Rationing minimizes a number of negative effects which occur and disturb measurements like uneven dye loading, leakage of FURA-2 and bleaching. FURA-2 dye provides the possibility to perform measurements for about 1 hour without significant bleaching.

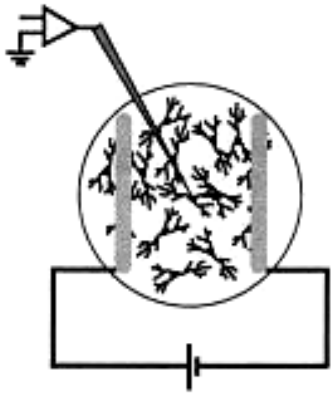


Figure 7: Field stimulation setup – a culture of hippocampal neurons is placed between parallel oriented platinum wires electrodes (6 mm apart), which are connected to a stimulus isolator and a pulse generator. Adapted from (Gärtner and Staiger, 2002).

Single cell imaging was performed with an imaging system composed by an inverted epifluorescence microscope (Eclipse TE300; Nikon, Tokyo, Japan) equipped with a 20x air objective, a monochromador (Polychrome II; TILL Photonics, Martinsried, Germany), a CCD camera (C6790; Hamamatsu Photonics, Japan) and a computer with analisys software (Aquacosmos, Hamamatsu Photonics). Cells were used between 8-10 DIV. Hippocampal cultured cells were loaded with the high affinity Ca^{2+} probe FURA-2 AM (5 μM – dissolved in DMSO from Molecular Probes, Leiden, The Netherlands) and tetramethylrhodamine ($TMRM^{+}$ – 50 nM dissolved in methanol from Molecular Probes, Leiden, The Netherlands) in a quench mode, allowing estimation of mitochondrial depolarization and repolarization, and distinguishing between loss of the Ca^{2+} probe attributable to membrane rupture and a true Ca^{2+} recovery (Ward et al., 2005). Loading of the probes was done at 37ºC during 30 minutes in buffer containing the following (in mM): 135 NaCl, 5 KCl, 0.4 KH_2PO_4, 2 $CaCl_2$, 1 Na_2SO_4, 20 HEPES and 15 mM glucose (pH 7.4). After rinsing with fresh buffer with $TMRM^{+}$ to maintain equilibrium of the probe, coverslips were mounted in a nonperfused 600 μl chamber and thermostatized to 37ºC. Individual cells were identified as region of interest (ROIs) for the determination of fluorescence time courses in the three different wavelengths: 340, 380 and 550 nm. The electrical field stimulation (EFS) started 5 minutes after the initiation of the experiment, with the following characteristics: every minute (trains of 10 s with pulses of 1 ms duration, 50 mA amplitude, applied at a frequency of 10 Hz), synchronized with the openness of the shutter, with 7 seconds of interval. The square wave was generated by a Stimulator Hugo Sachs Elektronik (Harvard Apparatus 215/I) and monitored by an oscilloscope Multimetrix X03002. *In situ* calibration of

Ca^{2+} response was performed at the end of every individual experiment, by determining maximal and minimal 340/380 ratios for each individual cell. This was achieved by adding a high concentration of Ca^{2+} ionophore, 4-BR-A23187 (15 μM) in a buffer with 2 mM of Ca^{2+} followed by Ca^{2+}-free buffer with 5 mM EGTA. Calibration will adjust values relatively to these maximal and minimal reference values, providing an easier way to compare results obtained in different laboratories regardless of the characteristics of equipment used to perform calcium imaging.

3.19. REAL TIME RT-PCR MEASUREMENT OF MRNA ENCODING CD11B AND A_{2A} RECEPTORS

RNA was extracted using RNeasy Protect mini kits (Qiagen, Hilden, Germany) or TRIzol (Invitrogen, Taastrup, Denmark) from total hippocampus samples, as previously described (Babcock et al., 2003; Meldgaard et al., 2006). Complementary DNA (cDNA) was synthesized from 0.4 μg of total RNA by reverse transcription, as previously described (Meldgaard et al., 2006). Real-time PCR and detection of product accumulation was performed using an *iCycler* (Bio-Rad, Denmark). Each PCR consisted of 2x RealQ master mix (Ampliqon, Denmark), diluted cDNA and the relevant primer/probe mix (see Table 7 with sequence of primers used – 10 μM of each PCR primer A_{2A}R cDNA – TAG-Copenhagen A/S, Denmark) and green dye 100-fold (SYBR, Green I nucleic acid gel stail, Cambrex, Denmark) and fluorescein 10 μM (Bio-Rad, Denmark). Amplification of CD11b and "housekeeping" gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) was done using previously published primer and probe sequences (see Table 7) and FAM (carboxyfluorescein)- or HEX (Hexachloro-Fluorescein)--labeled TaqMan probes instead of SYBR Green, respectively (Meldgaard et al., 2006). The PCR cycling for A_{2A}R was initiated by activating the *Taq polymerase* for 15 minutes at 95 °C; thereafter, two-step PCR protocol was run for 40 cycles. Each PCR A_{2A}R cycle consisted of 10 s denaturation at 95 °C, followed by annealing and elongation for 1 min at 62 °C. The PCR cycling for CD11b was initiated by activating the *Taq polymerase* for 15 minutes at 95 °C; thereafter, a two-step PCR protocol was run for 45 cycles. Each PCR cycle consisted of 10 s of denaturation, at 95 °C, followed by annealing and elongation for 1 min at 60 °C. The "housekeeping" gene (HPRT1) was targeted for an internal control gene (Meldgaard et al., 2006).

The test gene ($A_{2A}R$ or CD11b) data were normalized using the HPRT1 data and the mean value of duplicate sample extracts was used for determining the relative RNA levels. The relative RNA level was calculated as the ratio of the sample mean-value to the baseline sample ("calibrator") mean-value. The calibrator sample was included for testing on each PCR plate. The cDNA (diluted) used for real-time PCR standard curves was derived from a pool RNA sample of a non-operated mice.

Table 7: Primers used in the Real Time RT-PCR technique

Oligomer	Sequence
Sense PCR primer $A_{2A}R$ -sense	CGAATTCCACTCCGGTACAATGG
AntiSense PCR primer $A_{2A}R$ –antisense	ATGACAGCACCCAGCAAATCG
Sense PCR primer CD11b-sense	CGGAAAGTAGTGAGAGAACTGTTTC
AntiSense PCR primer CD11b –antisense	CTTATAATCCAAGGGATCACCGAATTT
FAM- labeled TaqMan probe (sense) CD11b –probe	TCTGTGATGACAACTAGGATCTTCGCAGCA
Sense PCR primer HPRT1 –sense	GTTAAGCAGTACAGCCCCAAAATG
AntiSense PCR primer HPRT1 -antisense	AAATCCAACAAAGTCTGGCCTGTA
HEX-labeled TaqMan probe (sense) HPRT1 -probe	AGCTTGCTGGTGAAAAGGACCTCTCGAAGT

PCR primers were designed to target exon-exon junctions of the respective genes. Design was done using Beacon Designer 2.0 (Premier Biosoft International); melting point for the $A_{2A}R$ and CD11b (Fenger et al., 2006) was 60ºC, and for probes it was set 10ºC higher.

3.20. DRUGS

- $A\beta_{1-42}$ and $A\beta_{42-1}$ were purchased from American Peptides (Sunnyvale, USA).
- SCH58261 was generously provided by Scott Weiss, Vernalis, UK. SCH58261 was prepared as a 5 mM stock solution in 100% DMSO and aliquots stored at -20ºC. KW6002 was generously provided by Christa E. Müller and a fresh solution was ressupended daily (dissolved in 5% Tween 80 in saline), for ip administration.
- Lipopolysaccharide (LPS) from Escherichia coli O26:B6; 8-Br-cAMP, 5-iodotubercidin (dissolved in 100% DMSO), H-89 (dissolved in water), scopolamine, MK 801 (dissolved in 0.9% NaCl) and FCCP and oligomycin from Streptomyces diastatochromogenes (dissolved in ethanol) were purchased from Sigma-Aldrich, Portugal.
- SB202190 (dissolved in DMSO) was purchased to Tocris, UK.
- Kainate (dissolved in 0.9% NaCl) was from Ocean products, Canada.
- Pentobarbital sodium (dissolved in water or 0.9% NaCl) was purchased to Braün, Portugal.
- Buprenorphine (Temgesic®) from Schering-Plough, Denmark.
- Ketamine (20 mg/ml- Rompun® VET) was purchased to Bayer, Denmark.
- Xylazine (50 mg/ml - Ketaminol® VET) was purchased to Intervet, Denmark.

3.21. DATA PRESENTATION

Results are presented as means±S.E.M. values of n experiments. To test the significance of the effect of a drug *versus* control, an unpaired Student's *t* test was generally used considering a statistical difference for a $P<0.05$. In experiments with more than two groups it was used one-way analysis of variance (ANOVA), followed by Duncan's or Newman-Keuls multiple comparison test**.** A value of $P<0.05$ was considered to represent a significant difference. In the neuroinflammation chapter, in the perforant pathway lesion results, a two-way analysis of variance (ANOVA) was used to detect a possible interaction between the mRNA CD11b and $A_{2A}R$ through time. A value of $P<0.05$ was considered to represent a significant difference.

4. RESULTS

4.1. MODIFICATION UPON AGING OF THE DENSITY OF PRESYNAPTIC MODULATION SYSTEMS IN THE HIPPOCAMPUS

4.1.1. INTRODUCTION

There is increasing evidence indicating that dysfunction and loss of nerve terminals might represent one of the earliest modifications in the course of neurodegenerative diseases (Wishart et al., 2006). For instance, in Alzheimer's disease, the loss of synaptic markers, in contrast to neuronal loss, is the parameter that correlates better with memory dysfunction (Selkoe, 2002). Likewise, in Parkinson's disease, modification of firing patterns of cortico-striatal pathways (Bézard et al., 2003) and loss of dopaminergic terminals (Herkenham et al., 1991) occur early in the asymptomatic phase of the disease. This synaptic dysfunction and damage has also been recognized as an early event in the course of different other neurodegenerative diseases such as Huntington's (Li et al., 2001a), prion's diseases (Ferrer, 2002), HIV infection (Garden et al., 2002), schizophrenia (Glantz et al., 2006), temporal lobe epilepsy (Ratte and Lacaille, 2006) or motor neuropathies (Raff et al., 2002). This central and initial role of synaptic dysfunction in neurodegenerative diseases has been the main driving force to conceive presynaptic neuromodulation systems as candidate targets to restraint the early modifications in these diseases. Thus, drugs activating presynaptic modulators such as adenosine A_1 receptors (Fredholm et al., 2005b) or cannabinoid CB_1 receptors (van der Stelt and Di Marzo, 2005) might afford protection against different neurodegenerative diseases. Also, antagonists of adenosine $A_{2A}R$ (Fredholm et al., 2005b), metabotropic group 5 receptors (Flor et al., 2002) or ATP P_2Y_1 receptors (Franke et al., 2006) also confer neuproprotection in different animal models of brain degenerative diseases. However, the study of novel neuroprotective strategies has mostly been carried out using young adult animals, whereas most neurodegenerative diseases are prevalent in the elderly. The extrapolation of the conclusions reached in young adult animals to the context of disease progression in the elderly can only be tentatively suggested provided that there is no significant aging-related modification of the targeted presynaptic modulation systems. As a first step to tackle this question, it was explored by Western blot analysis how the density of different presynaptic markers and of receptors triggering different presynaptic modulation systems changed with aging in rat hippocampal nerve terminals.

4.1.2. ONTOGENY OF PRE-SYNAPTIC MARKERS AND RECEPTORS

When investigating the age-related changes of the density of proteins integrating the vesicular excoytotic machinery, it was found that the density of these proteins, often used as synaptic markers, was different in the different age groups. In fact, as shown in Figure 8A, the density of synaptophysin (a protein present in synaptic vesicles – see Pinheiro et al., 2003, and references therein) as well as the density of SNAP-25 (a membrane protein located in the active zone of nerve terminals – see Pinheiro et al., 2003, and references therein) were larger (P<0.05, n=5-8) at 6 and 12 months compared to 2 months (12–15% for synaptophysin and 21–24% for SNAP-25). In contrast, the density of both proteins decreased (P<0.05, n=4–8) at 18 months (12.4±4.2% for synaptophysin and 13.3±4.6% for SNAP-25, n=5) and 24 months (28.3±3.5% for synaptophysin and 48.5±5.1% for SNAP-25, n=6). When the densities of two cytoplasmatic proteins (α-tubulin or GAPDH) were used as normalizing factors instead of total protein, synaptic proteins still showed a significant decrease upon aging (18–24 months). However, upon normalization with α-tubulin and GAPDH, the levels of the studied synaptic proteins were no longer significantly different from control after 6–12 months because α-tubulin and GAPDH densities were also higher at 6–12 months compared to 2 months (Fig. 8B). This increase of the density of α-tubulin and GAPDH might be rather non-specific and reflect changes in levels of proteins related with 'house-keeping' functions at 6–12 months of age. Therefore, it is concluded that there is a modification of the density of the tested proteins associated with the vesicular release apparatus which might slightly increase during adulthood and then significantly decreases upon aging. This aging-related decrease of presynaptic markers is in general agreement with the majority of studies reporting a decrease in synaptophysin mRNA and protein density and in the number of elements immuno-positive for synaptophysin with aging in the hippocampus and various cortical structures (Masliah et al., 1993; Eastwood et al., 1994; Saito et al., 1994; Chen et al., 1995; King and Arendash, 2002; Frick and Fernandez, 2003; Rutten et al., 2005), albeit some studies reported lack of modification (Calhoun et al., 1998; Nicolle et al., 1999a; Eastwood et al., 2006) and even increases (Himeda et al., 2005; Benice et al., 2006) in the density of this presynaptic marker.

It was next investigated if there was an age-related different modification of the different types of nerve terminals. This was tested by probing the density of markers of different types of

nerve terminals, namely vesicular glutamate transporters (vGLUT1 and 2), vesicular GABA transporters (vGAT) and vesicular acetylcholine transporters (vAChT). As illustrated in Figure 8C, there was an age-related continuous decrease of vGLUT1 (significant after 18 months) and of vGLUT2 (significant after 12 months). This same pattern was observed for vAChT (Fig. 8E), whose density tended to continuously decrease with age (significant after 18 months), supporting a loss of cholinergic innervation upon aging in the hippocampus (see Sarter and Bruno, 2004). In contrast, it was found that the age-related change in the density of vGAT displayed a biphasic profile, with an increase at 6 months and a decrease at 24 months (Fig. 8D). However, if vGAT density was normalized by comparison with the density of cytoplasmatic markers (α-tubulin and GAPDH), the initial increase of vGAT density at 6 months was offset (since the density of α-tubulin and GAPDH increased at 6–12 months, see Fig. 8B) but the decrease at 24 months was still significant ($P<0.05$). This constitutes the first description of age-related changes in the density of these vesicular transporters that are widely used to discriminate between different types of nerve terminals. The results obtained indicate that there is a different age-related modification of the density of these markers of different nerve terminals, suggesting that there is an initial decrease of excitatory nerve terminals (glutamatergic and cholinergic) later followed by GABAergic nerve terminals but with lower amplitude. This suggests a potential imbalance between excitation and inhibition in hippocampal circuits in favor of inhibition, which would agree with the proposed imbalance towards inhibition as a substrate of aging-associated cognitive impairment (Wong et al., 2006). However, it should be noted that electrophysiological evaluation of excitatory *versus* inhibitory inputs into cortical pyramidal neurons did not reveal an imbalance with aging (Wong et al., 2000), although previous morphological studies showed that the age-related decline of inhibitory buttons is region-selective within the hippocampus (Shi et al., 2004). In this respect, it is important to keep in mind that the present study only investigated global changes of the density of different presynaptic markers in the whole hippocampus. Since it is known that there are different age-related changes in different hippocampal circuits (Barnes, 1994), further studies should be designed to investigate if there are different age-related changes of these presynaptic markers in different hippocampal areas. Therefore, although tempting, further studies are warranted to test if an imbalance between the strength of excitatory and inhibitory innervations may underlie age-related neurophysiological changes in the different hippocampal circuits.

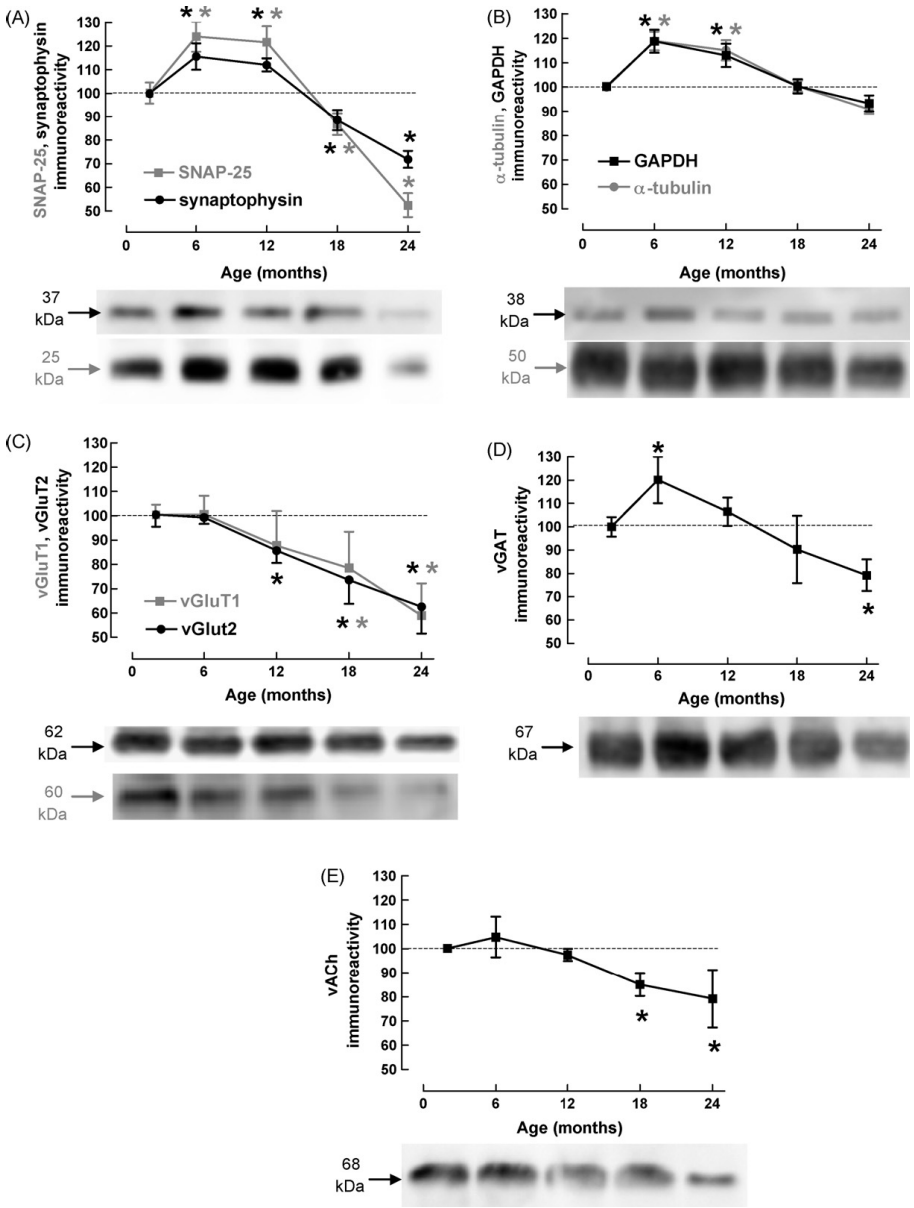


Figure 8: Age-related changes in the density of presynaptic markers (synaptophysin and SNAP-25 (A)), general cytoplasmatic markers (α-tubulin and glyceraldehyde-3-phosphate dehydrogenase, GAPDH (B)), markers of glutamatergic terminals (vesicular glutamate transporters types 1 and 2, vGLUT1 and 2 (C)), markers of GABAergic terminals (vesicular GABA transporter, vGAT (D)) and markers of cholinergic terminals (vesicular acetylcholine transporter, vAChT (E)) in nerve terminals purified from the hippocampus of rats with different ages (2, 6, 12, 18 and 24 months). Each panel displays the percentage density of each protein compared to its density at 2 months and the data was derived from Western blot analysis similar to that indicated below each graph where all age groups were simultaneously evaluated. The results are mean±S.E.M. of 4–8 animals in each age group. * $P<0.05$ compared to 2 months.

The known increased excitability of principal excitatory neurons, which is one of the hallmarks of aging in the hippocampus, might also result from the different intrinsic efficiency of presynaptic modulation systems. It was now confirmed that there was an age-related reduction in the density of inhibitory A_1R (Fig. 9A), in agreement with previous observations (Pagonopoulou

and Angelatou, 1992; Cunha et al., 1995a; Cheng et al., 2000). This powerful A_1R-mediated presynaptic inhibitory system mostly affects excitatory rather than inhibitory transmission in cortical circuits (reviewed in Fredholm et al., 2005b) and it was previously shown that the A_1R-mediated inhibition of excitatory transmission in the hippocampus is decreased in aged rats (Sebastião et al., 2000). Thus, the reduced density of A_1R in aged rats may contribute for the increased excitability of principal neurons upon aging (Barnes, 1994). On the other hand, it also suggests that strategies targeting A_1R to manage neurodegenerative diseases (reviewed in Fredholm et al., 2005b) may be less efficient in aged animals.

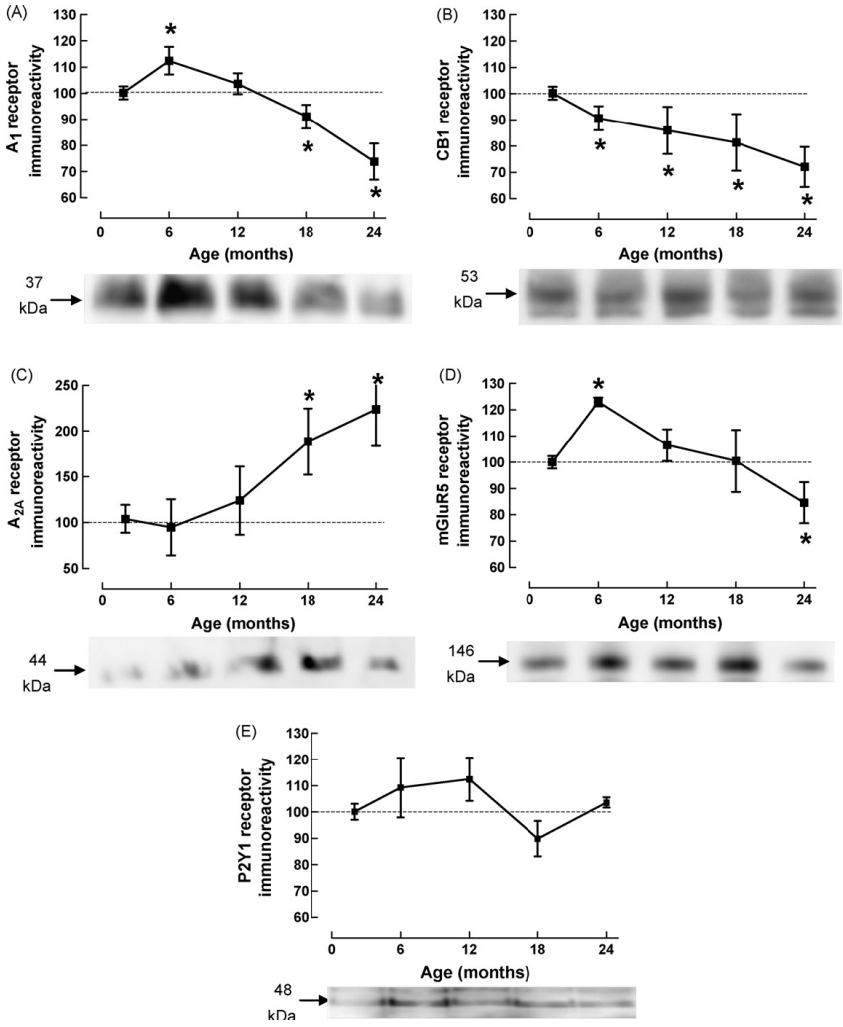


Figure 9: Age-related changes in the density of receptors operating presynaptic modulation systems that have been targeted as neuroprotective strategies, namely adenosine A_1R (A), cannabinoid CB1 receptors (B), adenosine A_{2A}R (C), glutamate metabotropic group 5 receptors (mGluR5 (D)) and purinergic P_2Y_1 receptors (E) in nerve terminals purified from the hippocampus of rats with different ages (2, 6, 12, 18 and 24 months). Each panel displays the percentage density of each receptor compared to its density at 2 months and the data was derived fromWestern blot analysis similar to that indicated below each graph where all age groups were simultaneously evaluated. The results are mean±S.E.M. of 4-8 animals in each age group. * P<0.05 compared to 2 months.

The density of cannabinoid CB_1R, which operates another presynaptic inhibitory modulation system (van der Stelt and Di Marzo, 2005) was also found to be decreased with aging (Fig. 9B). This is in agreement with the age-related decreased expression of CB_1R receptors in the hippocampus (Berrendero et al., 1998), although different age-related changes in CB_1R protein density were reported in different cortical regions (Berrendero et al., 1998; Liu et al., 2003; Mato and Pazos, 2004). In contrast to A_1R, CB_1R inhibit both excitatory and inhibitory transmission in the hippocampus (van der Stelt and Di Marzo, 2005), although the neuroprotective effects associated with CB_1R were ascribed to glutamatergic rather than GABAergic effects (Monory et al., 2006). However, this decrease in the density of CB_1R with aging suggests that the efficacy of targeting CB_1R to manage neuronal dysfunction in the elderly might be lower than that anticipated based on the studies carried out in young adults. In contrast to the age-related change in the density of the receptors operating these two main presynaptic inhibitory systems, the density of the adenosine $A_{2A}R$ was found to be significantly increased (Fig. 9C). $A_{2A}R$ trigger a presynaptic facilitation system which increases glutamatergic transmission and plasticity (reviewed in Fredholm et al., 2005b) and the present results confirm previous observations that the increased density of hippocampal $A_{2A}R$ mainly occurs from middle-aged to aged animals (Rebola et al., 2003b). This age-related increase of the density of $A_{2A}R$ is particularly interesting in view of the ability of $A_{2A}R$ antagonists to restore memory dysfunction associated with aging (Prediger et al., 2005) and neurodegenerative diseases (Dall'Igna et al., 2007). Another facilitation system in which antagonism affords neuroprotection is operated by metabotropic glutamate receptor 5 (mGluR5), although their mechanism of action is still unclear due to their pre-, post- and non-neuronal localization (reviewed in Flor et al., 2002). It has previously been reported that the global ability of mGluR5 to recruit phosphoinositide turnover is blunted (Nicolle et al., 1999b), whereas their post-synaptic effects on membrane properties are largely preserved in aged rats (Jouvenceau et al., 1997) in agreement with the preservation of mGluR5 density in the aged hippocampus (Jouvenceau et al., 1997; Nicolle et al., 1999b; Simonyi et al., 2005). It was now observed that the density of mGluR5 increased at 6 months and was maintained throughout aging, with only a marginal reduction observed at 24 months (Fig. 9D). In fact, the normalization of mGluR5 density using cytoplasmatic markers (α-tubulin and GAPDH) offsets any significant (P>0.05) modifications of the density of mGluR5. This suggests that the synaptoprotective effects associated with the control of mGluR5 action might essentially be preserved with aging. Finally, the last presynaptic

modulation system investigated in this study was the P_2Y_1 receptor. The group "Purines at CNC" recently found that the blockade of this receptor affords a robust neuroprotection against damage of hippocampal neurons in different models of excitotoxicity (unpublished results) in agreement with the neuroprotection afforded by P_2Y_1 receptor antagonists in *in vivo* models of ischemia (Franke et al., 2006). It was now found that the density of presynaptic hippocampal P_2Y_1 receptors is similar (P>0.05) in all age groups tested (Fig. 9E). This indicates that this presynaptic modulation is preserved upon aging, suggesting that it may be an interesting target to control age-related neurodegenerative processes. When evaluated globally, the results obtained prompt a trend in terms of age-related changes in the density of presynaptic modulation systems. In fact, there seems to be a clear reduction in the density of receptors operating inhibitory systems (A_1R and CB_1R) paralleled by a trend towards the preservation (mGluR5) or clear increase of presynaptic facilitation systems ($A_{2A}R$). This is a remarkable agreement with the hallmark of increased excitability that characterizes principal neurons in hippocampal circuits of aged rats. However, the age-related changes described in this study are total changes in the density of receptors and transporters, which do not discriminate between intracellular and membrane bound proteins. Hence, the present findings do not provide information about eventual changes in receptor reserve. Therefore, this tentative relation based on the age related changes in the density of protein receptors still needs to be explored at the functional level. Another interesting inference derived from the data presented is that the neuroprotective strategies targeting presynaptic modulation systems that seem best fitted to be transposed to the management of neurodegenerative diseases in the elderly are these based on the use of antagonists. In fact, antagonists of mGluR5, $A_{2A}R$ and P_2Y_1R are proposed as candidate neuroprotective strategies and the density of these presynaptic receptors is either preserved or increased on aging. In contrast, agonists of A_1R and CB_1R are proposed as candidate neuroprotective strategies and the density of these presynaptic receptors is decreased. This prompts the question of whether the age-related decrease of these inhibitory receptor systems is due to their intense recruitment to counteract age-related deleterious changes in hippocampal circuits. Conversely, the participation of each of the facilitation receptors in the age-related changes of neuronal circuits is still an open question.

4.2. BLOCKADE OF ADENOSINE A_{2A} RECEPTOR PREVENTS SYNAPTOTOXICITY AND MEMORY DYSFUNCTION CAUSED BY β-AMYLOID PEPTIDES VIA P38 MAPK KINASE PATHWAY

4.2.1. INTRODUCTION

Alzheimer's disease (AD) is the most common chronic neurodegenerative disease and is clinically characterized by a progressive impairment of cognitive functions such as learning and memory. Although the traditional neuropathologic hallmarks of AD are the presence of neurofibrillary tangles and the accumulation of the senile plaques resulting from β-amyloid peptide (Aβ) aggregation, the neurochemical parameter that correlates better with memory dysfunction in AD are the levels of soluble Aβ, mainly $A\beta_{1-42}$ (Selkoe, 2001). Also, the morphological trait that is most precociously observed and correlates better with early memory impairment in AD is the dysfunction and loss of synapses in the limbic cortex, namely in the hippocampus (Coleman et al., 2004). In fact, synapses seem to be the primordial target of toxic Aβ peptides oligomers (rather than fibrils), leading to a synaptic failure that underlies the memory impairment (Hardy and Selkoe, 2002; Selkoe, 2002; Klein et al., 2004). Thus, the early $A\beta_{1-42}$ synaptotoxicity and associated mechanisms constitute major targets in the development of novel therapeutic strategies for AD.

Adenosine is a neuromodulator that can either inhibit or facilitate synaptic transmission through inhibitory A_1 or facilitatory A_{2A} receptors ($A_{2A}Rs$), respectively, both of which are predominantly located in synapses in the limbic and neocortex (Fredholm et al., 2005b). Given the ability of A_1Rs to inhibit calcium entry into neurons, glutamate release and NMDA receptor activation, A_1Rs have been considered promising candidate targets to prevent neuronal damage; however, their rapid down-regulation and functional desensitization upon insults indicates that A_1Rs mostly act as hurls for initiation of neurodegeneration (de Mendonça et al., 2000). More recently, major interest has been devoted to $A_{2A}Rs$ since their blockade affords neuroprotection against chronic insults in the adult brain (Cunha, 2005; Chen et al., 2007), which also trigger major increases in the extracellular levels of adenosine (de Mendonça et al., 2000). This is in notable agreement with the ability of caffeine (a non-selective adenosine receptor antagonist) to afford protection against cognitive impairment in different animal models, an effect that mainly

seems to involve $A_{2A}Rs$ (reviewed in Cunha, 2008a; Takahashi et al., 2008). Likewise, caffeine consumption also inversely correlates with the incidence of AD (Maia and de Mendonça, 2002) and prevents memory impairment in animal model of AD (Arendash et al., 2006; Dall'Igna et al., 2007), an effect mimicked by selective antagonists of $A_{2A}Rs$ (Dall'Igna et al., 2007). Interestingly, $A_{2A}R$ in the limbic cortex are mostly located in synapses (Rebola et al., 2005a) and can control synaptic damage (Silva et al., 2007). This suggests that $A_{2A}Rs$ may selectively control synaptotoxicity associated with memory impairment, which would provide a molecular basis to support a neuroprotective action of $A_{2A}Rs$.

The present study tested the ability of $A_{2A}Rs$ to prevent $A\beta_{1-42}$-induced synaptotoxicity and memory impairment and investigated the underlying mechanisms.

4.2.2. PHARMACOLOGICAL BLOCKADE OF ADENOSINE A_{2A} RECEPTORS PROTECTS FROM $A\beta_{1-42}$-INDUCED SYNAPTOTOXICITY AND MEMORY IMPAIRMENT

As previously reported (Dall'Igna et al., 2007), the icv administration of β-amyloid peptides, in our experiments $A\beta_{1-42}$ (2 nmol), caused a time-delayed (within 2 weeks) memory impairment. Thus, $A\beta_{1-42}$-treated rats displayed a decreased spontaneous alternation (-27.1±1.7%. n=9, $P<0.001$ compared to water-injected controls) in the Y maze test (Fig. 10B). As an additional control, the non-amyloidogenic reverse peptide $A\beta_{42-1}$ (2 nmol) was used, which did not cause memory impairment (n=4, data not shown). Furthermore, either $A\beta_{1-42}$ or $A\beta_{42-1}$ failed to modify the locomotor activity tested in the open field (Fig. 10A).

The histological analysis of hippocampal sections of rats two weeks after the injection of $A\beta_{1-42}$ revealed a preservation of cresyl violet staining of Nissl bodies and absence of neuronal loss evaluated by Fluoro-jade C (Fig. 10C), microgliosis evaluated by CD11b immunoreactivity and astrogliosis evaluated by GFAP immunoreactivity (Fig. 10D, showing CA3, which is identical to CA1), which is indistinguishable to that found in hippocampal sections from control rats. However, it was observed that the injection of the peptide decreased the density of a synaptic marker, synaptophysin. Immunohistochemical analysis revealed a decrease of synaptophysin immunoreactivity in hippocampal sections from rats compared to controls (Fig. 10E). This decrease of synaptophysin immunoreactivity was confirmed by quantitative Western blot analysis. As illustrated in Figure 10F, the synaptophysin immunoreactivity was lower

(-25.7±4.3%, n=7, $P<0.001$) in hippocampal membranes from rats treated with $A\beta_{1-42}$ when compared to controls.

It was then tested if the blockade of adenosine A_{2A} receptors ($A_{2A}Rs$) prevents the synaptotoxicity and memory dysfunction observed two weeks after the icv administration of $A\beta_{1-42}$. This was carried out using a selective $A_{2A}R$ antagonist (SCH58261) in a dose (0.05 mg/kg, ip) that has previously been shown to afford brain neuroprotection without peripheral or locomotor effects (see Cunha et al., 2006; Dall'Igna et al., 2007). As illustrated in Figure 10C, SCH58261 (0.05 mg/kg) completely prevented the decrease of synaptophysin immunoreactivity caused by $A\beta_{1-42}$. In fact, synaptophysin immunoreactivity in hippocampal sections was indistinguishable in control conditions and in $A\beta_{1-42}$-injected rats that were treated daily with SCH58261 (Fig. 10E). Accordingly, the Western blot analysis confirmed that the decrease of the density of the synaptophysin upon $A\beta_{1-42}$-injection was prevented by SCH58261 (Fig. 10F). In parallel, SCH58261 (0.05 mg/kg) was also able to significantly ($P<0.001$) prevent the decrease of spontaneous alternation upon $A\beta_{1-42}$-injection. In contrast, SCH58261 did not modify synaptophysin immunoreactivity (Fig. 10E) or spontaneous alternation in control rats (Fig. 10B) nor did it affect locomotion in control or $A\beta_{1-42}$-treated rats (Fig. 10A).

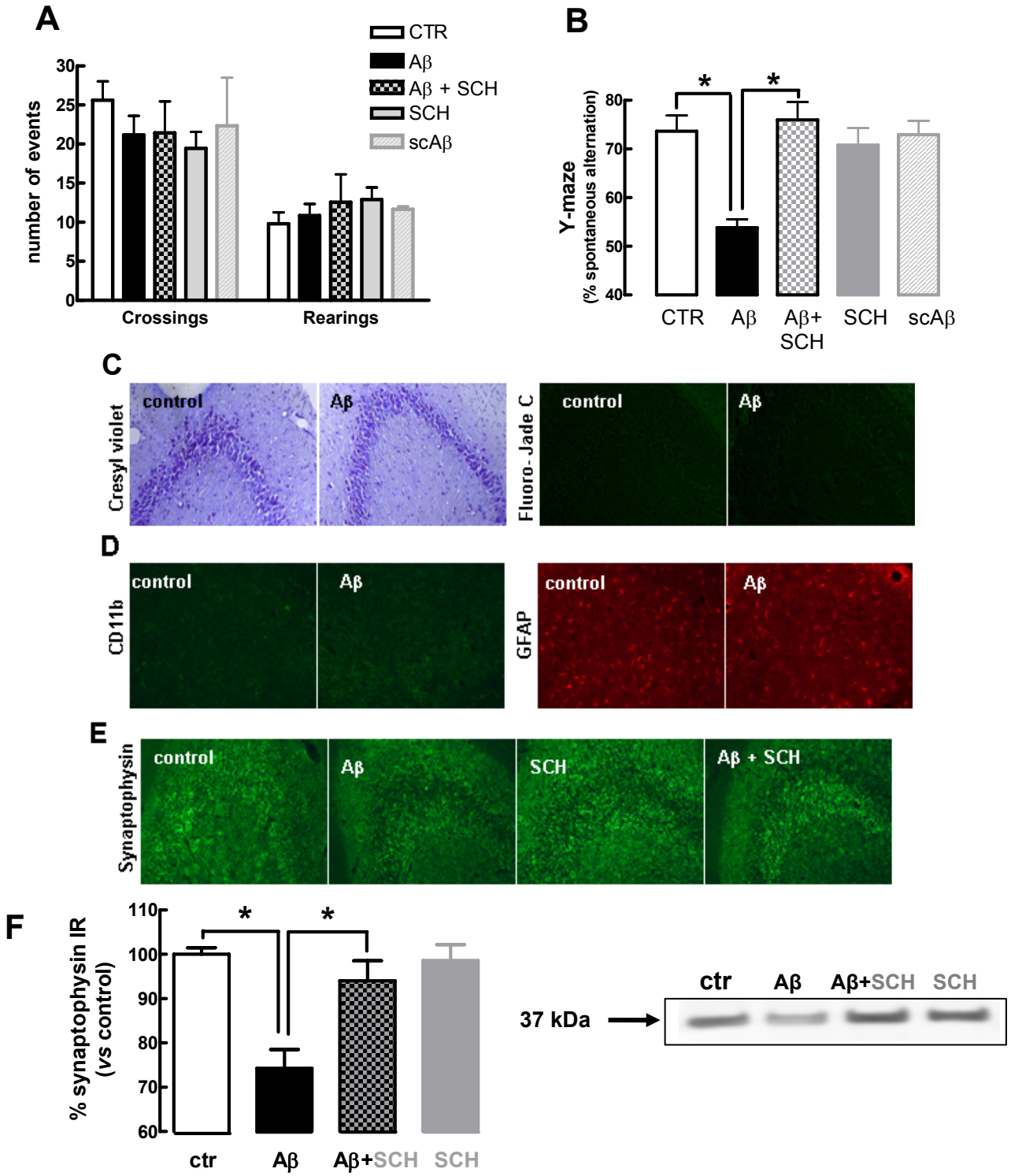


Figure 10: Blockade of adenosine A_{2A} receptors prevents β-amyloid-induced decrease of spontaneous alternation and synaptotoxicity. Rats were treated (2 nmol, icv) with β-amyloid peptide 1-42 fragment ($A\beta_{1-42}$) or distilled water. The A_{2A} receptor antagonist SCH58261 (0.05 mg/kg, ip) was administered daily starting 30 minutes before Aβ (or water) and rats were behaviorally analyzed after 15 days. (A) Spontaneous locomotion evaluated in an open field arena. B) Spontaneous alternation in the Y-maze test. Behavioral tests are expressed as mean± SEM from 9 rats in each group, * $P<0.001$ (C) Cresyl violet staining of Nissl bodies in hippocampal sections from control (water-injected) rats and $A\beta_{1-42}$-injected rats and Fluoro-jade C staining of neuronal death in hippocampal sections from control (water-injected) rats and $A\beta_{1-42}$-injected rats. (D) CD11-b marker of microgliosis and GFAP marker of astrogliosis in hippocampal sections from water-injected and Aβ-injected rats (E) Immunohistochemical labeling with anti-synaptophysin in hippocampal sections from rats injected with water (control), $A\beta_{1-42}$ (Aβ), SCH58261 (SCH) and Aβ + SCH. Images are representative of 5 experiments. (F) Western blot comparing synaptophysin immunoreactivity in hippocampal membranes from rats with the indicated treatment. Data are mean±SEM from 7 experiments; * $P<0.001$.

4.2.3. GENETIC INACTIVATION OF A_{2A} ADENOSINE RECEPTORS ABOLISHES AB_{1-42} -INDUCED SYNAPTOTOXICITY AND MEMORY DEFICITS

The memory impairment and synaptotoxicity observed in rats could also be reproduced upon $A\beta_{1-42}$ administration in C57BL6 mice, the genetic background of $A_{2A}R$ knockout (KO) mice. Thus, two weeks after the icv administration of $A\beta_{1-42}$ (2 nmol), wild type (WT) mice displayed a decreased memory performance, measured as a decreased (-23.0±1.7%, n=7, $P<0.001$) spontaneous alternation measured in the Y maze (Fig. 11B), without modification of locomotor activity (Fig. 11A), and a decreased density of two synaptic markers, synaptophysin (-26.7±3.7%, n=4, $P<0.001$) and SNAP-25 (-25.8±2.3%, n=4, $P<0.001$) (Fig. 11C), when compared to water-injected (i.e. control) mice. Furthermore, the histological analysis of hippocampal sections of $A\beta_{1-42}$-treated WT mice showed the absence of neuronal loss evaluated by Fluoro-jade C, microgliosis evaluated by CD11b immunoreactivity, and astrogliosis evaluated by GFAP immunoreactivity (Fig. 11D, showing CA3 area).

The key role of $A_{2A}Rs$ in controlling $A\beta_{1-42}$-induced synaptotoxicity and memory impairment, was confirmed by testing the effects of $A\beta_{1-42}$ in $A_{2A}R$ KO mice with the same genetic background (littermates of the above tested wild type mice). The genetic inactivation leads to a decrease of number of crossings (30±7, n=14, $P<0.05$) and rearings (7±2, n=14, $P<0.05$) when compared to the WT mice (Fig. 11A). However this decrease in the number of crossings and rearings does not affect the Y-maze alternation, upon comparison of saline-injected WT and KO mice (Fig. 11B). As shown in Figure 11, both the $A\beta_{1-42}$-induced synaptotoxicity and accompanying memory impairment were not present in $A_{2A}R$ KO mice. Thus, $A\beta_{1-42}$-injected $A_{2A}R$ KO mice did not display a decrease of spontaneous alteration in the Y maze (Fig. 11B) or a decrease in the density of the synaptic markers, synaptophysin or SNAP-25 (Fig. 11C). Furthermore, water- or $A\beta_{1-42}$-injected $A_{2A}R$ KO mice displayed neither cell death, nor microgliosis nor astrogliosis (Fig. 11D).

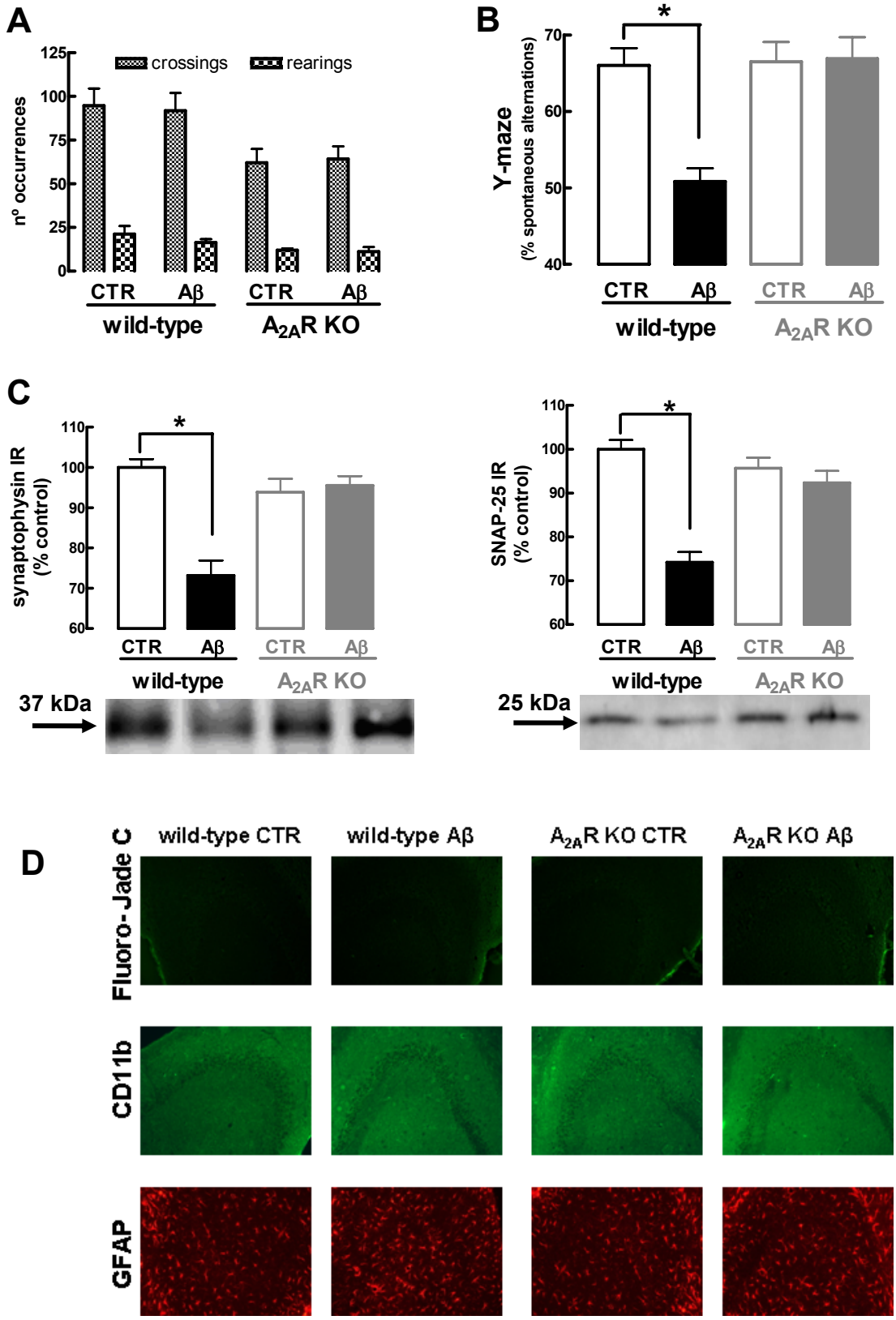


Figure 11: Genetic inactivation of adenosine A_{2A} receptors prevents β-amyloid-induced decrease of spontaneous alternation and synaptotoxicity. C57BL6 and $A_{2A}R$ KO were treated with $A\beta_{1-42}$ (2 nmol, icv) or distilled water. Mice were behaviorally analyzed after 15 days. (A) Spontaneous locomotion evaluated in an open field arena and (B) Spontaneous alternation in the Y-maze test. Data are from 7 mice in each experimental group. (C) Western blot comparing synaptophysin and SNAP-25 immunoreactivity in hippocampal membranes obtained from water injected wild-type (WT) mice, Aβ-injected WT, water injected adenosine $A_{2A}R$ knockout (KO) mice, $A\beta_{1-42}$-injected $A_{2A}R$ KO. Results are mean±SEM of 4 experiments. (D) Fluoro-jade C staining of neuronal death, CD11-b marker of microgliosis and GFAP marker of astrogliosis in hippocampal sections from saline-injected and Aβ-injected mice WT and $A_{2A}R$ KO. * P<0.001.

4.2.4. BLOCKADE OF A_{2A} RECEPTORS PREVENTS AB_{1-42}-INDUCED DYSFUNCTION OF PURIFIED NERVE TERMINALS

The observations that $A\beta_{1-42}$ triggered an A_{2A}R-sensitive synaptotoxicity prompted the hypothesis that this A_{2A}R-sensitive $A\beta_{1-42}$-induced toxicity could be replicated in enriched nerve terminals (synaptosomes). Previous studies have already reported that the exposure of synaptosomes to β-amyloid peptides triggers mitochondrial dysfunction (Mattson et al., 1998), which has been argued to be a key feature of Alzheimer's disease (Moreira et al., 2006). Accordingly, the synaptosomes exposed for 2 hours to 500 nM $A\beta_{1-42}$ displayed a decrease (-8.3±3.6 % compared to control, n=4, $P<0.001$) in MTT reduction (Fig. 12A), which measures the redox status of synaptosomes, indicative of synaptosomal viability (Mattson et al., 1998; Silva et al., 2007). Furthermore, a decrease of the membrane mitochondrial potential (-11.5±2.5 %, n=8, $P<0.05$) in $A\beta_{1-42}$-treated synaptosomes was also observed (Fig. 12B).

Upon blockade of A_{2A}Rs with SCH58261 (50 nM), there was a prevention of the $A\beta_{1-42}$-induced disruption of the functionality (Fig. 12A) and mitochondrial membrane potential of synaptosomes (Fig. 12B), whereas SCH58261 was devoid of effects in control synaptosomes.

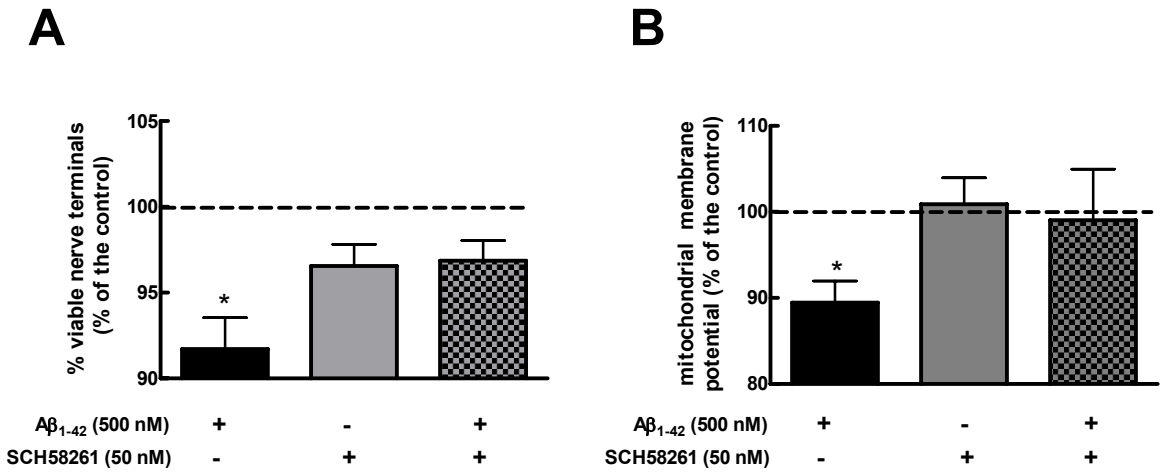


Figure 12: The decrease of synaptosomal viability and mitochondrial potential induced by $A\beta_{1-42}$ is prevented by A_{2A} receptor blockade with SCH58261. (A) Synaptosomal viability was measured through a MTT assay. The results are expressed as the mean±SEM from 4 different experiments. (B) Measurement of mitochondrial potential in synaptosomes from a functional experiment after adding FCCP and oligomycin (see methods in sub-chapter 3.15). The results are expressed as the mean±SEM from 8 different experiments. * $P<0.05$.

4.2.5. BLOCKADE OF A_{2A} RECEPTORS PROTECTS HIPPOCAMPAL NEURONS FROM AB_{1-42}-INDUCED TOXICITY

To investigate the mechanism involved in the $A_{2A}R$-mediated control of $A\beta_{1-42}$-induced neurotoxicity, a cell culture model was used, namely primary cultures of hippocampal neurons. Cultured hippocampal neurons were exposed for 12, 24 and 48 h to 500 nM $A\beta_{1-42}$ and neuronal death was analyzed by double labeling with Syto-13 and PI (Fig. 13A, B). After 12 h of exposure to $A\beta_{1-42}$ hippocampal neurons did not present any significant decrease (-1.0±1.0 %, n=5, $P>0.05$) of either cell viability (Fig. 13A) or number of apoptotic-like neurons (Fig. 13B) when compared to control neurons (either not exposed to $A\beta_{1-42}$ or exposed to the non-amyloidogenic $A\beta_{42-1}$ peptide). In fact, a decrease of cell viability (-9.0±2.0 %, n=5, $P<0.001$) was only observed 24 h after $A\beta_{1-42}$ exposure (Fig. 13A), which was accompanied by an increased number of apoptotic-like neurons (5.0±1.0 %, n=5, $P<0.001$) (Fig. 13B). This $A\beta_{1-42}$-induced neuronal death was larger after 48 h of exposure to $A\beta_{1-42}$, as evaluated by the decreased number of viable neurons (-12.3±3.7 %, n=5, $P<0.001$) (Fig. 13A) and the increased number of apoptotic-like neurons (9.0±2.0 %, n=5, $P<0.001$) (Fig. 13B), indicating a time-dependent evolving profile of $A\beta_{1-42}$-induced neurodegeneration. As occurred *in vivo* and in native brain preparations, this $A\beta_{1-42}$-induced neurotoxicity was prevented by the $A_{2A}R$ antagonist, SCH58261 (50 nM), which did not affect neuronal viability in control neurons (Fig. 13C, D).

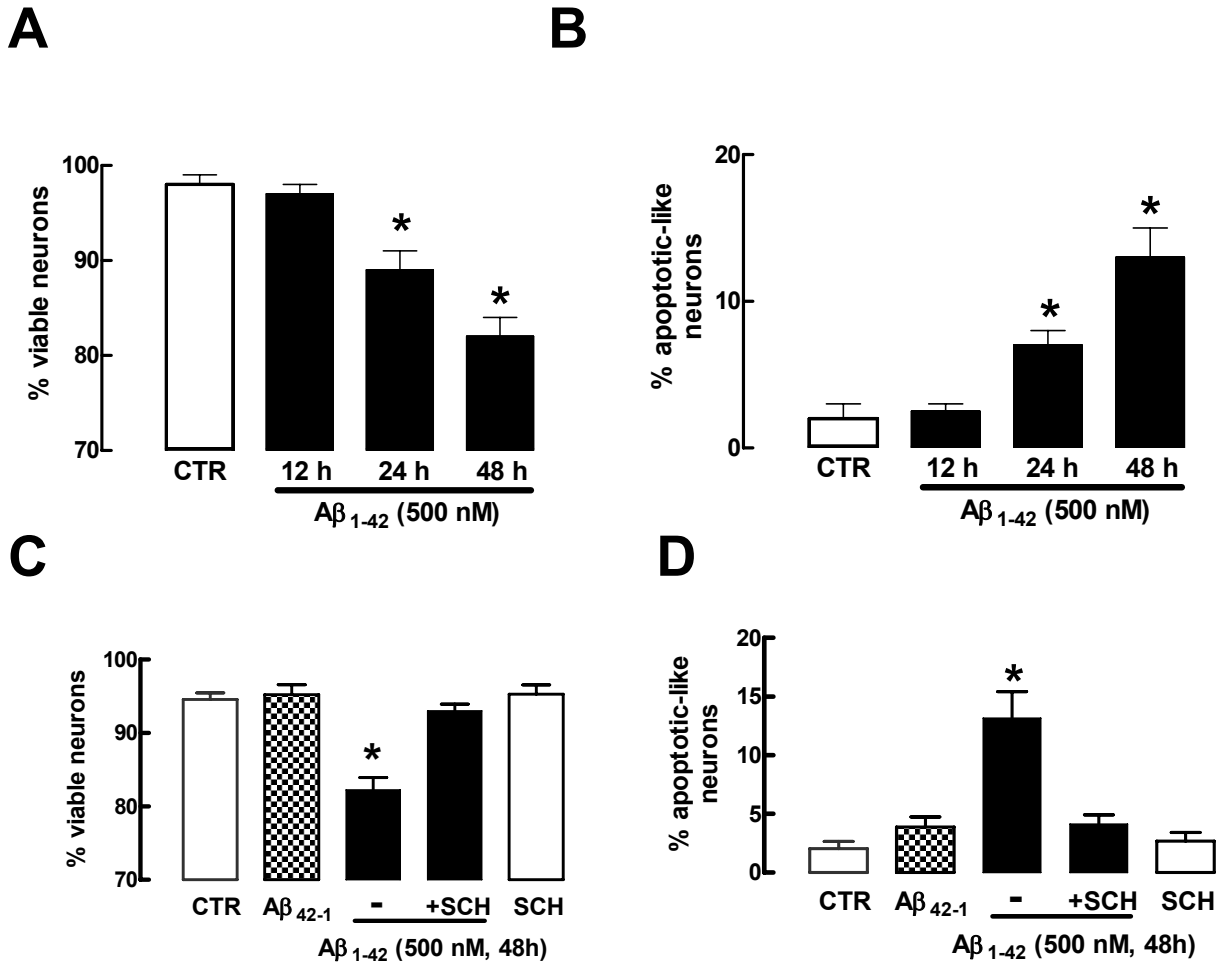


Figure 13: Temporal analysis of neuronal death by $A\beta_{1-42}$ and neuroprotective effect of SCH58261. Hippocampal neurons were pre-incubated with 50 nM of SCH58261 for 15 minutes before the addition of 500 nM $A\beta_{1-42}$. (A, B) Aβ-induced neuronal death is time-dependent. (C, D) Blockade of $A_{2A}R$ with SCH58261 prevents neuronal death upon 48 h of incubation with Aβ. Neurons were double-labeled with Syto-13 and PI fluorescent probes. A total of about 300 cells per coverslip were counted. Results are means±SEM duplicate coverslips from 5 independent hippocampal cultures. * $P<0.05$.

Thus, it was next investigated if the exposure of cultured neurons to $A\beta_{1-42}$ caused an initial synaptotoxicity preceding neuronal death. Since neurons incubated for 12 h with $A\beta_{1-42}$ did not display loss of viability or damage, it was tested whether $A\beta_{1-42}$-induced synaptotoxicity would be present after 12 h of exposure to $A\beta_{1-42}$, by evaluating the double staining of MAP-2 and synaptophysin or SNAP-25. As shown in Figures 14 and 15, there was a retraction of MAP-2-labeled segments and a decrease in the number of synaptophysin-immunoreactive spots after 12 h of exposure to $A\beta_{1-42}$, *i.e.* at the time when neuronal damage is not yet present (see Fig. 13). To quantify this $A\beta_{1-42}$-induced synaptotoxicity, Western blotting analysis was used, which showed a decrease in the density of synaptophysin (-30.3±7.5%, n=6, $P<0.05$) and SNAP-25 (-37.0±6.6%, n=6, $P<0.05$) upon exposure to $A\beta_{1-42}$. As occurred *in vivo*, this initial and evolving $A\beta_{1-42}$-induced synaptotoxicity in neuronal cultures was also prevented by $A_{2A}R$ blockade with the selective $A_{2A}R$ antagonist, SCH58261 (50 nM) (Fig. 14D).

This observation that SCH58261 prevents $A\beta_{1-42}$-neurotoxicity but is devoid of effects in controls suggests that the levels of extracellular adenosine might be increased upon exposure to $A\beta_{1-42}$, which is in accordance with the general concept that noxious stimuli are expected to increase the extracellular levels of adenosine (Fredholm et al., 2005b). Thus, it was tested if the exposure of cultured neurons to $A\beta_{1-42}$ leads to an increase in the extracellular levels of adenosine. As shown in Figure 14E, the incubation of hippocampal neurons with $A\beta_{1-42}$ (500 nM) caused an over 100% increase of the extracellular concentration of adenosine (104.7±38.8 nM, n=5, $P<0.05$) after 3 h that is persistent until 48 h of incubation.

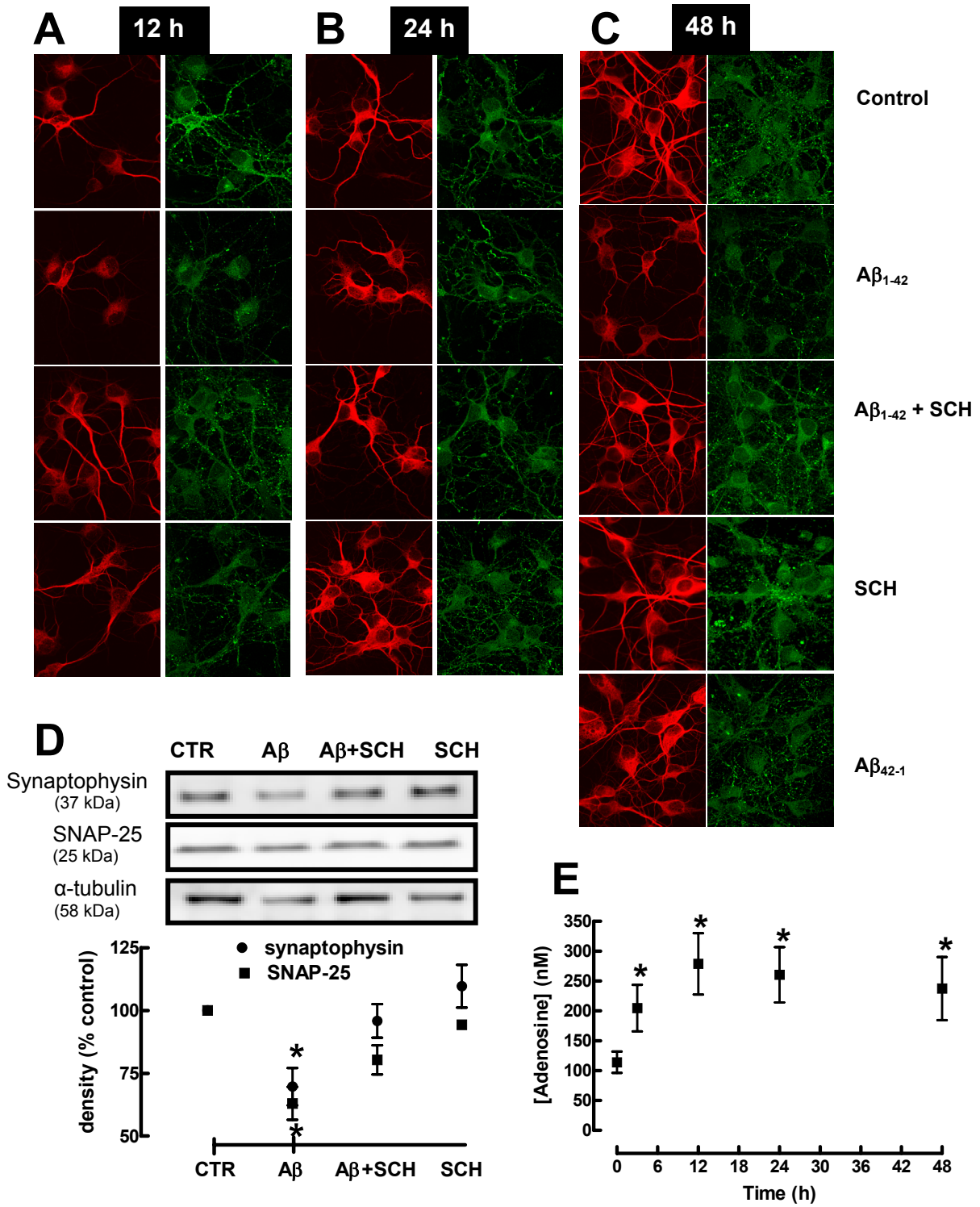


Figure 14: Temporal analysis of the effects of SCH58261 (SCH) on the decrease of the immunoreactivity for synaptophysin triggered by $A\beta_{1-42}$ (Aβ). Confocal analysis of hippocampal neurons double-labeled for **MAP-2 (red)** and **synaptophysin (green)**. Hippocampal neurons were pre-incubated for 15 minutes with 50 nM of SCH58261 before 500 nM of $A\beta_{1-42}$. (A) – 12 hours; (B) – 24 hours; and (C) – 48 hours of incubation. Pre-incubation of hippocampal neurons with SCH58261 prevented the decrease of the immunoreactivity for both proteins caused by $A\beta_{1-42}$ at all time points of incubation. Magnification 400×. (D) Displays a Western blot (15 µg protein loaded in each lane) comparing the density of synaptophysin and SNAP-25 immunoreactivity in membranes of hippocampal cultured neurons of the different groups incubated with saline, Aβ, SCH and SCH plus Aβ. Results are expressed as mean±SEM of synaptophysin or SNAP-25 immunoreactivity in 6 independent cultures. (E) HPLC quantification of the extracellular adenosine levels in the incubation medium of hippocampal neurons incubated with Aβ throughout time. The results are expressed as mean±SEM from 5 independent cultures. * $P<0.05$.

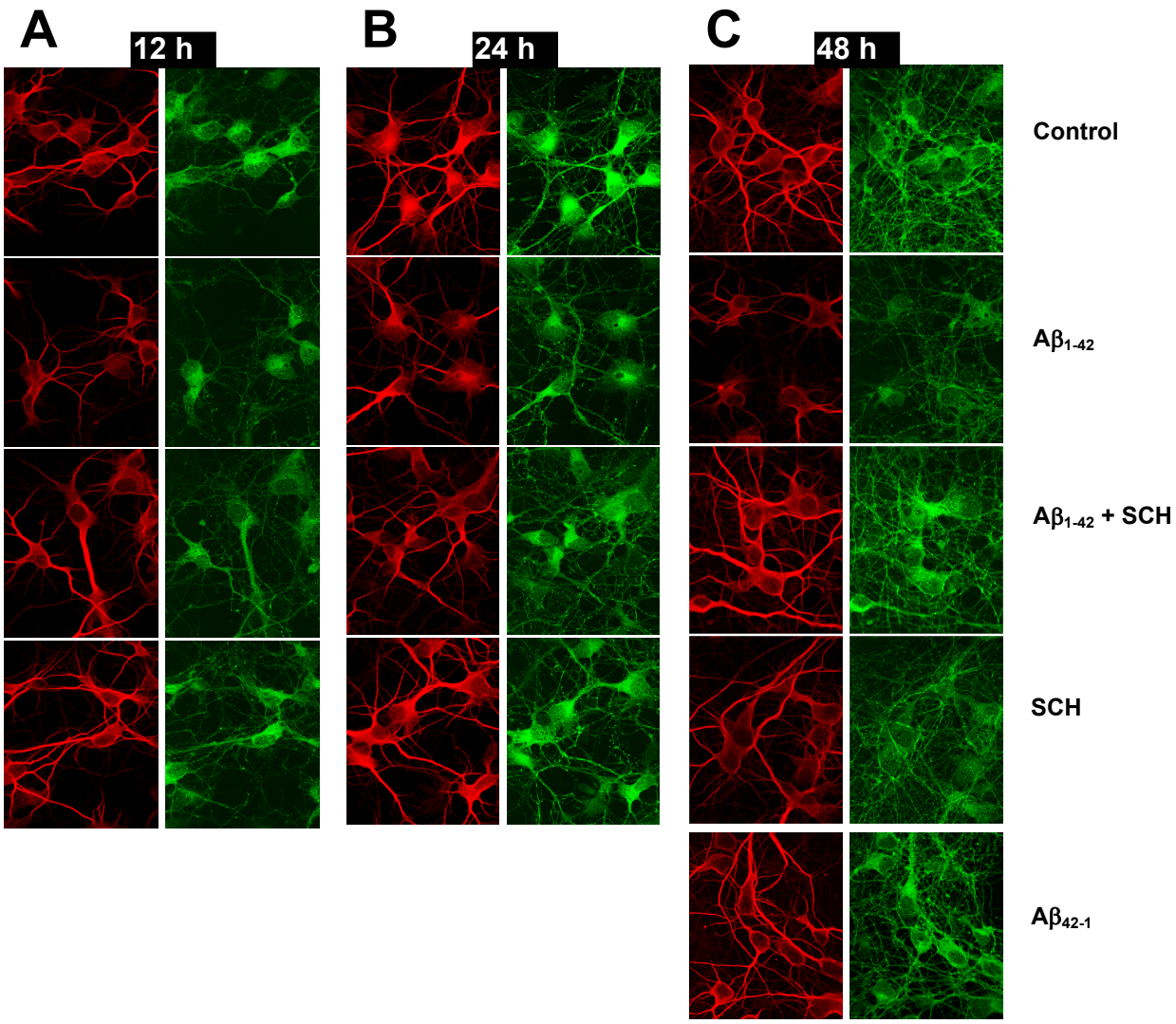
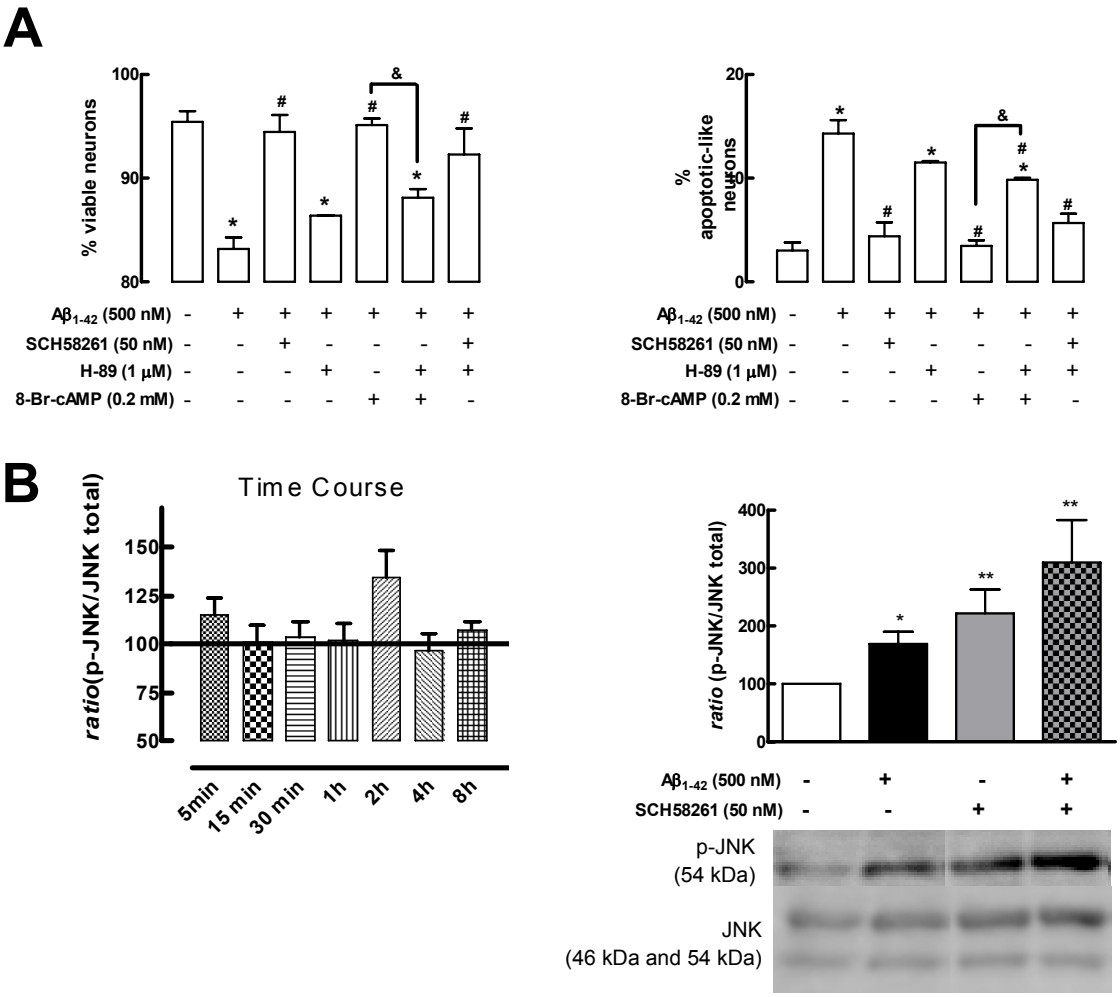


Figure 15: Temporal analysis of the effects of SCH58261 on the decrease of the immunoreactivity for SNAP-25 triggered by $A\beta_{1-42}$. Confocal analysis of hippocampal neurons double-labeled for MAP-2 (red) and SNAP-25 (green). Hippocampal neurons were pre-incubated for 15 minutes with 50 nM of SCH58261 before 500 nM of $A\beta_{1-42}$. (A) – 12 hours; (B) – 24 hours; and (C) – 48 hours of incubation. Pre-incubation of hippocampal neurons with SCH58261 prevented the decrease of the immunoreactivity for both proteins caused by $A\beta_{1-42}$ at all time points of incubation. Magnification 400×.

4.2.6. SIGNALING PATHWAYS INVOLVED IN THE NEUROPROTECTION AFFORDED BY A_{2A} RECEPTOR BLOCKADE AGAINST AB_{1-42}-INDUCED NEUROTOXICITY

Since one of the main transducing systems operated by A_{2A}Rs involves cAMP/protein kinase A (PKA) pathway (Fredholm et al., 2005b), it was investigated whether the neuroprotective effects afforded by SCH58261 involved this pathway. As observed in Figure 16A, the manipulation of the cAMP/PKA pathway influences $A\beta_{1-42}$-induced neurotoxicity, as described by others (Parvathenani et al., 2000; Gong et al., 2004; Shrestha et al., 2006). In fact, the activation of PKA with the cell-permeable cAMP analogue 8-Br-cAMP (200 μM) attenuated $A\beta_{1-42}$-induced neurotoxicity, an effect prevented by the PKA inhibitor H-89 (1 μM) (Fig. 16A). However, the neuroprotection by SCH58261 persisted even in the presence of H-89, ruling out the participation of cAMP/PKA pathway on the effects resulting from blockade of A_{2A}Rs (Fig. 16A).



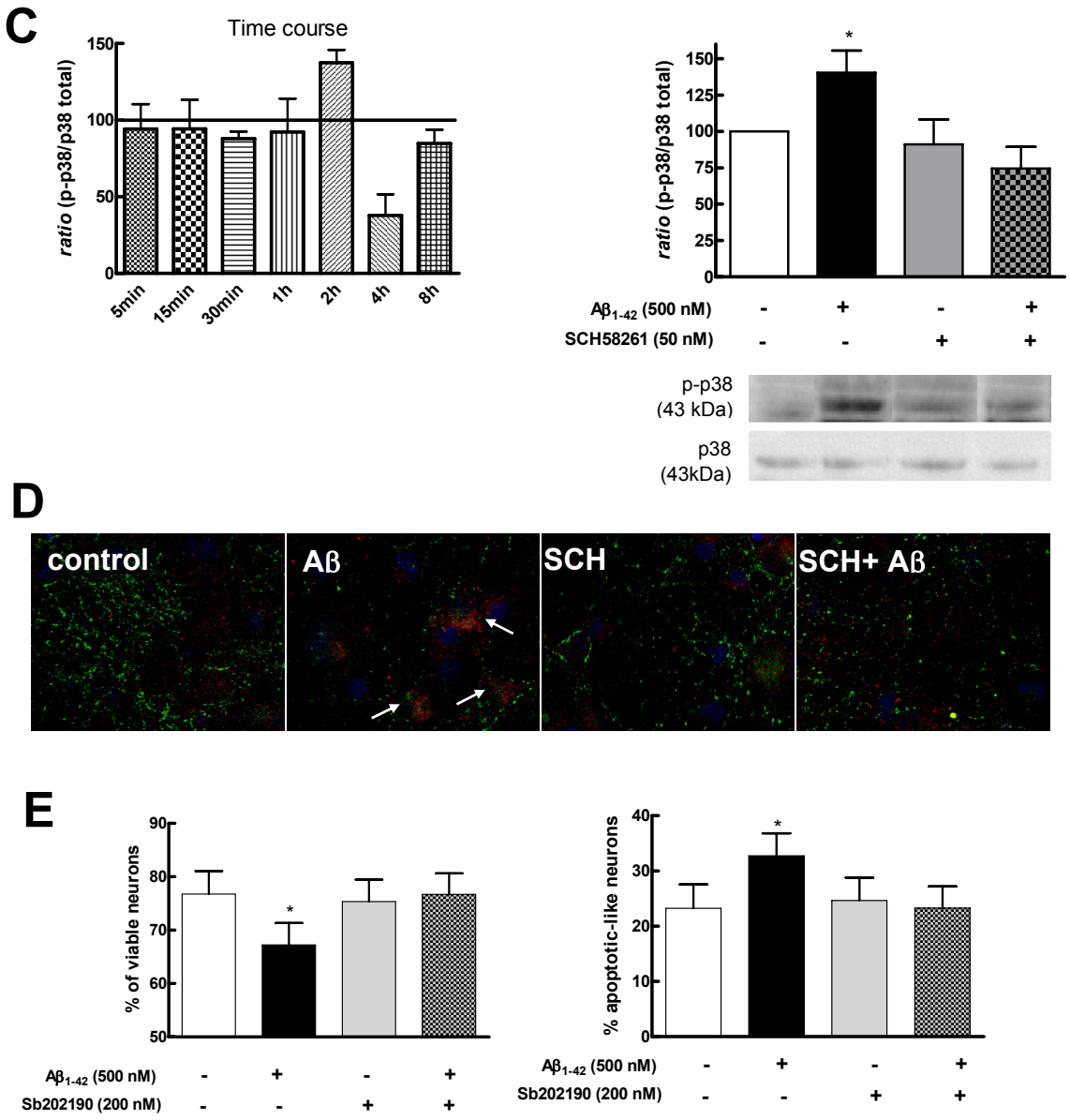


Figure 16: Signaling pathways involved in $A\beta_{1-42}$-induced toxicity and P38-MAPK dependent SCH58261 protection (A) Neuroprotection by SCH58261 did not involve cAMP-PKA pathway. Culture hippocampal neurons were incubated with 1 µM of H-89 15 minutes before addition of 200 µM of 8-Br-cAMP and/or 50 nM SCH58261. After 15 minutes 500 nM of $A\beta_{1-42}$ were added and incubated for 24 hours. (B) Increase in JNK phosphorylation in hippocampal neurons after $A\beta_{1-42}$ incubation and blockade of $A_{2A}R$. Hippocampal neurons were incubated with $A\beta_{1-42}$ for 2 hours and *ratio* p-JNK/JNK total was determined through a Western blot analysis (15 µg protein loaded in each lane), the results are presented expressed as the average results (mean±SEM) from 6 independent cultures. (C) Increase in p38 MAPK phosphorylation in hippocampal neurons after $A\beta_{1-42}$ incubation and prevention upon blockade of $A_{2A}R$. Hippocampal neurons were incubated with $A\beta_{1-42}$ for 2 h and the *ratio* p-p38/p38 MAPK total was determined through Western blot analysis (80 µg protein/lane). Data are mean±SEM from 7 independent cultures. (D) Increase in p38 MAPK phosphorylation in hippocampal neurons after $A\beta_{1-42}$ incubation and prevention upon blockade of $A_{2A}R$. Confocal analysis of hippocampal neurons triple-labeled for p-p38 (red), synaptophysin (green) and DAPI (blue). (E) P38 MAPK inhibitor SB202190 prevents neuronal death induced by $A\beta_{1-42}$. Hippocampal neurons were incubated with SB202190 (200 nM) 30 min after $A\beta_{1-42}$ (500 nM) incubation of 24 h. Data are mean±SEM from 5 independent cultures. Neuronal death was assessed by double-labeling with Syto-13 and PI after 24 h of incubation. Viable neurons presented intact nuclei stained with Syto-13 and apoptotic neurons presented shrunken nuclei stained simultaneously with Syto-13 and PI. * $P<0.05$ *versus* control; ** $P<0.01$ *versus* control; # $P<0.05$ *versus* $A\beta_{1-42}$; & $P<0.05$ *versus* $A\beta_{1-42}$ + 8-Br-cAMP.

It is also suggested that deregulation of the MAPK pathways, namely of JNK and p38 MAPK family of proteins, might play a role in the intracellular mechanisms of neurodegeneration, in particular in $A\beta_{1-42}$-induced neurotoxicity (Minogue et al., 2003; Muñoz et al., 2007; Troy et al., 2001; Wang et al., 2004; Zhu et al., 2005), and $A_{2A}Rs$ can also signal through the MAPK pathway (reviewed in Fredholm et al., 2005b). To test the involvement of JNK and p38 MAPK in the $A_{2A}R$-mediated protection against $A\beta_{1-42}$-induced neurotoxicity, it was first investigated the time course of $A\beta_{1-42}$-induced activation of p38 MAPK and JNK (evaluated as their degree of phosphorylation) to determine the time points where this process occurs (Fig. 16B and C). It was found that after 2 hours of incubation with $A\beta_{1-42}$ (500 nM), there was an increase of JNK (69±21%, n=16). At this time point, $A_{2A}R$ blockade with SCH58261 (50 nM) increased the $A\beta_{1-42}$-induced JNK phosphorylation (209.7±73.6%, n=6, $P<0.01$) whereas it abolished the $A\beta_{1-42}$-induced p38 MAPK phosphorylation (Fig. 16B, C), which was confirmed by an immunocytochemical approach (Fig. 16D). Corroborating the key role of p38 MAPK in the $A\beta_{1-42}$-induced neurotoxicity (Zhu et al., 2005; Muñoz et al., 2007; Origlia et al., 2008), it was found that the p38 MAPK inhibitor SB202190 (200 nM) prevented the $A\beta_{1-42}$-induced loss of neuronal viability and increased number of apoptotic-like neurons (Fig. 16D).

4.2.7. DISCUSSION

The present results provide the first demonstration that the blockade of a membrane receptor that is enriched in hippocampal synapses, namely $A_{2A}Rs$, can abolish the cascade of events triggered by Aβ to culminate in memory dysfunction characteristic of AD. These results are relevant for two different reasons: 1) they provide evidence that the control of a presynaptic modulation system that prevents synaptotoxicity via p38 MAPK pathway and also prevents memory dysfunction, strengthening the hypothesis that synaptic dysfunction is a precocious core modification of AD; 2) they provide further evidence that $A_{2A}Rs$, whose density is increased in AD (Albasanz et al., 2008), are a novel promising target to control AD.

The initials phases of AD are mainly characterized by the enhanced levels of soluble forms of β-amyloid peptides (Aβ) and the loss of synapses in particular cortical areas; (Hardy and Selkoe, 2002; Selkoe, 2002; Coleman et al., 2004). Thus, the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ are

elevated early in dementia and strongly correlate with cognitive decline (e.g. Lue et al., 1999; Naslund et al., 2000); also altered levels of Aβ in the cerebro-spinal fluid predict the evolution from prodromal mild cognitive impairment into AD (e.g. Hampel et al., 2004). Likewise, the loss of synapses in frontal cortical and hippocampal regions occurs early in AD (Scheff et al., 2006; Scheff et al., 2007) and seems to be the morphological parameter that correlates better with cognitive impairment (e.g. DeKosky and Scheff, 1990; Terry et al., 1991). This early synaptotoxicity is also observed in different transgenic animal models of AD (e.g. Hsia et al., 1999; Jacobsen et al., 2006; Mucke et al., 2000; Oddo et al., 2003; Wu et al., 2004), most of which are designed to enhance the production of Aβ (reviewed in Gotz and Ittner, 2008). Likewise, it was now observed that the intracerebroventricular administration of $A\beta_{1-42}$ caused a parallel loss of memory performance and a loss of synaptic markers. Notably, this $A\beta_{1-42}$-induced memory impairment appears to be selectively associated with a synaptic disruption; in fact, the only morphological change found in the hippocampus of Aβ-injected rodents displaying impaired memory performance was the loss of synaptic markers, whereas neither overt neuronal damage, nor astrogliosis nor microgliosis were observed. Accordingly, in cultured neurons (where peripheral, vascular, glial or immune influences are not present), it was also found that the exposure to Aβ caused first a synaptotoxicity (see also Roselli et al., 2005; Calabrese et al., 2007; Shankar et al., 2007), which is only later followed by an overt neuronal damage. Further strengthening that Aβ causes a direct effect on nerve terminals, it was showed that Aβ indeed directly impairs synaptosomal function, as observed by others (e.g. Mattson et al., 1998; Arias et al., 2002). Altogether these observations indicate that Aβ, which can bind to synaptic proteins (e.g. Lacor et al., 2007) and accumulates in synapses in AD patients (Takahashi et al., 2002; Gylys et al., 2004; Fein et al., 2008), causes a primordial synaptotoxicity that precedes overt neuronal damage.

This tight relation between synaptotoxicity and memory dysfunction is further strengthened by the key observation of the present study, *i.e.* that the blockade of $A_{2A}Rs$ simultaneously prevents the synaptotoxicity and the memory impairment caused by Aβ administration. It remains to be demonstrated if the early synaptotoxicity triggered by Aβ is the primary cause that leads to memory impairment. Although this ability of $A_{2A}R$ blockade to prevent both Aβ-induced synaptotoxicity as well as memory impairment indicates that manipulations that preserve synapses are sufficient to abrogate the delayed neuronal death. Thus, it was now observed that both the pharmacological blockade and the genetic inactivation of

A_{2A}Rs abrogate the memory impairment and the synaptotoxicity caused by Aβ administration. Furthermore, the initial synaptotoxicity that precedes overt neuronal damage upon exposure of cultured neurons to Aβ was also prevented by A_{2A}R blockade. Finally, the direct Aβ-induced impairment of nerve terminal function was also prevented by A_{2A}R blockade. All these observations are in agreement with the predominant localization of A_{2A}Rs in synapses in cortical regions (Rebola et al., 2005a). These synaptic A_{2A}Rs play a key role in the control of NMDA-dependent synaptic plasticity (Rebola et al., 2008), which is severely hampered early in AD (e.g. Roselli et al., 2005; Shankar et al., 2007; Venkitaramani et al., 2007). Thus, synaptic A_{2A}Rs normalize the function of these glutamatergic synapses (reviewed in Cunha, 2008b), which are dysfunctional in AD (see Bell et al., 2007) and their blockade prevents synaptotoxicity caused by different stimuli (Cunha et al., 2006; Silva et al., 2007) that leads to subsequent overt neurodegeneration upon stressful conditions (Silva et al., 2007). This implies that the ability of A_{2A}Rs to control memory impairment should be particularly evident when synaptotoxicity is the cause of memory impairment. Overall, this supports the notion that the prevention of synaptic impairment upon A_{2A}R blockade may underlie the ability of A_{2A}R antagonists to prevent Aβ-induced memory impairment; this illustrates that the control of synaptic dysfunction may be an overlooked strategy to alleviate memory dysfunction associated with neurodegenerative conditions (see Coleman et al., 2004; Wishart et al., 2006).

This putative relevance of targeting A_{2A}Rs to control memory impairment associated with neurodegenerative conditions is strongly supported by the ability of caffeine to counteract the development of neurodegenerative conditions and in particular the development of cognitive deficits (reviewed in Cunha, 2008a; Takahashi et al., 2008). In fact, although it is doubtful that caffeine is a cognitive enhancer, its long term consumption effectively prevents memory impairment caused by different perturbing conditions (see Cunha, 2008a; Takahashi et al., 2008) such as upon aging (e.g. Ritchie et al., 2007; Costa et al., 2008a) or upon Alzheimer's disease (Maia and de Mendonça, 2002). The known mechanisms of action of non-toxic doses of caffeine are the antagonism of adenosine receptors (low concentrations) and inhibition of phosphodiesterases (higher concentrations) (Fredholm et al., 1999). Animal studies indicate that the impact of chronic caffeine consumption to prevent memory deterioration caused by different insults is mimicked by antagonists of A_{2A}Rs rather than A_1Rs (reviewed in Cunha, 2008a; Takahashi et al., 2008). Accordingly, it has been previously shown that the beneficial effects of caffeine on Aβ-induced neurotoxicity and memory impairment are mimicked by antagonists of

A_{2A}Rs but not of A_1Rs (Dall'Igna et al., 2003; Dall'Igna et al., 2007). Thus, it is tempting to propose that the promising beneficial effects of caffeine consumption as a strategy to prevent the burden of AD might be related to the synapto-protective effect afforded by A_{2A}R blockade. It should be pointed out that this proposal does not exclude other possible concurring mechanisms by which caffeine may afford protection in AD, such as the control of Aβ production (Arendash et al., 2006), the control of the disruption of the blood-brain barrier (Chen et al., 2008) or the control of neuroinflammation (Angulo et al., 2003). Thus, although the present data combining the use of fractionated nerve terminals, cultured neurons and *in vivo* models strongly argues for the predominant importance of synaptic A_{2A}Rs in the control of Aβ-induced neurotoxicity, it does not exclude the possibility that other mechanism may also contribute for the neuroprotection against Aβ-induced neurotoxicity and memory impairment.

Finally, this study also determined the transducing system operated by A_{2A}Rs to control Aβ-induced neurotoxicity. In fact, whereas the ability of A_{2A}R blockade to afford neuroprotection against different chronic noxious stimuli is well established, the mechanisms by which A_{2A}Rs contribute for neurodegeneration are still unresolved (discussed in Cunha, 2005; Chen et al., 2007). For historical reasons there is a general consensus that A_{2A}Rs signal through activation of the adenylate cyclase/cAMP/protein kinase A pathway (Fredholm et al., 2005b). However, this is unlikely to be the relevant transducing system related to the control by A_{2A}Rs of neurodegeneration since enhanced cAMP levels afford neuroprotection against Aβ-induced neurotoxicity (see also Parvathenani et al., 2000; Gong et al., 2004; Shrestha et al., 2006), whereas it is the blockade of A_{2A}Rs (expected to <u>decrease</u> cAMP levels) that affords neuroprotection. Accordingly, the neuroprotection afforded by A_{2A}R blockade against Aβ-induced neurotoxicity was insensitive to the protein kinase A inhibitor H-89, which prevented the neuroprotection afforded by enhanced cAMP levels. Several other transducing pathways have been documented to allow protection in AD models, amongst which stem the MAPK pathways (e.g. Muñoz et al., 2007; Origlia et al., 2008; Zhu et al., 2005) and, accordingly, it was confirmed that Aβ triggered the activation of both JNK and p38 MAPKs. Interestingly, it was observed that A_{2A}R blockade prevented the Aβ-induced activation of p38, whereas it actually enhanced the phosphorylation of JNK. JNK can have pro- or anti-apoptotic functions, depending on cell type, nature of the death stimulus, duration of its activation and the activity of other signaling pathways (Liu and Lin, 2005). Given that the inhibition of p38 activation is sufficient to prevent Aβ-induced neurotoxicity, as also observed by others (Muñoz

et al., 2007; Origlia et al., 2008; Zhu et al., 2005), this indicates that A_{2A}R signals through p38 MAPK to control neurodegeneration. Indeed, previous studies have documented the ability of A_{2A}Rs to control the MAPK pathways in a cAMP-independent manner (e.g. Schulte and Fredholm, 2003; Gsandtner et al., 2005; reviewed in Fredholm et al., 2005b) and it has previously been suggested that the control by A_{2A}Rs of the ischemia-induced brain damage was related the ability of A_{2A}R antagonists to blunt the ischemia-induced accumulation of phosphorylated forms of p38 (Melani et al., 2006). Thus, the present results indicate that A_{2A}Rs control Aβ-induced neurotoxicity through the control of p38 MAPK phosphorylation. However, this conclusion should not over-shadow the documented involvement of other transducing systems and intracellular mediators, which interact with p38 MAPK in the demise of Aβ-induced neurotoxicity still remains to be elucidated.

In summary, the blockade of A_{2A}Rs leads to memory impairment and synaptotoxicity prevention via p38 MAPK pathway, which is indirectly confirmed by the beneficial effects of caffeine and might be a straightforward candidate for clinical trials upon introduction into clinical practice of A_{2A}R antagonists as novel anti-parkinsonian drugs (Schwarzschild et al., 2006).

4.3. SELECTIVE LOSS OF CHOLINERGIC AND GLUTAMATERGIC TERMINALS IN A β-AMYLOID PEPTIDES MODEL OF ALZHEIMER'S DISEASE

4.3.1. INTRODUCTION

In an early stage of Alzheimer's disease, there is synaptic and neuronal loss that is related to accumulation of Aβ monomers and Aβ oligomers (Hardy and Selkoe, 2002; Selkoe, 2002). In particular, Aβ oligomers have been described as the earliest effectors to adversely affect synaptic structure and plasticity (Selkoe, 2001; Coleman et al., 2004). In this way, they compromise aspects of learning and memory, including long-term potentiation (Selkoe, 2002). Local inflammatory changes, neurofibrillary degeneration, and neurotransmitter deficits all contribute to the memory impairment, but available evidence suggests that these alterations develop as a consequence of early Aβ accumulation (Selkoe, 2002). As reported in the previous chapter, in an intracerebroventricular Aβ model, there is a loss of synapses. It was now investigated if there was a loss of terminals i.e. if synaptotoxicity affected some particular type of synapses. For that purpose, the different types of nerve terminals were distinguished according to their composition of vesicular transporters.

Vesicular neurotransmitter transporters (VNTs) are the final arbiters of neurotransmitter entry into the secretory vesicle, i.e. they specify the type and quantity of the vesicular content of neurotransmitters. VNTs located in different types of vesicles ultimately determine the type of neurotransmitters released in each neuron. VNTs shuttle neurotransmitters from the cytosol into synaptic vesicles (Gasnier, 2000; Chaudhry et al., 2008), influencing the dynamics of neurotransmission in neuronal network. VNTs include thoses of glutamate (vGLUT1–3) (Takamori, 2006), acetylcholine (vAChT) (Erickson et al., 1996b), monoamines (vMATs) (Erickson et al., 1996a; Liu and Edwards, 1997) and glycine/GABA (vGAT/vIAAT) (Chaudhry et al., 1998).

VGLUT are specific markers for neurons that use glutamate as a neurotransmitter (e.g. Gabellec et al., 2007). VGLUTs are regulated developmentally and determine functionally distinct populations of glutamatergic neurons (Liguz-Lecznar and Skangiel-Kramska, 2007). VGLUT1 and vGLUT2 are expressed in the CNS in a largely complementary manner with only

a limited overlap (Fremeau et al., 2001; Fujiyama, 2001; Herzog, 2001; Kaneko and Fujiyama, 2002; Varoqui et al., 2002). VGLUT1 is mainly present in the cortex, hippocampus, amygdala and subiculum (Fremeau et al., 2001; Hisano et al., 2002; Liguz-Lecznar and Skangiel-Kramska, 2007). VGLUT2 is present in olfactory bulb, cortex, dentate gyrus of hippocampus, thalamus, hypothalamus and brain stem (Fremeau et al., 2001; Fujiyama et al., 2001; Herzog et al., 2001; Kaneko and Fujiyama, 2002). VGLUT3 is localized in a limited number of glutamatergic neurons in several regions, such as the neocortex, hippocampus, olfactory bulb, hypothalamus, substantia nigra, raphe nuclei (Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002; Herzog et al., 2004). VGLUT3 is also present in a population of symmetrical synapses, such as in hippocampal and cortical GABAergic neurons (Herzog et al., 2004), cholinergic neurons in the striatum and serotonergic neurons in the raphe nuclei (Gras et al., 2002; Schafer et al., 2002). Thus, the identification of glutamatergic terminals has mainly relied on the use of vGLUT1 and 2 as markers, since vGLUT3 can also be localized in non-glutamatergic neurons, namely in some GABAergic and cholinergic neurons.

Glycine/GABA transporters (vGAT/vIAAT) (Chaudhry et al., 1998) exhibit characteristic substrate specificities and are often used as phenotypic markers of GABAergic neurons (Takamori et al., 2000). VGAT is responsible for the uptake of GABA into synaptic vesicles (McIntire et al., 1997; Takamori et al., 2000) and is present in nerve endings of inhibitory neurons containing GABA, but also in glycinergic neurons in the brain (e.g. hippocampus and striatum) and retina (McIntire et al., 1997; Chaudhry et al., 1998; Jellali et al., 2002).

VAChT provides a specific marker for cholinergic neurons, which enables the study of cholinergic transmission in experimental models (Butcher et al., 1992; Bejanin et al., 1994; Erickson et al., 1994; Roghani et al., 1994; Woolf et al., 2001). VAChT is responsible for transport of acetylcholine into synaptic vesicles for regulated exocytotic release (Parsons et al., 1993) and its pattern of expression, vAChT mRNA, is consistent with anatomical, pharmacological, and histochemical information on the distribution of functional cholinergic neurons in the brain and peripheral tissues of the rat (Schafer et al., 1994).

Thus, probing the modification of the density and distribution of each of these VNTs by immunocytochemistry and Western blot in synaptosomes prepared from injected rats with water or $A\beta_{1-42}$ after 15 days is expected to allow identifying if any particular type of nerve terminal is more susceptible to exposure to $A\beta_{1-42}$.

4.3.2. BEHAVIORAL ANALYSIS

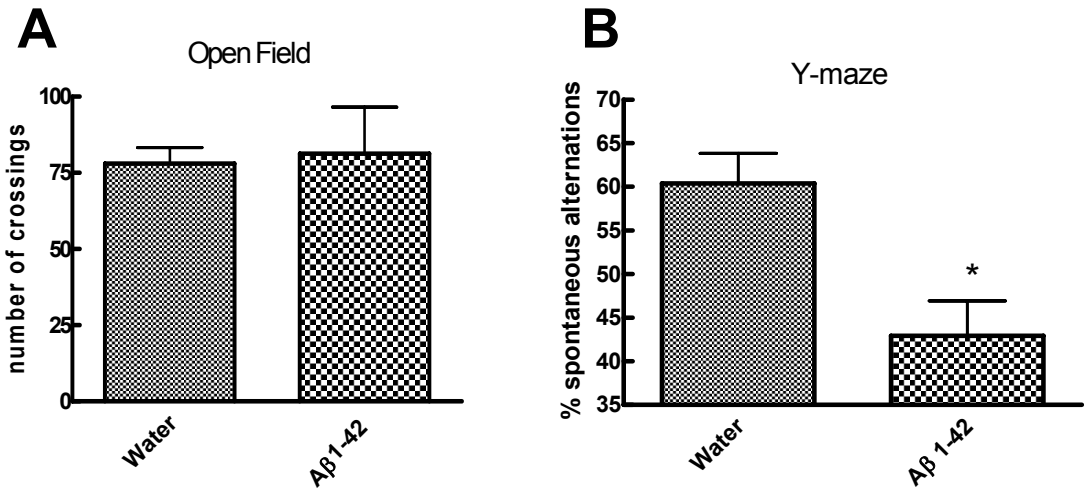


Figure 17: Behavioral analysis after fifteen days of administration of 2 nmol $A\beta_{1-42}$ into the brain ventricles of mice. Panel (A) shows the number of crossings and rearings of water- and Aβ-infused mice exposed for 5 minutes to an open field arena. Panel (B) shows a decrease on the number of alternations in the Y-maze test of mice infused with 2 nmol of $A\beta_{1-42}$ when compared to the vehicle. The data are mean±SEM of 8-10 animals per experimental group, * $P<0.05$.

As illustrated in Figure 17, no differences were observed in the open field test between the animals of the different groups, neither in terms of horizontal nor vertical explorations. The Y-maze test revealed a decrease of spontaneous alternation of the $A\beta_{1-42}$-injected mice, when compared to the water-injected mice (-17.5±4.0 %, n=8, $P<0.05$).

After the behavioral analysis, the mice were sacrificed either for a Western blot or immunocytochemmistry evaluation.

4.3.3. WESTERN BLOT ANALYSIS OF VESICULAR TRANSPORTERS

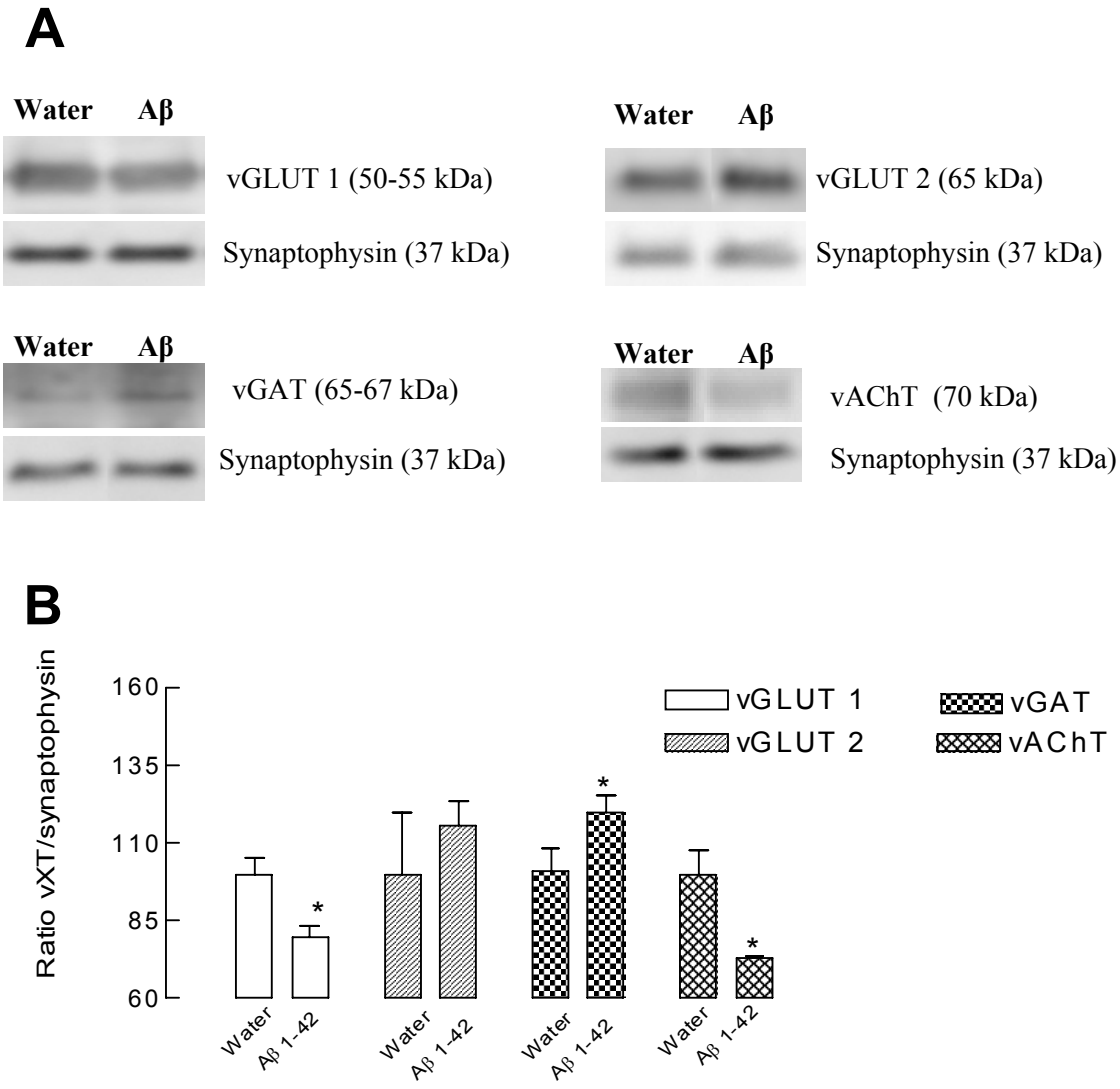


Figure 18: Western blot quantification of different vesicular transporters in synaptosomes from control (water-injected) and $A\beta_{1-42}$-injected mice. Panel (A) – Representative images of Western blot analysis for the different vesicular transporters tested, namely glutamate transporter 1 (vGLUT1), glutamate transporter 2 (vGLUT2); GABA transporter (vGAT) and cholinergic transporter (vAChT). Panel (B) – Average modification of the density of the different vesicular transporters, expressed as the *ratio* between the density of different vesicular transporters and synaptophysin that stains the synaptosomal population. There is a decrease of vGLUT1 and vAChT in $A\beta_{1-42}$-injected animals. The data are mean±SEM of n=4-5, * $P<0.05$.

Western blot analysis showed a decrease of vGLUT1 (20.1±3.6%, n=5, $P<0.05$) and vAChT (30.9±3.7%, n=4, $P<0.05$), an increase of vGAT (20.7±5.8%, n=5-7, $P<0.05$) and no difference in vGLUT2 between the Aβ-injected and control mice (Fig. 18).

4.3.4. IMMUNOCYTOCHEMICAL ANALYSIS OF VESICULAR TRANSPORTERS

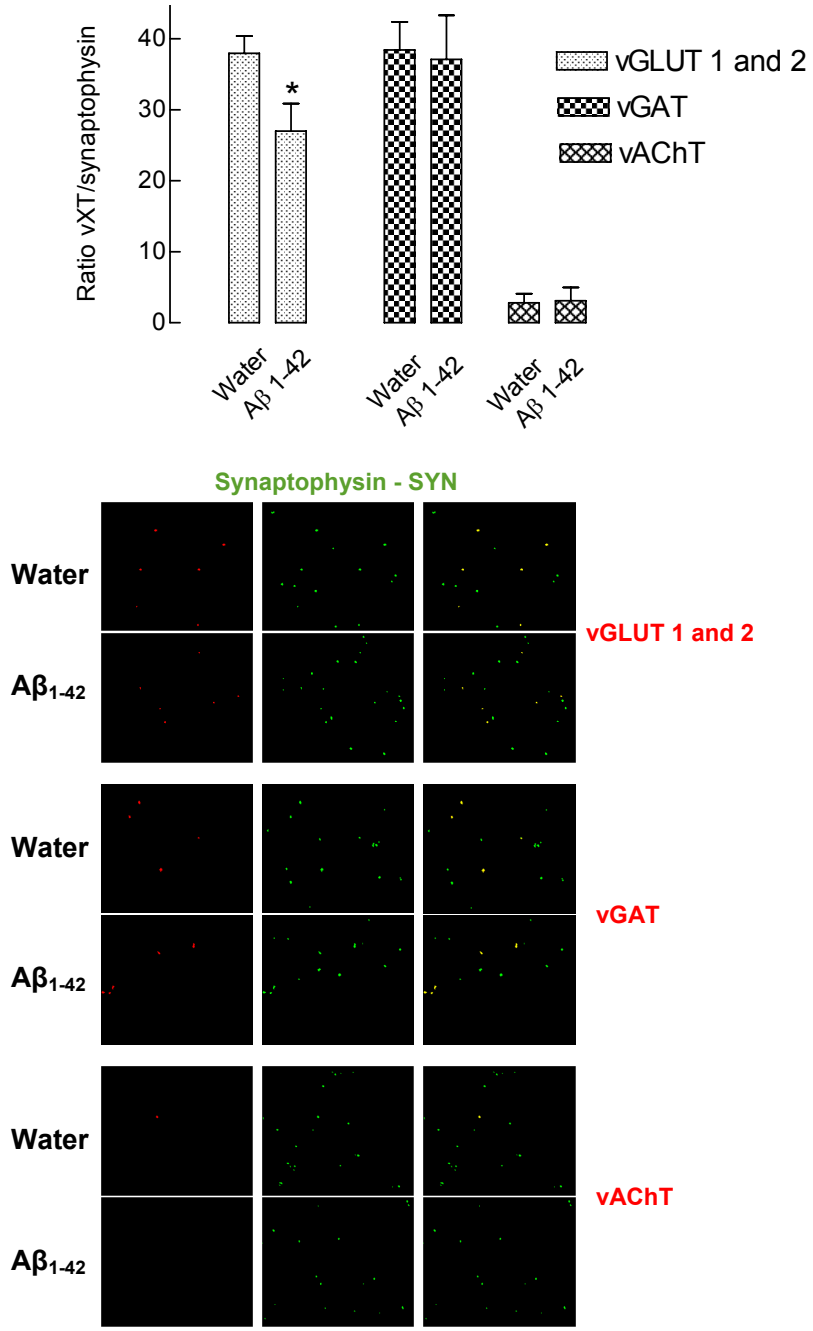


Figure 19: Immunocytochemical quantification of different vesicular transporters in nerve terminals from control (water-injected) and $A\beta_{1-42}$-injected mice. Panel (A) – Representation of *ratio* between the different vesicular transporters tested [namely glutamate transporter 1 (vGLUT 1), glutamate transporter 2 (vGLUT2); GABA transporter (vGAT) and cholinergic transporter (vAChT)] and synaptophysin, that stains the synaptosomal population. Panel (B) – Representative images of immunocytochemistry for the different vesicular transporters, in which vesicular transporters are in red and synaptophysin is in green. There is a decrease of vGLUT1 and 2 in $A\beta_{1-42}$-injected animals. The data are mean±SEM of n=5, * $P<0.05$.

An immunocytochemistry approach was used to confirm the Western blot results, showing a decrease of glutamatergic nerve terminals (-10.9±3.9%, n=5, $P<0.05$) and no differences of the relative numbers of GABAergic and cholinergic nerve terminals between the Aβ-injected animals when compared to the control animals (Fig. 19).

4.3.5. DISCUSSION

Studies in transgenic models of early-staged amyloid pathology have suggested that the amyloid pathology progresses in a neurotransmitter-specific manner where cholinergic terminals appear most vulnerable, followed by glutamatergic terminals and finally by somewhat more resistant GABAergic terminals (Bell and Cuello, 2006). It is now reported that Aβ-injected mice display an asymmetric loss of nerve terminals similar to that occurring in transgenic models of Alzheimer's disease. Thus in this Aβ-model, it was now observed that there is a loss of glutamatergic synapses, observed both by Western blot and immunocytochesmistry analysis, a loss of cholinergic synapses and an increase of GABAergic synapses, which was only observed by Western blot. The differences between the results can be explained by the different sensitivity of the techniques. Because of the low percentage of the cholinergic terminals in the hippocampus (2.8±1.3%, n=6, $P<0.05$), it is difficult to perceive differences between the groups of animals, even when the total of nerve terminals counted is approximately 900 (in 10 different fields).

Possible explanations for the asymmetric loss of nerve terminals in AD may be due to different metabolic demands or to alterations of the levels of trophic factors. The metabolic demands of each type or nerve terminals can be different, and GABAergic nerve terminals have the lowest metabolic demand (Wurtman, 1992; Mark et al., 1997; Casu et al., 2002). Alteration of trophic support and function has been proposed to explain the specific vulnerability of the cholinergic neurons (Woolf et al., 2001; Counts and Mufson, 2005). This observed vulnerability of the cholinergic terminals is in accordance with the cholinergic hypothesis associated to AD pathology, which proposes that AD is caused by a reduced synthesis of acetylcholine (reviewed in Bartus, 2000). The cholinergic hypothesis has not maintained widespread support, largely because medications intended to restore acetylcholine deficiency have not been very effective (Wenk, 2003). A cholinergic vulnerability occurs in other neurodegenerative diseases such as Huntington's disease (HD); thus vAChT mRNA and protein levels are decreased in the striatum

and cortex of R6/1 mice (animal model of HD) and HD patient samples (Smith et al., 2006). VAChT is a protein often described as essential for mnemonic processes (Gutierrez et al., 1997; Woolf et al., 2001). Accordingly, vAChT mutant mice (mice with a targeted mutation in the vAChT gene that reduces transporter expression) display a deficit in memory encoding necessary for the temporal order version of the object recognition memory (Prado et al., 2006; de Castro et al., 2009). Also, in a model of experimental sepsis and in a model of cerebral trauma there is a decreased cholinergic innervation that occurs in parallel with memory deficits (Dixon et al., 1999; Semmler et al., 2007).

A decrease of glutamatergic nerve terminals was also observed in the present model of AD which is in accordance with the modification of vGLUTs in AD (Masliah et al., 2000). A recent post-mortem study in AD patients also found a reduction of the proteins levels of vGLUT1 in both the parietal and occipital cortices (Kirvell et al., 2006). In transgenic mice expressing human mutated amyloid precursor protein and presenilin-1, there was an age-dependent decline in vGLUT1 levels (Minkeviciene et al., 2008). As in AD, it has been observed a decrease in vGLUT1 in brains of schizophrenic and Parkinson's disease patients (Eastwood and Harrison, 2005; Kashani et al., 2007). VGLUT1 expression could contribute to synaptic efficacy by influencing not only the amount but also the rate of filling (Sulzer and Pothos, 2000). There has been a wide interest in the dysfunction of glutamatergic synapses and glutamate excitotoxicity in AD (Greenamyre and Young, 1989). In support of this notion, there are reports of reduced levels of excitatory amino acid transporter protein 1 (EAAT-1) in the platelets (Zoia et al., 2004) and EAAT-2 in the frontal cortex (Li et al., 1997), as well as reduced glutamate uptake activity in the frontal and temporal cortices of AD brains (Procter et al., 1988), which may lead to elevated extracellular glutamate concentration favoring excitotoxicity. Aβ accumulation leads to increased sensitivity to glutamate-induced excitotoxicity mediated by NMDA receptors have been specifically linked to Aβ-induced degeneration (Mattson et al., 1992; Mattson, 2004). Furthermore, long-term alterations in efficacy of glutamate transmission contribute to memory mechanisms (Bliss and Collingridge, 1993; Malenka and Bear, 2004).

In contrast, GABAergic terminals seem to be resilient to degeneration which is in agreement with human studies performed post-mortem in AD patients (Bell et al., 2006). Similarly to AD, there is an increase of vGAT in a genetic model for Down Syndrome (Belichenko et al., 2009) and an increase of vGAT mRNA levels in preproenkephalin-negative neurons in an unilateral injection of 6-hydroxydopamine (a model of PD) (Wang et al., 2007a).

An increased inhibition in the hippocampus was shown to be responsible for the failure to induce long-term potentiation (LTP) (Kleschevnikov et al., 2004). GABAergic (inhibitory) synaptic transmission will have important consequences for glutamatergic (excitatory) synaptic plasticity (Chevaleyre and Castillo, 2003). An imbalance, whether of excitatory or inhibitory in neurotransmission will influence the normal function of neuronal circuits and consequently memory processes (Hensch and Fagiolini, 2005; Trevelyan and Watkinson, 2005; Akerman and Cline, 2007).

There is an asymmetric loss of cholinergic and glutamatergic synapses in an $A\beta_{1-42}$-induced AD model.

4.4. BLOCKADE OF A_{2A} RECEPTORS DOES NOT PREVENT β-AMYLOID PEPTIDES-INDUCED LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL AND CALCIUM DEREGULATION IN HIPPOCAMPAL NEURONS

4.4.1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is characterized by a progressive cognitive decline resulting from selective neuronal dysfunction, synaptic loss and neuronal cell death (McGeer and McGeer, 1995; Sisodia and Price, 1995; Selkoe, 2002). Aβ peptide is known to play a major role in the pathogenesis of the disorder of AD (Selkoe, 2002). Aβ has been shown, in general, to cause synaptic dysfunction and can render neurons vulnerable to excitotoxicity and apoptosis (Mattson and Chan, 2003). In fact, synaptic mitochondria may initiate neuronal death in response to insults such as Aβ that elevates synaptic levels of intracellular Ca^{2+}, consistent with the early degeneration of distal axon segments in neurodegenerative disorders (Brown et al., 2006). A possible explanation for synaptic mitochondria having a role in cell death, could be their increased susceptibility to Ca^{2+} overload than nonsynaptic mitochondria (Brown et al., 2006), which in turn escort synapses to be particularly sensitive to metabolic perturbation and exacerbated Aβ-toxicity (Mattson et al., 1998; Arias et al., 2002).

$A_{2A}R$ blockade can afford neuroprotection against chronic insults in the adult brain (Cunha, 2005; Chen et al., 2007). Also, a retrospective study showed that caffeine consumption inversely correlates with the incidence of AD (Maia and de Mendonça, 2002). Caffeine and other $A_{2A}R$ antagonists prevent memory impairment in animal models of AD (Arendash et al., 2006; Dall'Igna et al., 2007). In sub-chapter 4.2, it was also observed a prevention of memory impairment and synaptotoxicity in an Aβ model of AD, either by a pharmacologic blockade or genetic inactivation of $A_{2A}R$. The pharmacological blockade of $A_{2A}R$ prevents Aβ-induced synaptotoxicity and the later on neuronal death in hippocampal cultures (sub-chapter 4.2). $A_{2A}R$ are located at hippocampal synapses (Rebola et al., 2005a), one of the main regions affected in AD (Coleman et al., 2004). This synaptic localization might indicate a neuprotection role for

$A_{2A}R$ in Aβ-induced synaptotoxicity (sub-chapter 4.2.4), like it was reported for staurosporine-induced synaptotoxicity (Silva et al., 2007).

To study the underlying mechanisms by which synaptic $A_{2A}R$ have a neuroprotective role when synaptotoxicity occurs, it was decided to use synaptosomes. Synaptosomes were exposed acutely or during 2 hours to Aβ and it was explored if the mitochondrial membrane potential and calcium levels were affected.

To further study the role of $A_{2A}R$ in neuronal function that is impaired in AD, hippocampal neurons in culture were pre-exposed to Aβ and subject to electric field stimulation to test if mitochondrial membrane potential and calcium levels were affected in intact neurons.

4.4.2. IMAGING OF SYNAPTOSOMES

Hippocampal synaptosomes were purified with a Percoll 45% followed by incubation of 2 hours with $A\beta_{1-42}$ (5 μM) in HEPES buffered medium (HBM) with 2 mM $CaCl_2$. Changes in intracellular calcium concentration and mitochondrial membrane potential were monitored with FURA-2 and tetramethyl rhodamine methyl ester ($TMRM^+$), respectively, as previously described (Oliveira et al., 2006). Since these two parameters are related, particular attention was devoted to calcium imaging to optimize the recording conditions.

In a pilot study, synaptosomes where exposed acutely to $A\beta_{1-42}$ 5 μM, as was previously done by Dougherty and collaborators (Dougherty et al., 2003). $A\beta_{1-42}$ (5 μM) was added at 2 minutes to a synaptosomes preparation, and calcium was measured for 20 minutes.

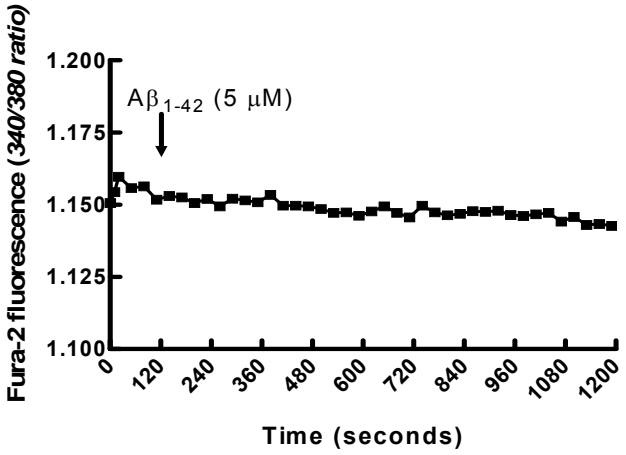


Figure 20: Calcium changes after addition of $A\beta_{1-42}$ 5 μM at 2minutes to a synaptosomal preparation. Synaptosomes were first loaded with FURA-2 (5 μM). Calcium responses represent the average of 70 synaptosomes of a single experiment. $A\beta_{1-42}$ did not cause any calcium changes throughout the experiment.

In a single experiment, it was observed that Aβ failed to modify intracellular free calcium levels in single synaptosomes (Fig.20), in contrast to what was reported by Dougherty and collaborators (Dougherty et al., 2003). So, it was decided to change the experimental protocol from an acute stimulation with $A\beta_{1-42}$ (5 μM) to a prolonged exposure to the peptide, during 2 hours. After this pre-treatment, baseline calcium levels were measured for 5 minutes before challenging the synaptosomes with KCl 20 mM.

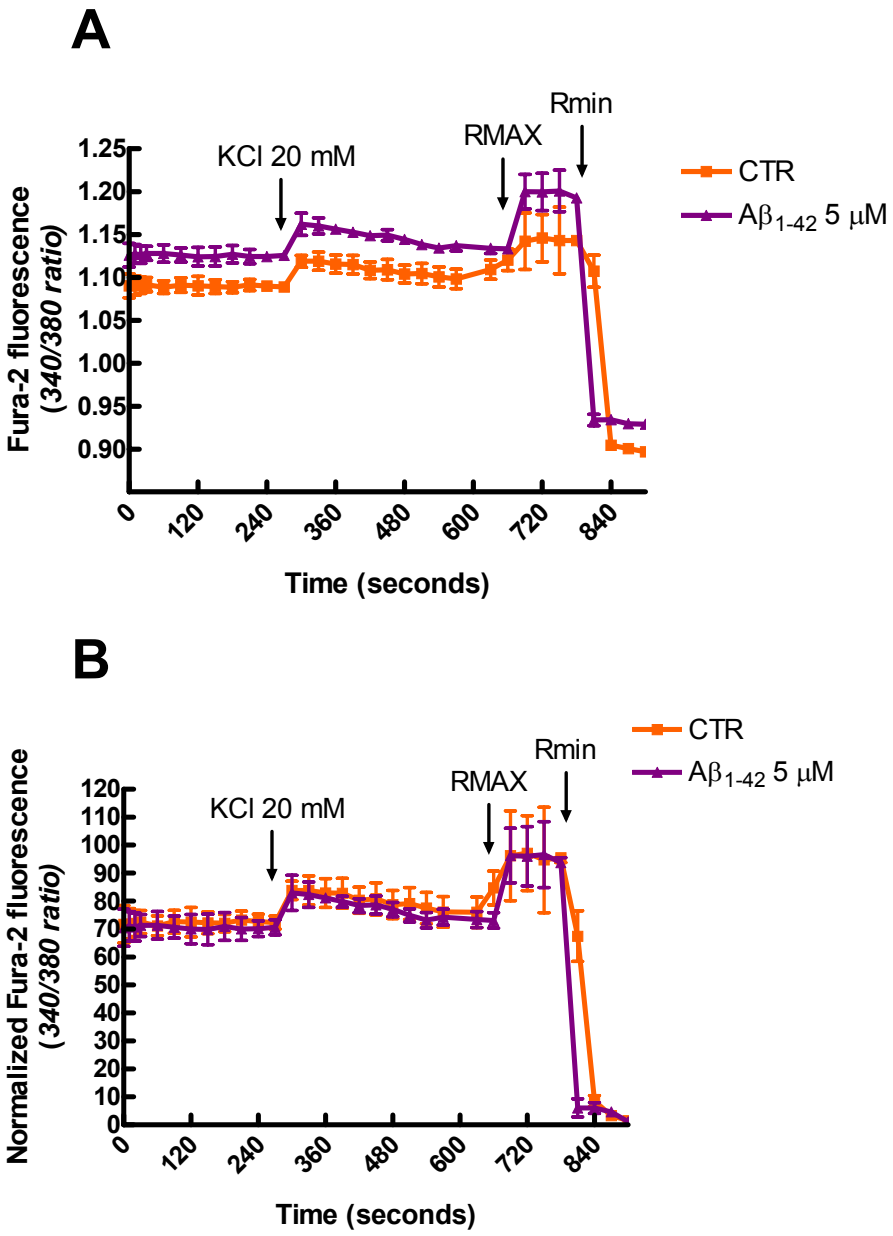


Figure 21: Calcium elevation after addition of KCl 20 mM at 5 minutes after pre-incubation of synaptosomes for 2h without (control – CTR) or with $A\beta_{1-42}$ (5 μM). A – Calcium signals from fura-2 *ratio* without normalization. B – Normalized calcium signals from fura-2 *ratio*. Synaptosomes were loaded with 5 μM FURA-2. For determination of RMAX with 4-BR-A23187 (15 μM) was added at 11 minutes and for determination of Rmin with EGTA (5 mM) was added at 13 minutes. Calcium responses represent the average of synaptosomes per experiment (n=4).

Without normalization, it can be observed a tendency to increase the basal intracellular free calcium concentration with $A\beta_{1-42}$ incubation (Fig. 21A). However, after normalization the initial basal calcium was 70% (% calculated after normalization FURA-2 340/380 *ratio,* where 100% = RMAX and 0%= Rmin) for synaptosomes either pre-treated with vehicle or $A\beta_{1-42}$ (Fig. 21B). Thus no calcium basal alteration was observed with pre-treament with $A\beta_{1-42}$, after normalization. KCl was used as a functional marker for nerve terminals distinguishing them from contaminating gliossomes and as a functional stimulus to depolarize synaptosomes (Scott et al., 1980) that will evoke a transient raise in the intracellular free Ca^{2+} concentration. Stimulation with KCl increased calcium levels by 10% in synaptosomes either pre-treated with vehicle or $A\beta_{1-42}$ (Fig. 21A). Again, it was not possible to observe any $A\beta_{1-42}$-induced alterations.

Thus, using synaptosomal preparation and single analysis, it is not possible to study the influence of the $A_{2A}R$ upon the toxicity of $A\beta_{1-42}$, since no measurable effect of $A\beta_{1-42}$ was observed. This led us to attempt using a more complex system – hippocampal neurons in culture.

4.4.3. IMAGING OF HIPPOCAMPAL CULTURES AND ELECTRIC FIELD STIMULATION

A functional model of neuronal excitability was developed by exposing hippocampal cultures (8 DIV) to an electrical field stimulation (EFS) protocol mimicking repetitive action potential firing (every minute - trains of 10 s - pulses 1 ms, 10 Hz; 50 mA). Electrical inputs are expected to generate Ca^{2+} responses in the soma by triggering action potentials, which in turn open voltage-gated Ca^{2+} channels in the somatic plasma membrane (Jacobs and Meyer, 1997). Ca^{2+} signals have been shown to affect important checkpoints of the cell death process, such as mitochondria, thus tuning the sensitivity of cells to various challenges (Rizzuto et al., 2003; Giorgi et al., 2008). The Aβ peptide can be one of these challenges, because it may indirectly cause damage to mitochondria in neurons by inducing oxidative stress and cellular calcium overload (Keller and Mattson, 1998; Canevari et al., 2004; Mattson, 2004, 2006; Yang et al., 2008). Mitochondria are recognized to play a pivotal role in neuronal cell survival or death because they are regulators of energy metabolism and apoptotic pathways (Moreira et al., 2006). Morphologic, biochemical, and molecular biology studies suggest that mitochondria might be a convergence point for neurodegeneration (Martin, 2006). Thus modification of the functions and properties of mitochondria might render subsets of selectively vulnerable neurons intrinsically

susceptible to cellular aging and stress (Mancuso et al., 2007).

The evaluation of neuronal function induced by electrical stimulation, after a pre-exposition either to $A\beta_{1-42}$ or vehicle, relied on the measurement of 2 parameters that are correlated: time-span to occur a calcium deregulation (calculated as the time required to achieve an irreversible calcium elevation) and mitochondrial potential loss (calculated as the time required to lose the maintenance of mitochondrial membrane potential). When cells achieve a certain plateau, either referring to mitochondrial potential or calcium measurement, it means that the cell is going to die, since the decrease of mitochondrial potential or calcium elevation is irreversible (Oliveira et al., 2006). The measurement of these 2 parameters in cultured neurons relied upon the use of the same probes used in the synaptosomal experiments, namely FURA-2 for intracellular calcium levels and $TMRM^+$ to measure mitochondrial membrane potential.

To be sure that in fact EFS induced calcium elevation in neurons was related to action potentials and not just membrane depolarization, tetrodoxin (TTX) was tested. TTX blocks action potentials in nerves by binding to the pores of the voltage-sensitive sodium channels in nerve cell membranes (Narahashi et al., 1960; Narahashi et al., 1964).

Figure 22 shows that TTX inhibits the appearance of calcium elevation synchronized with the EFS and the delayed mitochondrial membrane potential dysfunction. This confirms that the calcium elevation synchronized with the EFS is due to action potentials. The application of a prolonged EFS protocol (EFS starting at 5 minutes and prolonged until the end of the experiment) leads to a faster loss of mitochondrial membrane potential and calcium deregulation when compared to TTX-incubated neurons, subjected to the same prolonged EFS protocol. In the acute EFS protocol (EFS starts at 5 minutes and lasts 5 minutes), neurons lose more rapidly calcium homeostasis and mitochondrial membrane potential, when compared to TTX incubated neurons. Upon comparison of an acute *versus* a prolonged EFS protocol, it is observed that the prolonged EFS leads to a faster calcium elevation and mitochondrial membrane potential loss, which results in a faster impairment of cell maintenance.

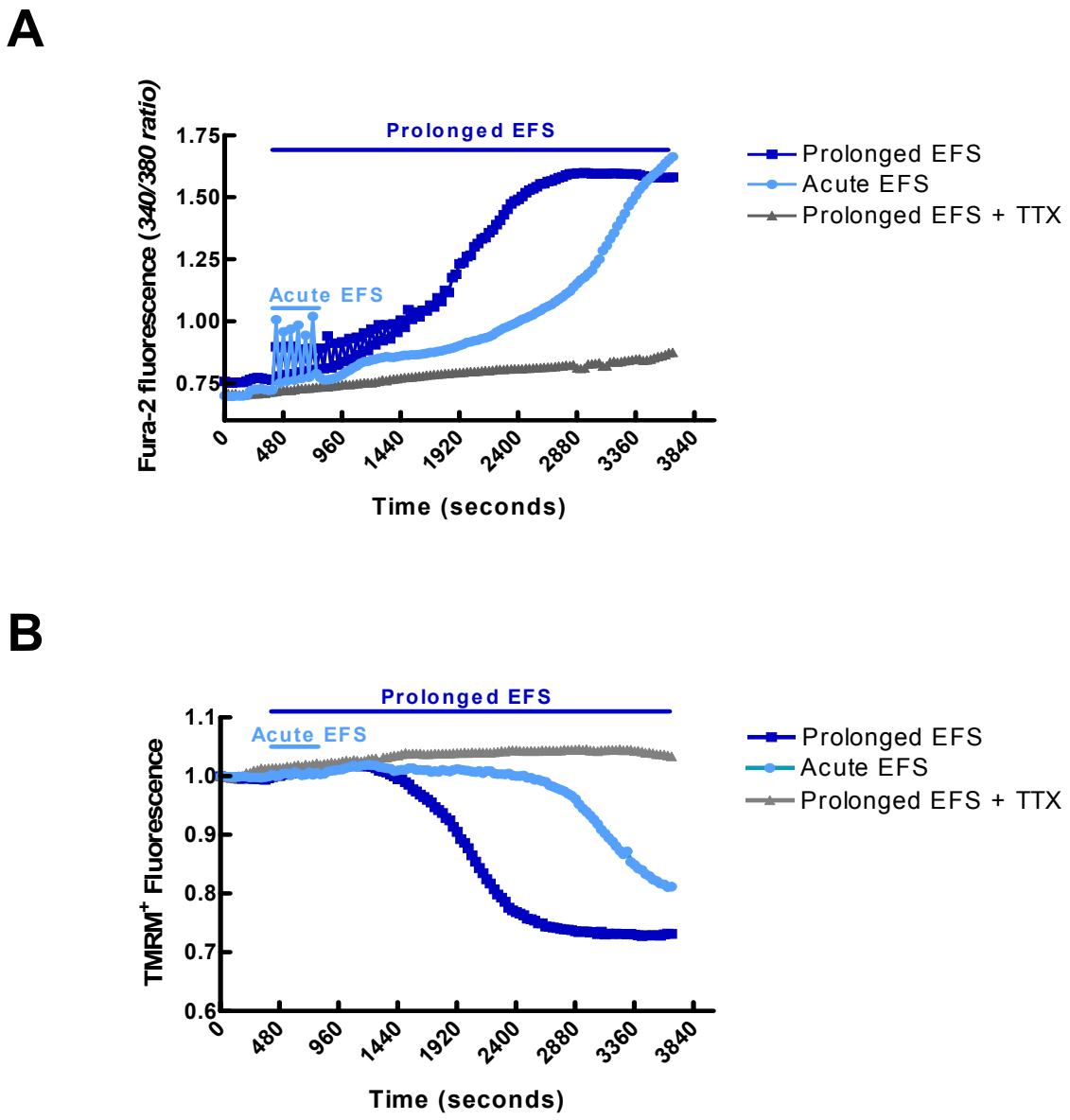


Figure 22: Effect of acute and prolonged electrical field stimulation on calcium transients and mitochondrial membrane potential in neurons and effect of tetrodoxin (TTX). A – Time course of calcium transients. B – Time course of mitochondrial membrane potential. Changes in intracellular calcium concentration and mitochondrial membrane potential were monitored with FURA-2 (5 µM) and tetramethyl rhodamine methyl ester ($TMRM^+$, 50 nM), respectively. Calcium transients were synchronized with acute EFS and the incubation of neurons with TTX (1 µM) 20 minutes before EFS abolished calcium elevation in neurons. Prolonged EFS (starting at 5 minutes and prolonged until the end of the experiment) leads to mitochondrial potential loss and calcium deregulation when compared to TTX incubated neurons. An acute protocol of EFS (starting at 5 minutes and lasting 5 minutes) leads to mitochondrial potential loss and calcium deregulation more slowly when compared to prolonged EFS. These results represent the average of neurons of a single experiment.

In this model, neurons and glial cells from the hippocampal culture (8-9 DIV) were distinguished by using phase-contrast optics, a bright-field image that allowed identification of neurons, which look quite different from the flatter astrocytes and also lie at a different focal plane above the astrocytic layer. This allowed dividing cells that compose the hippocampal

culture, in its major groups, namely neurons and glial cells. According to their different responses to prolonged EFS, neurons were further sub-divided into 3 sub-populations.

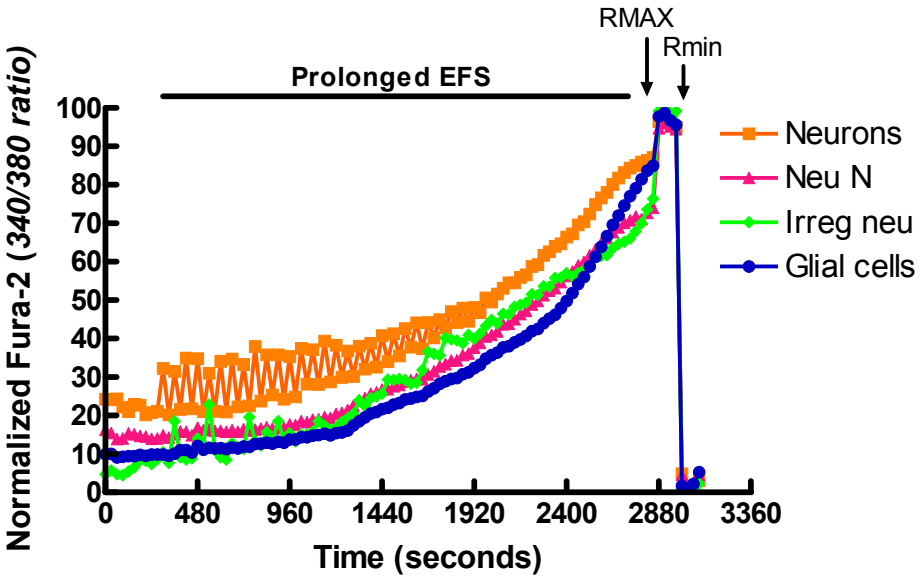


Figure 23: Representative experiment of calcium transients observed in hippocampal cultures (8-9 DIV) subject to a prolonged electrical field stimulation (EFS). Hippocampal cultures were loaded with FURA-2 (5 µM), whose response was calibrated at the end of experiment using 4-BR-A23187 (15 µM) followed by EGTA (5 mM). Calcium responses represent the average of the elements that constitute each sub-population: **neurons** - neurons that respond to EFS; **neu n** – neurons that do not respond to EFS and do not display spontaneous calcium transients; **irreg neu** – neurons that display spontaneous calcium transients independently of EFS; **glial cells**.

The first sub-population, corresponding neurons that respond to EFS, was labeled as **neurons**. The second sub-population is named **neu n,** corresponding to neurons that do not respond to the EFS and also do not display spontaneously calcium transients. The third sub-population is named **irreg neu**, corresponding to neurons that display spontaneous calcium transients, independently of EFS. Figure 23 displays examples of Ca^{2+} responses in the different sub-populations of cells in culture.

The effect of $A\beta_{1-42}$ was then tested by pre-incubation of cells during 48 hours with $A\beta_{1-42}$ (5 µM).

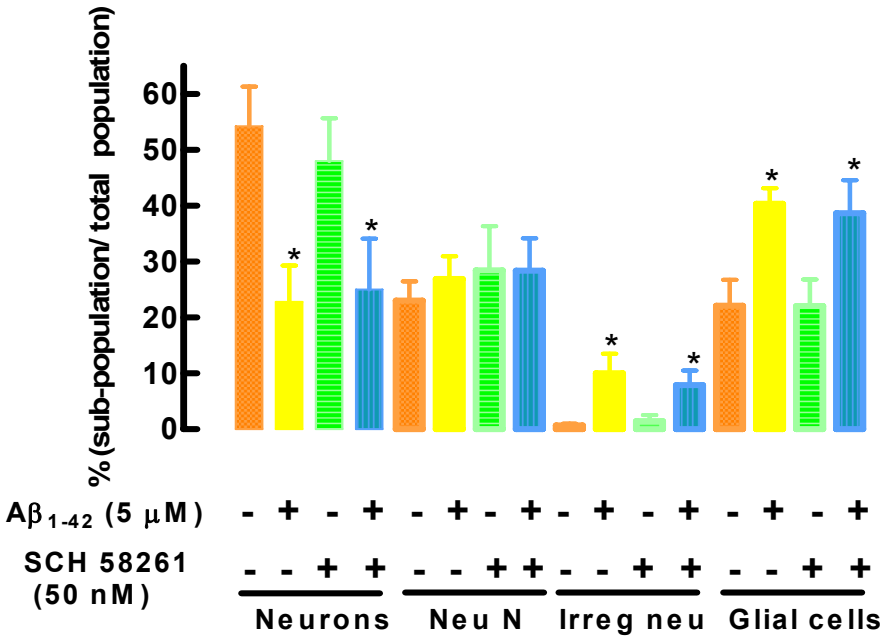


Figure 24: Modification of the number of the different cells in culture, upon exposure to Aβ and/or SCH58261 to block A_{2A}Rs. Cells were either treated with $A\beta_{1-42}$ (5 µM) or vehicle and each of the groups were either treated with the vehicle or with the A_{2A}R antagonist SCH58261 (50 nM, added 20 min before Aβ). Cells were then loaded with FURA-2 (5 µM) after 48 hours and then calcium responses to prolonged electrical field stimulation were evaluated. Each bar represents, on average, the percentage of the different sub-populations (neuron, Neu N, Irreg neu, Glial cells) concerning total population (sum of sub-populations) during the experiment (n=5 independent experiments). There is a decrease in the number of neurons and increase in the irregular neurons and glial cells upon pre-exposition to $A\beta_{1-42}$, which is not prevented upon the blockade of A_{2A}R. Data are mean±SEM of n=5 independent experiments, * P<0.05 compared to control (no added drugs).

Figure 24 shows that the pre-incubation with Aβ (5 µM) for 48 h caused the following changes, when compared to the control group: 1) decrease of the number of **neurons** (-29.9±6.1%, n=5); 2) increase of the number of **irreg**ular **neu**rons (+5.9± 2%, n=5); 3) increase of the number of **glial cells** (+22.5± 2.8%, n=5). However, the blockade of A_{2A}R with SCH58261 did not modify the effects of $A\beta_{1-42}$ on the number of each cell type. Also, SCH58261, *per se*, does not change cell distribution when compared to the control.

After characterization of cell population distribution in the 4 experimental groups subjected to EFS, the results were detailed for 2 sub-populations: **neurons** and **glial cells** population. Functional parameters of **neurons** were first analyzed, namely the number of synchronized responses to EFS and the time-span to occur calcium deregulation and loss of mitochondrial membrane potential.

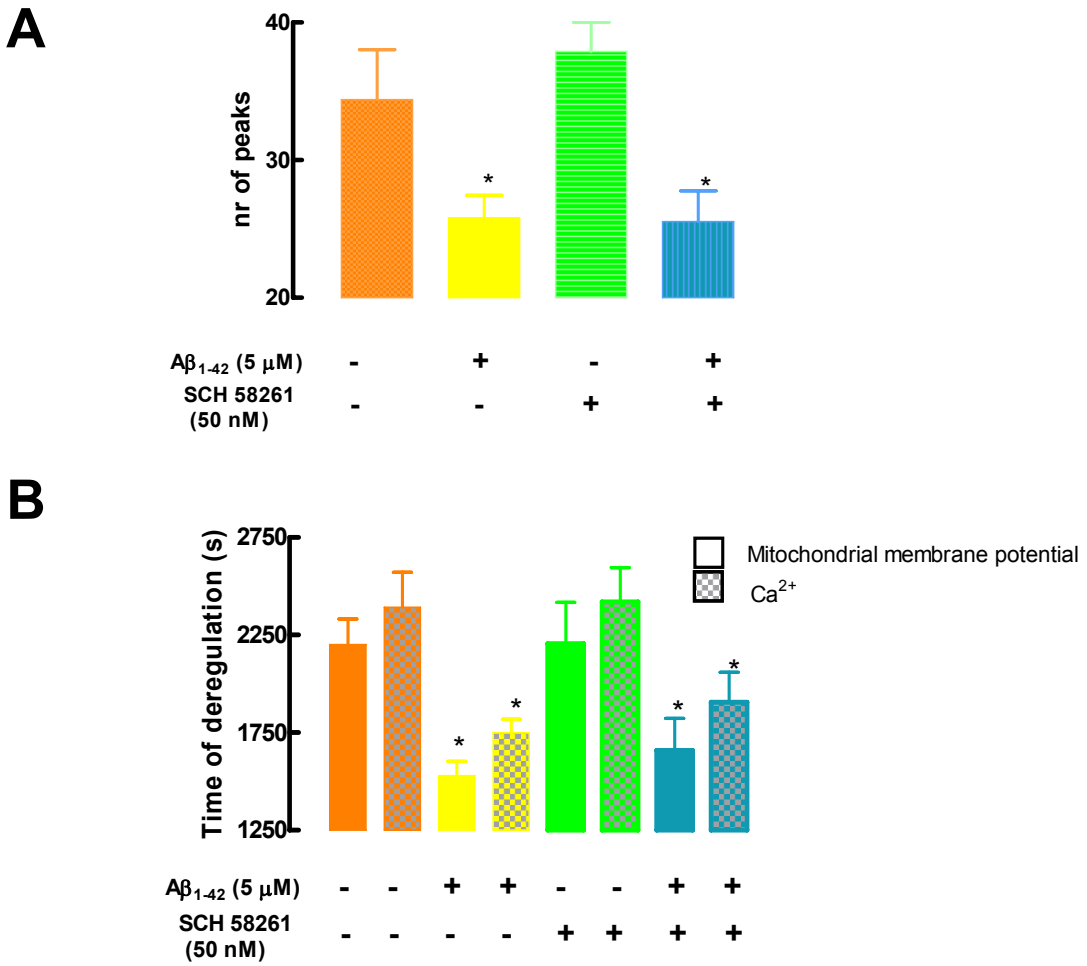


Figure 25: Functional parameters measured for neurons after a pre-incubation for 48 h with $A\beta_{1-42}$ (5 μM) or vehicle in the absence or presence of an $A_{2A}R$ antagonist (SCH58261) in hippocampal cultures subjected to prolonged EFS. A – Number of synchronized responses to EFS. **B** – Time-span to occur calcium deregulation and loss of mitochondrial membrane potential in neurons. Changes in intracellular calcium concentration and mitochondrial membrane potential were monitored with FURA-2 5 μM and tetramethyl rhodamine methyl ester ($TMRM^+$, 50 nM), respectively. There was a decrease of synchronized responses of neurons and a decrease of time-span to observe mitochondrial membrane potential loss and calcium deregulation, upon pre-exposure to $A\beta_{1-42}$, which is not prevented upon the blockade of $A_{2A}R$. Data are mean±SEM of n=5 independent experiments, * $P<0.05$ compared to control (no added drugs).

After a pre-treatment with $A\beta_{1-42}$, neurons exhibit a decrease of: 1) the number of synchronized responses to EFS observed during the experiment [$A\beta_{1-42}$: 25.8±1.6, (n=5) *vs* control: 34.4±3.6, (n=5)]; 2) the time-span to occur a calcium deregulation in neurons [$A\beta_{1-42}$: 1750±69 s, (n=5) *vs* control: 2393±179 s, (n=5)] and a loss of mitochondrial membrane potential maintenance in neurons [$A\beta_{1-42}$: 1530±73 s, (n=5) *vs* control: 2200±133 s, (n=5)] (Fig.25). SCH58261 had no significant effect on the Aβ-induced alterations in the sub-population **neurons** subjected to EFS nor did it modify the behavior of vehicle-treated neurons (Fig.25).

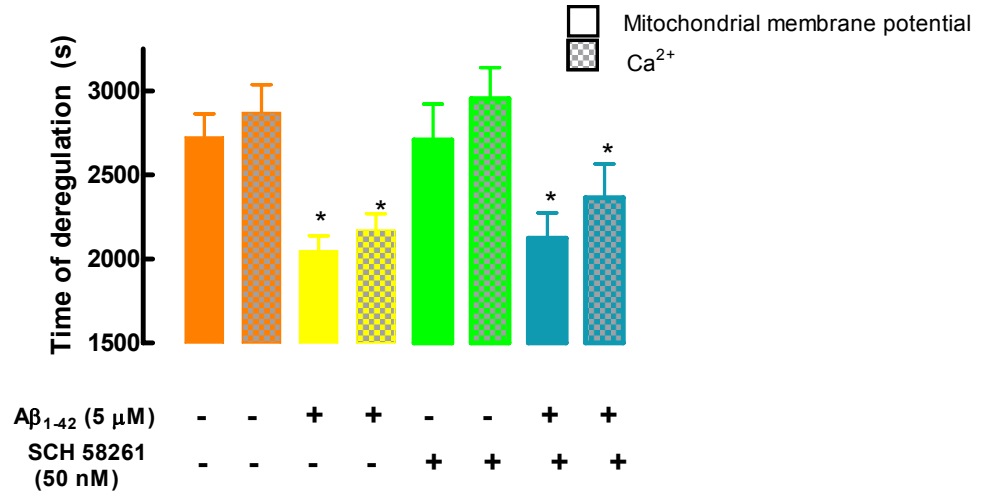


Figure 26: Functional parameters measured in glial cells after a pre-exposure for 48 h with $A\beta_{1-42}$ (5 µM) or vehicle, in the absence or presence of an $A_{2A}R$ antagonist (SCH58261) in hippocampal cultures subjected to prolonged EFS. Time-span to occur mitochondrial loss and calcium deregulation in glial cells. Changes in intracellular calcium concentration and mitochondrial membrane potential were monitored with FURA-2 (5 µM) and tetramethyl rhodamine methyl ester ($TMRM^+$, 50 nM), respectively. There is a decrease of the time-span to observe mitochondrial membrane potential loss and calcium deregulation, upon pre-exposure to $A\beta_{1-42}$, which is not prevented upon the blockade of $A_{2A}R$. Data are mean±SEM of n=5 independent experiments, * $P<0.05$ compared to control (no added drugs).

Pre-treatment with $A\beta_{1-42}$ caused a decrease in the time-span to occur a calcium deregulation in glial cells [$A\beta_{1-42}$: 2171±97 s, (n=5) *vs* control: 2871±165 s, (n=5)] and a loss of mitochondrial membrane potential maintenance in glial cells [$A\beta_{1-42}$: 2048±91 s, (n=5) *vs* control: 2726±137 s, (n=5)] (Fig.25). SCH58261 had no significant effect on the Aβ-induced alterations in the sub-population glial cells subjected to EFS nor did it modify the behavior of vehicle-treated glial cells.

As shown in Figures 25B and 26, it is possible to observe that mitochondrial membrane potential deregulation precedes calcium deregulation for neurons and glial cells, as previously reported for other models of toxicity, where both parameters were measured simultaneously (e.g. Oliveira et al., 2006).

4.4.4. DISCUSSION

Upon acute exposure of synaptosomal preparations to $A\beta_{1-42}$, it was not possible to detect any calcium changes throughout the experiment. This contrasts with the report of Dougherty and colleagues, showing calcium changes with a lower concentration of $A\beta_{1-42}$ in a subset of

hippocampal synaptosomes 17±10%, with sustained elevation in Ca^{2+} level evident for over 10 min of incubation (Dougherty et al., 2003). Experimental procedure differences might explain the lack of $A\beta_{1-42}$-effects in the acute preparation. The acute synaptosomal experiments were done in batch and volume chamber was 500 µl and Dougherty and colleagues used a rapid exchange Warner (36 µl volume) perfusion system attached to the microscope and subjected to perfusion with HBS containing Ca^{2+} at 3-5 ml/min.

In the prolonged exposure of synaptosomes to $A\beta_{1-42}$, it was also not possible to observe any calcium changes. The lack of effects induced by $A\beta_{1-42}$ can be explained if the calcium variations occur in a small percentage of synaptosomes. As an example of a type of nerve terminals that represent a small percentage of the total synaptosomal population is the cholinergic terminals sub-population. The cholinergic terminals represent circa 6% of the total number of nerve terminals in the hippocampus (Kuhar and Rommelspacher, 1974; Richardson, 1981). One of the hypotheses described to explain AD is a cholinergic deficit, associated with a loss of cholinergic nerve terminals, which was described in AD transgenic models and AD human patients (Bell et al., 2006). In an AD transgenic model, the cholinergic nerve terminals are the first synapses that become dysfunctional or disappear through time (Bell et al., 2006), as was also observed in sub-chapter 4.3. A possibility to overcome this problem is optimizing a protocol, where it is possible to distinguish functionally the different nerve terminals.

To further explore the influence of $A_{2A}R$, it was decided to develop a functional protocol (EFS) using hippocampal cultures, previously incubated with $A\beta_{1-42}$. Mechanistically $A\beta_{1-42}$ can impair calcium homeostasis, mitochondrial function, leading to energy hypometabolism and elevated reactive oxygen species production (Wang et al., 2007b). Additionally, $A\beta_{1-42}$ affects the balance of mitochondrial fission/fusion and mitochondrial transport, negatively impacting a host of cellular functions of neurons (Wang et al., 2007b).

The prolonged EFS protocol led to a transient elevation of calcium levels in neurons, dependent of voltage-gated Na^{+} channels. In less than one hour, the cells subjected to EFS achieved an irreversible calcium plateau and irreversible mitochondrial membrane potential loss that results later in cell death. This toxicity triggered by EFS occurs primarily in neurons and afterwards in astrocytes. These results may be explained by the consequent malfunction of neurons leading to an indirect toxicity in astrocytes.

After 48h of incubation with $A\beta_{1-42}$, the cells that survived are more susceptible to a second noxious stimulus, such as EFS, because they presented a decrease of the time-span

required for loss of mitochondrial membrane potential and calcium de-regulation, when compared to the control. It is possible that the cells that survived could be resistant to Aβ because of the existence, as reported by Simakova and Arispe, of a subpopulation of cells with resistance to Aβ, even at high concentrations and after long periods of treatment (Simakova and Arispe, 2007). However, the Aβ-resistant cells can be susceptible to a second stimulus, because upon Aβ-induced toxicity there is release of several noxious substances from dead cells, such as glutamate, leading to a posterior excitotoxicity, which will influence the survival of population (Reichmann et al., 2005; Koutsilieri and Riederer, 2007).

The blockade of $A_{2A}R$ in this protocol could not prevent the $A\beta_{1-42}$-induced toxicity. However, Aβ-induced toxicity required high concentration of $A\beta_{1-42}$ (5 μM) and prolonged prior incubation (48h) to achieve a significant effect in the different parameters measured in the EFS protocol. The $A_{2A}R$ antagonist was not able to prevent this exacerbated toxicity, however it prevents early synaptotoxicity and neuronal toxicity caused by a lower $A\beta_{1-42}$ concentration, like it was described in sub-chapter 4.2. It has also been reported that the $A_{2A}R$ antagonist prevents staurosporine-induced synaptotoxicity and neuronal death (Silva et al., 2007). Thus, it is possible that this inability of $A_{2A}R$ to prevent Aβ-induced neuronal or glial dysfunction in the present study might be related to a combined use of a supramaximal toxic Aβ stimulus together with the fact that the analysis was carried out in the cells that were more resistant to Aβ.

The $A\beta_{1-42}$ peptide does not cause any calcium changes in synaptosomes. In the EFS protocol, $A\beta_{1-42}$-induced toxicity altered normal firing characteristics of neurons, and decreased of time-span of calcium deregulation and membrane mitochondrial potential loss in neurons and glial cells. The $A_{2A}R$ antagonist SCH58261 had no significant effect on the $A\beta_{1-42}$ high concentration-induced alterations that were measured during EFS. It can be concluded that, at least for high concentration of $A\beta_{1-42}$, $A_{2A}R$ blockade does not prevent Aβ toxicity.

4.5. BLOCKADE OF ADENOSINE A_{2A} RECEPTORS PREVENTS MEMORY DYSFUNCTION CAUSED BY β-AMYLOID PEPTIDES BUT NOT BY SCOPOLAMINE OR MK-801

4.5.1. INTRODUCTION

Adenosine is a neuromodulator that can either inhibit or facilitate synaptic transmission through inhibitory A_1 or facilitatory A_{2A} receptors, respectively (Fredholm et al., 2005b). These adenosine receptors also have the ability to control neuronal damage after different insults: A_1 receptors constitute a hurl that increases the threshold for brain damage, whereas the blockade of adenosine A_{2A} receptors affords neuroprotection against chronic noxious brain insults (Cunha, 2005). A_{2A} receptor antagonists have recently received particular attention as novel strategies to prevent or restrain the development of neurodegenerative diseases, namely in Parkinson's disease, where A_{2A} receptor antagonists are the leading non-dopaminergic therapy currently in phase IIb trials (Schwarzschild et al., 2006). It was also recently shown that A_{2A} receptor antagonists can prevent memory impairment in animal models of aging (Prediger et al., 2005) and Alzheimer's disease (Arendash et al., 2006; Dall'Igna et al., 2007), in accordance with the ability of chronic consumption of caffeine (an adenosine receptor antagonist) to enhance memory performance in the elderly (e.g. Johnson-Kozlow et al., 2002), to attenuate memory decline in the elderly (Ritchie et al., 2007) and to decrease the risk of Alzheimer's disease (Maia and de Mendonça, 2002).

Recent studies indicate that the genetic manipulation of A_{2A} receptors modifies memory performance in rodents (Wang et al., 2006; Gimenez-Llort et al., 2007) and can also modulate long term potentiation (d'Alcantara et al., 2001; Rebola et al., 2008), a physiological correlate of learning and memory (Lynch, 2004). Thus, it remains to be determined if A_{2A} receptors play a general role in controlling memory performance or if the impact of A_{2A} receptors on memory impairment is restricted to particular conditions where insidious neurodegenerative processes underlie memory dysfunction. To tackle this question, it was compared the ability of A_{2A} receptor blockade to affect memory dysfunction caused by different experimental conditions, namely upon administration of a β-amyloid peptide (which triggers a slowly developing synaptic degeneration and memory dysfunction, (see Coleman et al., 2004) and upon

administration of either scopolamine (which triggers an acute memory impairment through a cholinergic block; see Hasselmo, 2006) or MK-801 (which triggers an acute memory impairment by blocking NMDA receptors; see Ellison, 1995).

4.5.2. MODEL OF Aβ-INDUCED MEMORY DYSFUNCTION

Soluble forms of β-amyloid peptides, namely $A\beta_{1–42}$, are considered the most likely culprit for the early development of AD (Hardy and Selkoe, 2002). Accordingly, cerebral microinjection of Aβ causes amnesia and is considered a suitable animal model to test new protective strategies eventually relevant to manage the early phases of AD (Coleman et al., 2004). It was now observed that 2 weeks after icv injection of $A\beta_{1–42}$ (2 nmol), rats displayed a decrease of spontaneous alternation in the Y-maze, with no significant modification of locomotion (Fig. 27). As previously observed to occur in mice (Dall'Igna et al., 2007), it was now confirmed that the selective A_{2A} receptor antagonist, SCH58261 (0.05 mg/kg, ip) prevented the $A\beta_{1-42}$-induced amnesia and did not modify *per se* either the spontaneous alternation or locomotion (Fig. 27). Likewise, another selective A_{2A} receptor antagonist, KW6002 (3 mg/kg, ip) also prevented the $A\beta_{1-42}$-induced amnesia and also did not modify *per se* either the spontaneous alternation or locomotion (Fig. 27).

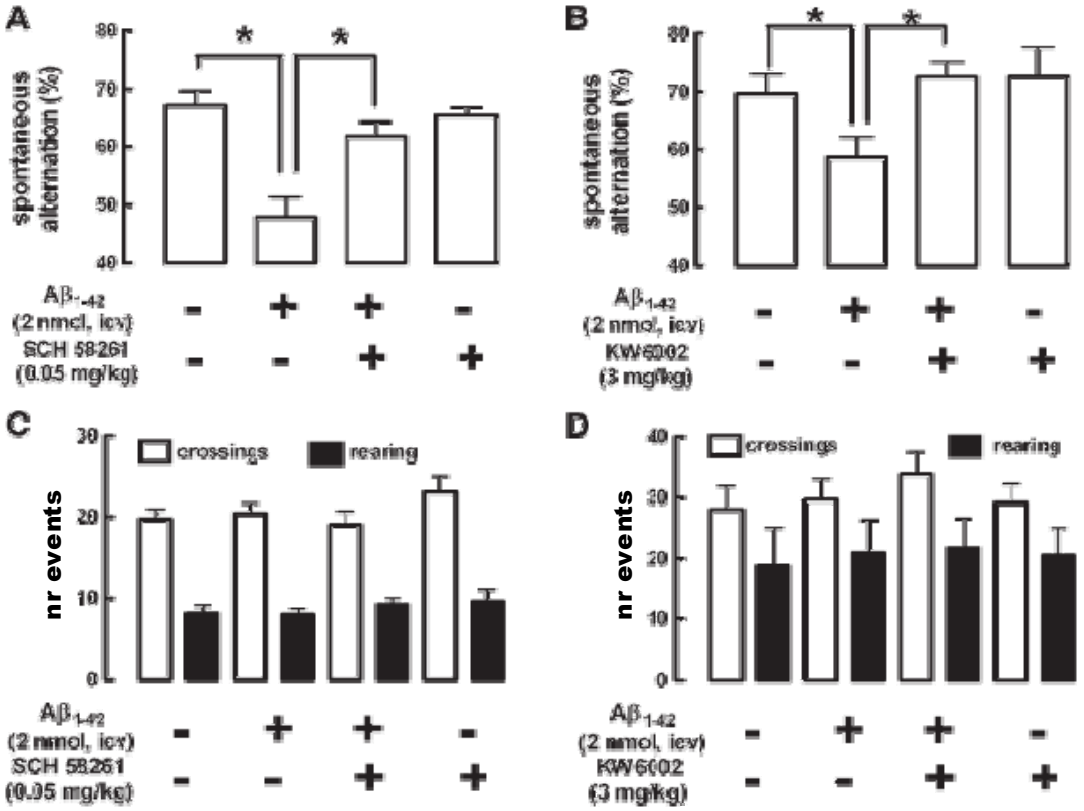


Figure 27: Blockade of adenosine A_{2A} receptors prevents β-amyloid-induced decrease of spontaneous alternation. Rats were treated (2 nmol, icv) with β-amyloid peptide 1-42 fragment ($A\beta_{1-42}$) or distilled water. The A_{2A} receptor antagonists SCH58261 (0.05 mg/kg, ip) or KW6002 (3 mg/kg, ip) were administered daily starting 30 min before Aβ and rats were behaviorally analyzed after 15 days. (A, B) Spontaneous alternation in the Y-maze test and (C, D) spontaneous locomotion evaluated in an open field arena are expressed as mean±S.E.M. The data from panels A and C are from 10 rats in each experimental group (* P<0.05 between the indicated columns) and the data from panels B and D are from 6 rats in each experimental group (* P<0.1 between the indicated columns).

4.5.3. MODEL OF SCOPOLAMINE-INDUCED MEMORY DYSFUNCTION

Scopolamine is a muscarinic receptor antagonist, which is a useful tool to induce an acute wide range memory impairment based on its ability to transiently induce a cholinergic-like deficit (e.g. Sitaram et al., 1978). Thirty minutes after the administration of scopolamine (2 mg/kg, ip), rats displayed a decreased spontaneous alternation in the Y-maze (Fig.28). As previously observed (e.g. Hooper et al., 1996), they also displayed an increased locomotion, which was only evident when measuring horizontal activity (Fig. 28). Previous studies have already demonstrated that spatial memory can be evaluated by measuring spontaneous alternation even in animals displaying a modified locomotion pattern (Drew et al., 1973; Anisman, 1975). Albeit, the scopolamine-induced decrease of spontaneous alternation had a

magnitude similar to the $A\beta_{1-42}$-induced depression, SCH58261 (0.05 mg/kg, ip) failed to prevent the scopolamine-induced depression of spatial memory (Fig. 28). SCH58261 also failed to prevent the scopolamine-induced hyperlocomotion (Fig. 28).

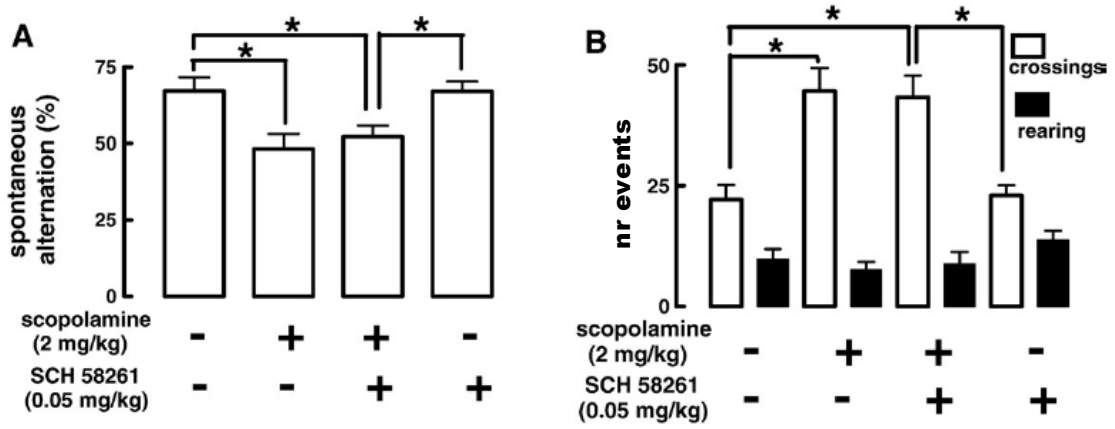


Figure 28: Blockade of adenosine A_{2A} receptors fails to modify the scopolamine induced decrease of spontaneous alternation. Rats were treated with scopolamine (2 mg/kg, ip) or saline and behaviorally analyzed after 30 min. The A_{2A} receptor antagonist SCH58261 (0.05 mg/kg, ip) was administered 30 min before scopolamine. (A) Spontaneous alternation in the Y-maze test and (B) spontaneous locomotion evaluated in an open field arena are expressed as mean±S.E.M. Data are from 7–8 rats in each experimental group. * P<0.05 between the indicated columns.

4.5.4. MODEL OF MK-801 -INDUCED MEMORY DYSFUNCTION

MK-801 is another pharmacological tool that causes an acute disruption of memory performance by virtue of its ability to inhibit NMDA receptors, which play a crucial role in the implementation of several forms of synaptic plasticity in the mammalian central nervous system (Collingridge, 1987).

Thirty minutes after the administration of MK-801 (0.25 mg/kg, ip), rats displayed a decreased spontaneous alternation in the Y-maze (Fig. 29). As previously observed (e.g. Pitkanen et al., 1995), they also displayed an increased locomotion (Fig. 29). As observed for scopolamine and in contrast to $A\beta_{1-42}$-induced decrease of spontaneous alternation, SCH58261 (0.05 mg/kg, ip) failed to prevent the MK-801-induced depression of spatial memory (Fig. 29). Likewise, another selective A_{2A} receptor antagonist, KW6002 (3 mg/kg, ip) also failed to prevent the MK-801-induced depression of spatial memory (Fig.29). Interestingly, it was found that both SCH58261 and KW6002 significantly (P<0.05) attenuated the MK-801-induced hyperlocomotion (Fig. 29), which is in agreement with previous observations showing that

endogenous adenosine has a differential ability to control different NMDA receptor-dependent central effects (see Dall'Igna et al., 2003).

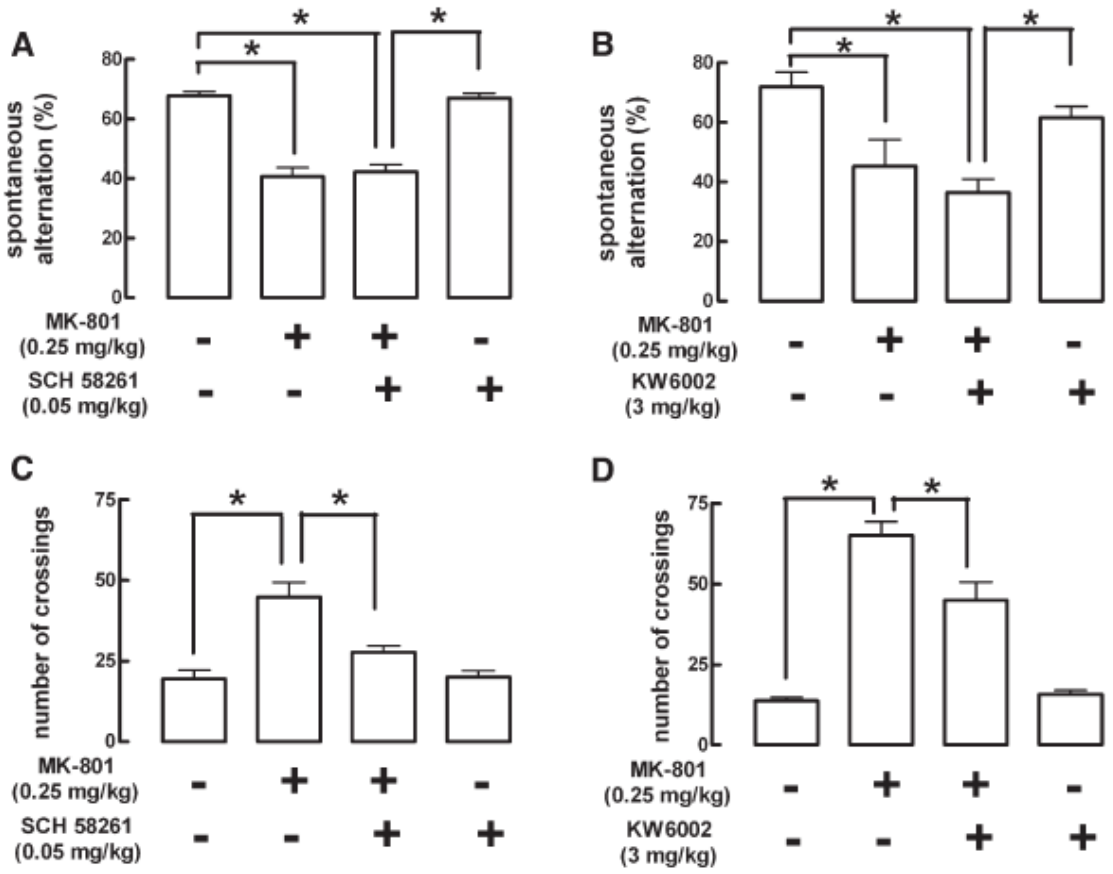


Figure 29: Blockade of adenosine A_{2A} receptors fails to modify the MK-801-induced decrease of spontaneous alternation. Rats were treated with MK-801 (0.25 mg/kg, ip) or saline and behaviorally analyzed after 30 min. The A_{2A} receptor antagonists SCH58261 (0.05 mg/kg, ip) or KW6002 (3 mg/kg, ip) were administered 30 min before MK-801. (A, B) Spontaneous alternation in the Y-maze test and (C, D) spontaneous locomotion evaluated in an open field arena are expressed as mean±S.E.M. Data are from 7–8 rats in each experimental group. * $P<0.05$ between the indicated columns.

4.5.5. DISCUSSION

The present results show that the prevention of memory deficits upon blockade of adenosine A_{2A} receptors is not a general feature. Instead, the beneficial effect resulting from A_{2A} receptor blockade is only observed in insidious slowly evolving conditions leading to memory deficits, such as upon β-amyloid-induced impairment of cognitive performance, and is not observed in experimental conditions that cause an acute deterioration of memory performance,

such as upon administration of scopolamine or MK-801. This is in notable agreement with the neuroprotective profile resulting from pharmacological or genetic inactivation of A_{2A} receptors in adult animals. In fact, A_{2A} receptor blockade is effective in affording neuroprotection against chronic insults causing a slowly evolving brain damage whereas it fails to afford protection against acute brain damage (reviewed in Cunha, 2005). The Aβ-induced cognitive disruption primarily results from a synaptic deterioration which then spreads to include a pattern of neuronal death (reviewed in Coleman et al., 2004). Therefore synaptically located modulatory systems, such as A_{2A} receptors (Cunha, 2005), may be particularly effective to counteract this insidious and slowly developing Aβ-induced cognitive impairment.

In contrast, single administrations of scopolamine or MK-801 cause memory deficits that are acute and transient and result from blockade of cholinergic and NMDA receptor-mediated signaling rather than causing synaptic loss (Ellison, 1995; Hasselmo, 2006). Thus, it is tempting to speculate that the beneficial role of A_{2A} receptor antagonists on memory disturbance might be limited to conditions where there is a slowly evolving loss of synaptic viability. This contention implies that A_{2A} receptors might not directly affect the basic pharmacology of memory but instead only impacts indirectly, by way of controlling neurodegeneration, on memory dysfunction. This would also indicate that A_{2A} receptor antagonists might be particularly interesting novel therapeutic tools to correct memory dysfunction without expectable effects in situations where memory performance is not compromised. This is in notable agreement with the effects on memory performance of the generic adenosine receptor antagonist, caffeine, which is the most widely consumed psychoactive drug worldwide (see Fredholm et al., 2005b): the chronic consumption of moderate doses of caffeine does not cause consistent mnemonic effects in healthy young adult animals or subjects, but it ameliorates memory performance in aged or in memory compromised animals or subjects (e.g. Johnson-Kozlow et al., 2002; Dall'Igna et al., 2007; Prediger et al., 2005; Ritchie et al., 2007). However, the contention that A_{2A} receptor blockade does not affect memory performance in healthy adult animals (and humans) should still be considered with caution. In fact, in spite of the lack of effect of A_{2A} receptor antagonists on memory performance in healthy adult animals (as now observed and in accordance with most studies; reviewed in Takahashi et al., 2008), two recent reports using transgenic approaches argued that A_{2A} receptors might directly control the unimpaired memory performance. Thus, the over-expression of A_{2A} receptors impaired working memory performance (Gimenez-Llort et al., 2007) and conversely the genetic deletion of A_{2A} receptors improved memory performance

(Wang et al., 2006) However, it remains to be defined if the effects of these genetic modifications of A_{2A} receptors might not be a result from a developmental effect on memory-related circuits. Another interesting observation emerged from the present study in relation to the ability of selective A_{2A} receptor antagonists to attenuate the hyperlocomotion caused by MK-801, which had previously been shown to occur for caffeine (Dall'Igna et al., 2003). This might result from the fact that the global effect of endogenous adenosine acting through A_{2A} receptors in the striatum is to maintain a fine-tuned balance between glutamatergic and dopaminergic signaling in the basal ganglia (reviewed in Schiffmann et al., 2007). Hence, A_{2A} receptor blockade may shift to the left the dose-response curve of MK-801 effect on locomotion, which causes motor activating effects at lower doses and motor depression at higher doses. Further studies will be required to establish the mechanistic basis of this observation.

In summary, the present results directly show that A_{2A} receptor antagonists are only effective to restore memory function in particular conditions associated with slowly developing memory failure that involve synaptic deterioration. In contrast, in situations of acute memory impairment due to transient inhibition of particular signaling systems, the blockade of A_{2A} receptors is unable to counteract memory deficits. Therefore, it is canvassed that the beneficial effect of A_{2A} receptor antagonists on memory performance may result from their neuroprotective properties rather to the direct ability of A_{2A} receptors to control any general mechanism underlying mnemonic processing. Particular attention should follow the observation that the neuroprotective effects of A_{2A} receptor antagonists on memory dysfunction can be observed at doses lower than those clearly affecting locomotion (see Fredholm et al., 2005b). This opens the possibility that it might be possible to overcome the undesired effects of A_{2A} receptor blockade associated with greater activity of the immune-inflammatory system (Sitkovsky et al., 2004) or the onset of psychotic symptoms resulting from the release of dopamine D2 receptor function (Weiss et al., 2003; Ferré et al., 2004).

4.6. POSSIBLE ROLE OF ADENOSINE IN THE CONTROL OF NEUROINFLAMMATION

4.6.1. INTRODUCTION

Inflammation processes in the body have two main purposes: tissue homeostasis and tissue defense against pathogens, through the production of cytotoxic substances, chemokines and cytokines (Lo et al., 1999; Rosenberg, 2002; Elward and Gasque, 2003; Carson et al., 2006; Grigoriadis et al., 2006). Until recently, the brain was considered a "privileged organ" because of the existence of the blood brain barrier (BBB) that creates a barrier between the brain parenchyma and the immune systemic cells (Medawar, 1918; Barker and Billingham, 1977). In the past years, neuroinflammation has begun to have a prominent role in neuroscience research, as testified by the exponential increase of number of papers about neuroinflammation from 2002 onwards. This lead to a shift of ideas regarding the type of cellular immune responses that can occur in the CNS. Current data indicates that CNS is both immune competent and actively interacts with the peripheral immune system (Bechmann, 2005; Carson et al., 2006). The inflammatory response in the CNS comprises a complex and integrated interplay between different cellular types of the immune system (macrophages, T and B lymphocytes, dendritic cells) and resident cells of the CNS (microglia, astrocytes, oligodendrocytes, neurons), as well as a complex orchestra of cytokines, adhesion molecules, chemokines and their receptors (Bernardino and Malva, 2007). Neuroinflammation can have a dual role: neuroprotective and/or neurototoxic (Schwartz and Moalem, 2001; Weiner and Selkoe, 2002; Elward and Gasque, 2003; Kerschensteiner et al., 2003; Schwartz, 2003; Marchetti and Abbracchio, 2005). At an early stage and under proper control it fulfills a beneficial role affording an effective defensive or clearance mechanism together with a trophic support to allow the recovery of the damaged area; however, if neuroinflammation evolves to a chronic non-controlled state, then it becomes a deleterious process that may contribute to the amplification of the brain damage (Cunha et al., 2007). Several neurodegenerative diseases display an associated neuroinflammation state, such as AD, Parkinson's disease, epilepsy, although it not yet clear if neuroinflammation is a cause or a consequence of these neurodegenerative diseases (Piehl and Lidman, 2001; Gao et al., 2003;

Hartmann et al., 2003; Liu and Hong, 2003; Andersen, 2004; Block and Hong, 2005; Marchetti and Abbracchio, 2005; Minghetti, 2005; Lucas et al., 2006).

Apart from its central neuromodulatory role, adenosine is also a potent modulator of inflammatory reactions in the periphery (Ohta and Sitkovsky, 2001). $A_{2A}R$ can have a dual role in inflammation depending on their localization, because the activation of $A_{2A}R$ in the periphery confers protection; however in the CNS it is the blockade of $A_{2A}R$ that is responsible for neuroprotection. In fact, the pharmacological activation of $A_{2A}R$ has been shown to confer a robust protection against tissue damage in different periphery organs such as: heart, blood vessels, kidney, liver, lung, intestines, stomach, joints and skin (reviewed in Jacobson and Gao, 2006). On the contrary, in the CNS the activation of $A_{2A}R$ only has an effect in an acute brain infection (Sullivan et al., 1999). Moreover upon chronic stimuli such as in a spinal cord injury, $A_{2A}R$ activation will be deleterious, and it will be the $A_{2A}R$ antagonists that are able to afford tissue protection and functional recovery (communication by Joel Linden at the Eighth International Adenosine and Adenine Nucleotide Symposium). In other chronic situations that involve neuroinflammation such as epilepsy (Lee et al., 2004), ischemia (Yu et al., 2004) and in a model of PD (Yu et al., 2008), the blockade of $A_{2A}R$ also prevented the neuronal damage and the events related with neuroinflammation. The mechanism by which the blockade of the $A_{2A}R$ is neuroprotective in neurodegenerative models is currently unclear (Cunha, 2005). However, several hypothetic mechanisms have been postulated, which may have different importance in diverse noxious conditions. It can involve control of glutamate that triggers excitotoxicity, the control of apoptosis or the control of neuroinflammation (Cunha, 2005). Presently, the role of $A_{2A}R$ in the control of neuroinflammation is still to be established since there are few papers in the literature and some of them are contradictory (see review Chen et al., 2007). To try to clarify the role of $A_{2A}R$ in neuroinflammation, different models were now tested. To follow neuroinflammation in these different models, particular attention was devoted to microglia cells because they participate in the onset and progression of CNS inflammatory responses (Kreutzberg, 1996; Streit et al., 1999). Microglia varies between 2 states: resting and activated (Kreutzberg, 1996; Streit et al., 1999). In the resting state, microglia monitors continuously the physiological integrity of their microenvironment and reacts rapidly in the event of pathological disturbances (Kreutzberg, 1996; Streit et al., 1999). In an activated state microglia are extremely plastic and react differently depending on the inflicted noxious stimuli (Kreutzberg, 1996; Streit et al., 1999). As a way to activate microglia, lypopolysaccharide (LPS) was used in cultured

microglia and in an *in vivo* study, since it is a standard way of inducing of inflammation (e.g. Gehrmann et al., 1995; Kreutzberg, 1996; Qin et al., 2007). Another model used in this study was the perforant pathway lesion because it provides excellent features for studying the activation and proliferation of microglial cells (Ladeby et al., 2005). The last model tested was the *in vivo* kainate-induced convulsion model. Systemic injection of kainate (KA) in rats results in activation of glial cells and inflammatory responses typically found in neurodegenerative diseases (Wang et al., 2005).

4.6.2. MICROGLIA CULTURE – *IN VITRO* MODEL

To try to understand the role of $A_{2A}R$ in cultured microglia cells upon stimulation with LPS, the activation state of microglia was monitored using different morphologic markers. It is possible to differentiate resting microglia from activated microglia because resting microglia exhibit a downregulation of many monocytoid markers, whereas activated microglia express numerous cell markers consistent with their monocytoid phenotype, including upregulated complement type 3 receptor (CR3)/MAC-1, MHC class I and II antigens and lectin binding site protein (Kreutzberg, 1996; Streit et al., 1999; Nelson et al., 2002).

Table 8: Antigenic features that may help to differentiate cells with monocytoid phenotype (from Nelson et al., 2002).

	Macrophage	IDCs	MDCs	Microglia-resting	Microglia-activated/culture
FcR	+/++	++	+/−	+	++
MHC II	+/++	+	++	+	++
CD1a	−	++	+	−	−
CD11b (CR3/MAC1)	+/++	+/++	−/+	+	++
CD11c (CR4)	+/++	+/++	−/+	+	+
CD14	+/++	+	−	−/+	+
CD80 (B7-1)	−/+	+/−	++	−/+	+
CD86 (B7-2)	+	+/−	++	−/+	+
CD45	+	−/+	−/+	−/+	+
CD68	++	−/+*	−/+*	−	+
CD54 (ICAM-1)	−/++	−/+	++	−/+	+/++

*If CD68 is present, only expressed as discrete juxtanuclear spots as opposed to within lysosomal structures of macrophages and microglia. IDCs = immature dendritic cells; MDCs = mature dendritic cells; CD1a = 49 kDa, MHC class I-like molecule which aids in presentation of non-peptide antigens to T cells; CD11b = 165 kDa, non-covalently linked to CD18 to form Mac-1 integrin, phagocytosis of iC3b coated particles; CD11c = 145 kDa, non-covalently linked to CD18 to form p150, 95 integrin, function similar to CD11b; CD14 = 53 kDa, binds complex of LPS and LPS binding proteins; CD80 = 60 kDa, Ig superfamily, co-stimulator for T-cell activation, ligand for CD28 and CD152 (CTLA-4); CD86 = 80 kDa, Ig superfamily, co-stimulator for T-cell activation, ligand for CD28 and CD 152 (CTLA-4); CD45 = multiple isoforms, tyrosine phosphatase which plays a critical role in T- and B-cell antigen receptor mediated signaling; CD68 = 110 kDa, unknown function; CD54 = 75-114 kDa Ig superfamily, cell-cell adhesion, receptor for Mac-1 and LFA-1.

The preparation of the cultures was adapted from a method described by Pocock and collaborators (Kingham and Pocock, 2000). The purity of the cultures was monitored using a monocytic marker, CD11b (Masumura et al., 2000; Ray et al., 2000; Kingham and Pocock, 2000; Nelson et al., 2002; Mokhtarian et al., 2003; Reichert and Rotshenker, 2003), and a marker for astrocytes (GFAP) (Pekny and Nilsson, 2005). The vast majority of the cells (95.0±3.5%, n=3) displayed CD11b immunoreactivity, with a small contamination with GFAP-stained cells. At 3 DIV, the microglia cells were stimulated during 4 hours with LPS (100 ng/ml), which triggers microglia activation (Gehrmann et al., 1995; Kreutzberg, 1996; Tomas-Camardiel et al., 2004; Qin et al., 2007). Two markers were chosen to monitor microglia activation: CD11b (OX42, ED7 or CR3) and ED1 or CD68 (macrosylianin) (e.g. Nelson et al., 2002; Fenger et al., 2006). Both are surface receptors recognizing a microbian pattern, and are therefore designated as scavenger receptors (Nelson et al., 2002). CD11b is often selected as marker to characterize microglia because it is present either in the resting state and activated state, although with an increased immunoreactivity in activated state (Kingham and Pocock, 2000; Masumura et al., 2000; Ray et al., 2000; Nelson et al., 2002; Mokhtarian et al., 2003; Reichert and Rotshenker, 2003). ED1 is considered an activation marker, with a distribution at the plasma membrane of microglia in the activation state (Ogata and Schubert, 1996; Chen et al., 2002; Nelson et al., 2002; Rogove et al., 2002; Faustmann et al., 2003; Taylor et al., 2003).

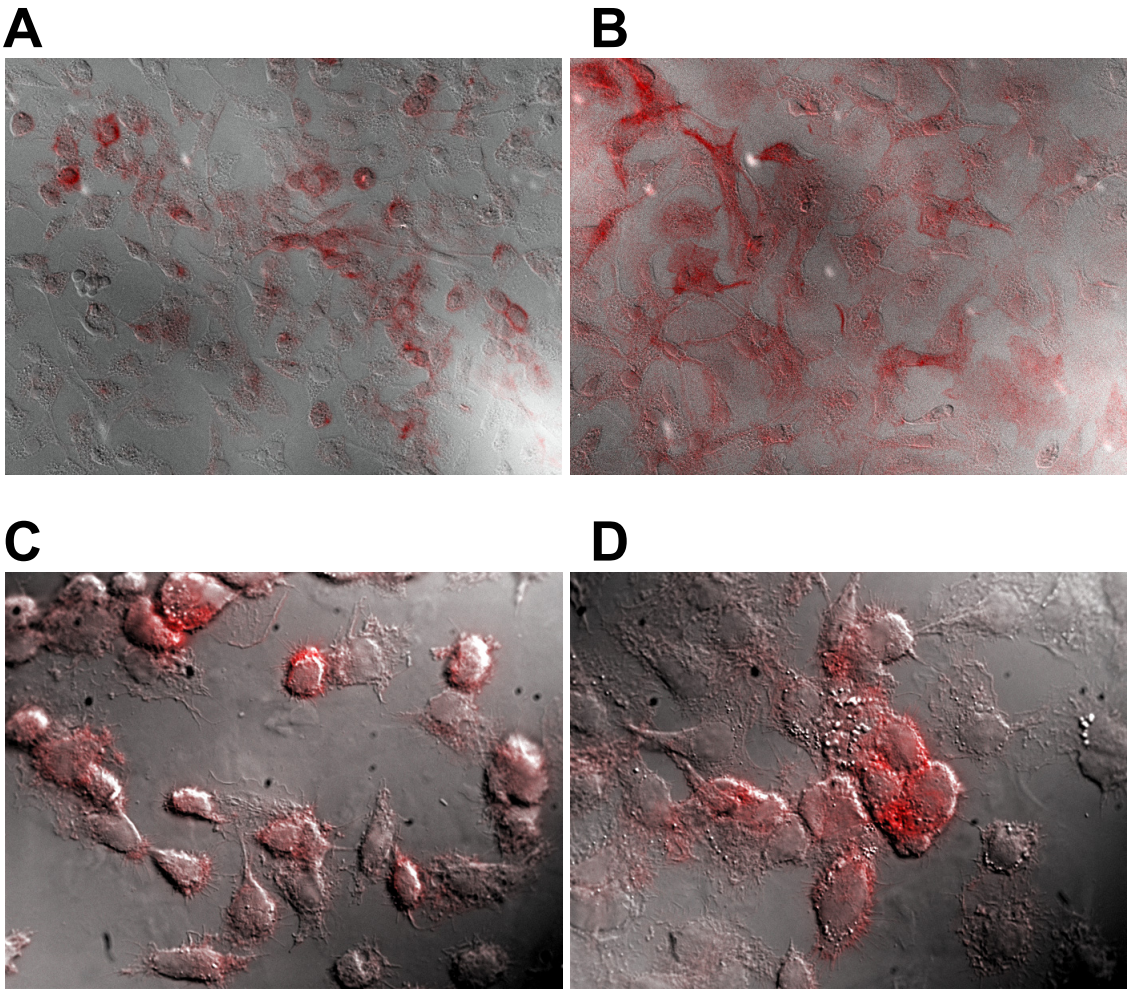


Figure 30: Increased CD11b immunoreactivity in cultured microglial cells triggered by LPS. Microglial cells labeled with CD11b (red) in combination with the respective light transmission image. Microglia cells were treated with LPS (100 ng/ml) or the vehicle (control) during 4 hours. Panels A and C – Control; Panels B and D – LPS. These pictures are representative of 3 independent experiments (A and B – 400x magnification; C and D – 1000x magnification).

In control conditions, the majority of microglia cells presented a ramified morphology with many short processes that extended into lamellipodia (Fig. 30A and C). When microglia was treated with LPS (100 ng/ml, 4h of exposure) most of the microglia cells acquired a large, round, and flat shape, identical to a fried egg (Wollmer et al., 2001) together with an increase of CD11b immunoreactivity (Fig. 30B and D). The change of morphology and up-regulation of CD11b immunoreactivity in microglial cells is associated with an increase of the cell body and cellular activation, when compared to the control (Fig. 30B and D).

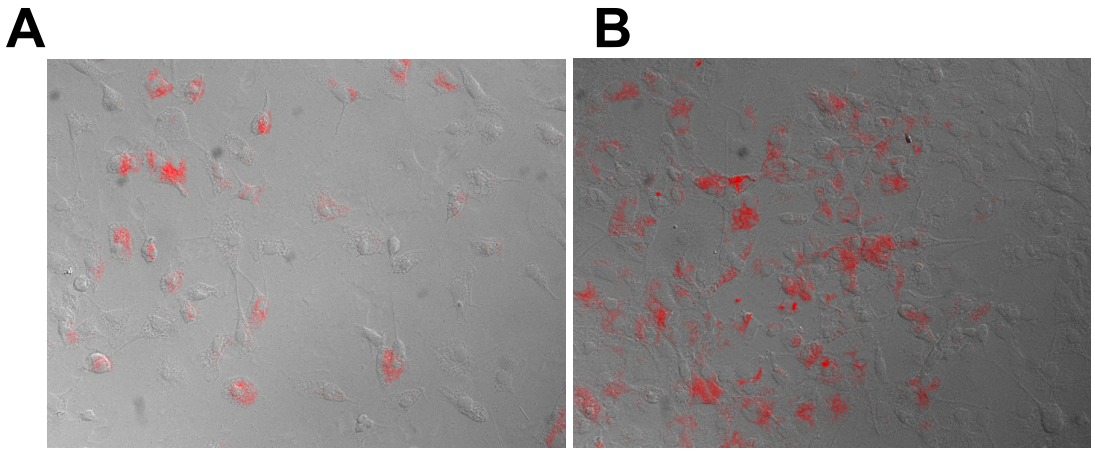


Figure 31: Increased ED1 immunoreactivity in cultured microglial cells triggered by LPS. Microglial cells labeled with ED1 (red) in combination with the respective light transmission image. Microglia cells were treated with LPS (100 ng/ml) or the vehicle (control) during 4 hours. Panel A – Control; Panel B – LPS. These pictures are representative of 3 independent experiments (A and B – 400x magnification).

As occurs with CD11b, there is also an increase of ED1 immunoreactivity in the microglial cells after stimulation with LPS, again consistent with an activation state (Fig. 31).

Until now, it was reported that microglia cells express A_1, A_{2A} and A_3, but not A_{2B} adenosine receptor mRNA (Moreau and Huber, 1999). Wittendorp and colaborators reported $A_{2A}R$ expression, in LPS-activated microglia (Wittendorp et al., 2004). Stimulation of $A_{2A}R$ in microglia cultures results in an activated phenotype (Fiebich et al., 1996; Gebicke-Haerter et al., 1996). However, the $A_{2A}R$ protein density in microglia cultures was never evaluated, so to detect the presence of $A_{2A}R$, it was performed an immunocytochemical approach in LPS-stimulated cultured microglia cells.

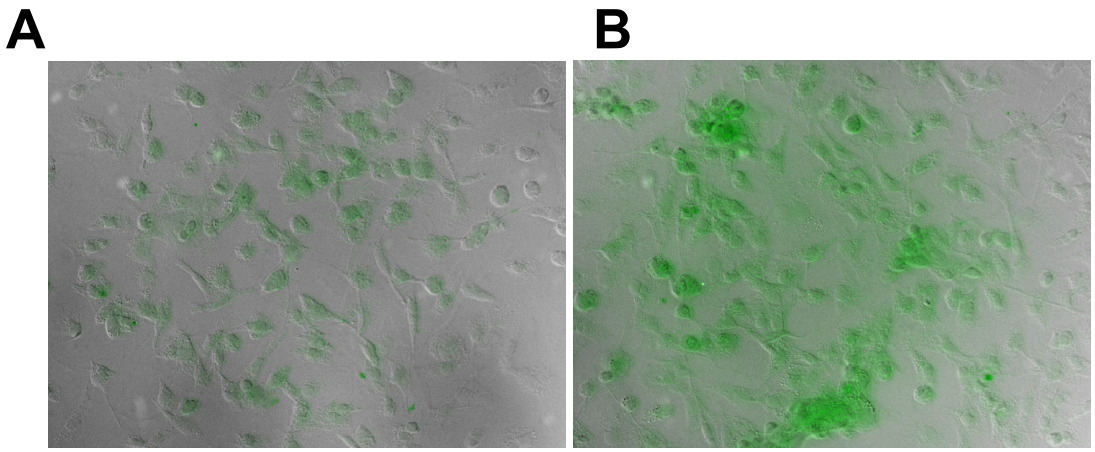


Figure 32: Up-regulation of $A_{2A}R$ immunoreactivity in LPS-activated cultured microglial cells. Microglial cells labeled with $A_{2A}R$ (green) in combination with the respective light transmission image. Microglia cells were treated with LPS (100 ng/ml) or the vehicle (control) during 4 hours. Panel A – Control; Panel B – LPS. These pictures are representative of 3 independent experiments (A and B - 400x magnification).

Figure 32 shows that resting microglia exhibit a weak staining of $A_{2A}R$; in contrast, there is a strong staining of $A_{2A}R$ in LPS-treated microglia cells. This indicates that $A_{2A}R$ are up-regulated in activated microglia and prompts the hypothesis that they might play a role in controlling the function of activated microglia.

4.6.3. LPS-ADMINISTRATION *IN VIVO* MODEL

To study the role of $A_{2A}R$ in an LPS *in vivo* model, a systemic injection of LPS was used. The $A_{2A}R$ antagonist SCH58261 was used as a pharmacologic tool to manipulate $A_{2A}R$ function.

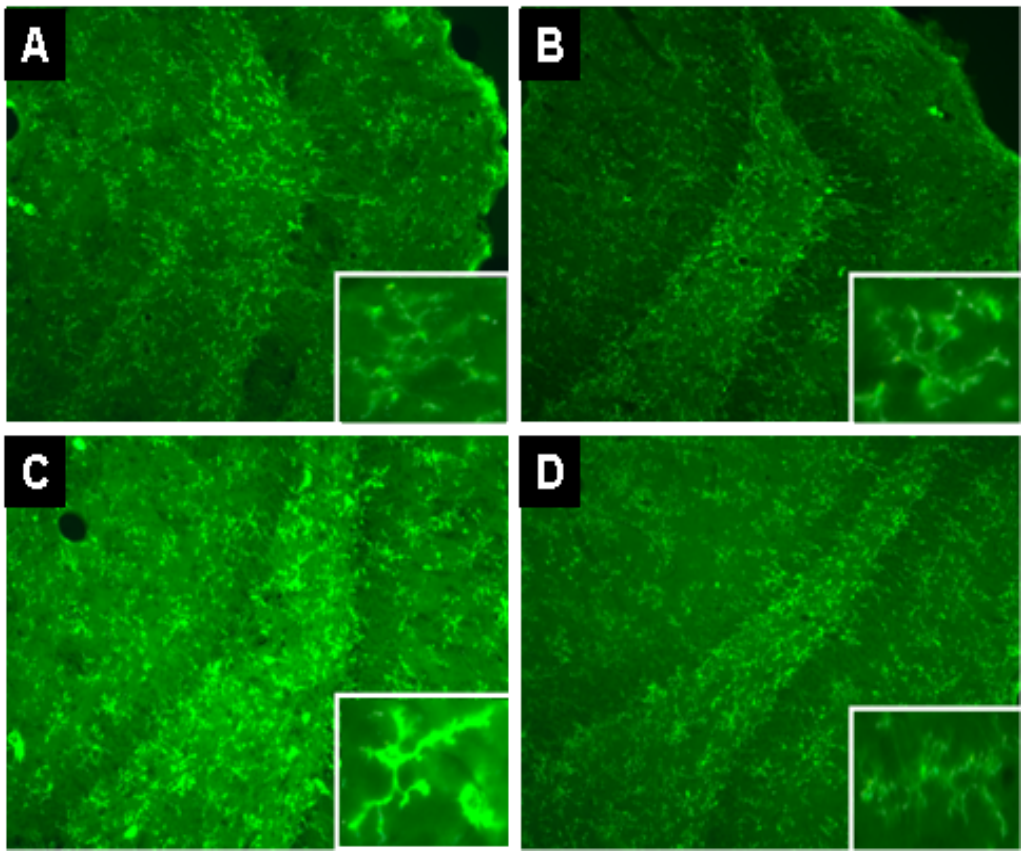


Figure 33: Effect of the admistration of LPS and/or the selective antagonist of $A_{2A}R$, SCH58261 on the appearance of reactive microglia in the rat dentate gyrus. Representative images slices of rat hippocampus dentate gyrus, sacrificed 4 hours after injection of saline solution or LPS injection through a immunocytochemical labeling with a mouse anti-rat CD11b antibody (a monocytyic marker) and a goat anti-mouse secondary antibody labeled with Alexa Fluor 488 (emits green). A – control – injected intracerebroventricular (icv) and intraperitoneally (ip) with vehicle 0.0001% DMSO. B – SCH58261 – $A_{2A}R$ antagonist (icv 5 µl of SCH58261 50 nM solution dissolved in 0.0001% DMSO, ip 0.9% NaCl). C – LPS (icv vehicle, ip LPS 200 µg/kg). D – LPS+SCH58261 (icv 5 µl of SCH58261 50 nM solution, ip LPS 200 µg/kg). n=3-4 animals per experimental groups (400x magnification).

As shown in Figure 33, it can be observed that 4h after LPS injection, microglia cells display an activated morphology (enlarged cell body and thick processes) and an increased immunoreactivity of CD11b when compared to the control (e.g. Kreutzberg, 1996; Nelson et al., 2002). Blockade of $A_{2A}R$, through SCH58261 administration, prevents microglia activation induced by LPS. The antagonist of $A_{2A}R$ is devoided of effects *per se*. These results confirm the role of $A_{2A}R$ in controlling the activation state of microglia cells.

4.6.4. PERFORANT PATHWAY LESION

Another model used to study the role of $A_{2A}R$ in neuroinflammation was the perforant pathway lesion. In this model, CD11b and $A_{2A}R$ mRNA expression levels were investigated through time.

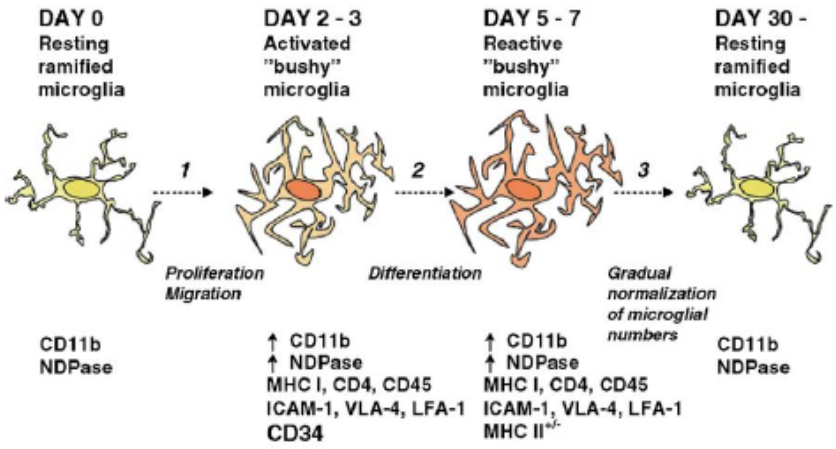


Figure 34: Schematic representation of microglial activation and expression of immunological markers in zones of anterograde axonal and terminal degeneration in the perforant path-denervated dentate gyrus (Ladeby et al., 2005).

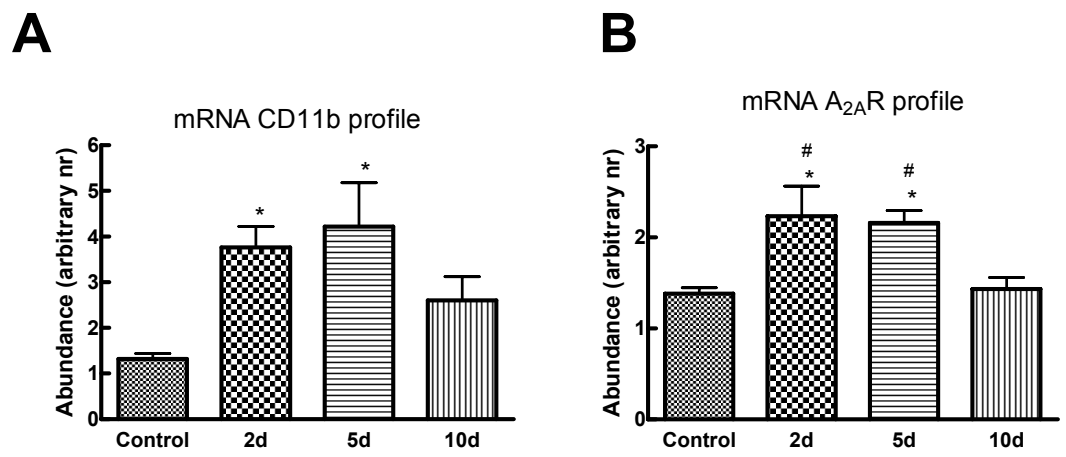


Figure 35: mRNA CD11b and A_{2A}R profiles are similar through time in a model of perforant pathway lesion. CD11b and A_{2A}R mRNA from perforant pathway-lesioned hippocampal samples (2, 5 and 10 days after lesioning) were measured through real time RT-PCR. The data are mean±SEM of n=5-6 animals per group, * P<0.05 *vs* control; # P<0.05 *vs* 10d.

This model of mechanical lesion causes a massive activation of microglia (Jensen et al., 1997). Microglia activation can be measured by increased CD11b mRNA (Fenger et al., 2006). In Figure 35, it can be observed that CD11b mRNA increased by 139±30.7% (* P<0.05 *vs* control, n=6) 2 days after the lesion. This increase is sustained until 5 days (187±65.4%; * P<0.05 *vs* control, n=6) and decreases after 10 days post-lesioning. Interestingly, the variation of A_{2A}R mRNA levels displayed a time course similar to that presented by CD11b. Thus A_{2A}R mRNA increased after 2 days of lesioning by 72.5±26.2% (* P<0.05 *vs* control, n=5); this increase was maintained after 5 days (56.0±9.0%; * P<0.05 *vs* control, n=5), whereas A_{2A}R mRNA levels decreased after 10 days. When comparing CD11b and A_{2A}R mRNA profiles through time, it can be observed that there is an interaction between the 2 genes (F=9.40; P<0.05) and time post-lesioning affects the results (F=44.7; P<0.05). This interaction confirms the possible existence of a relationship between the activation state of microglia cells in this model with A_{2A}R. To confirm the role of A_{2A}R, a pilot study was designed to test the effect of an A_{2A}R antagonist on the activation of microglia following perforant pathway lesioning.

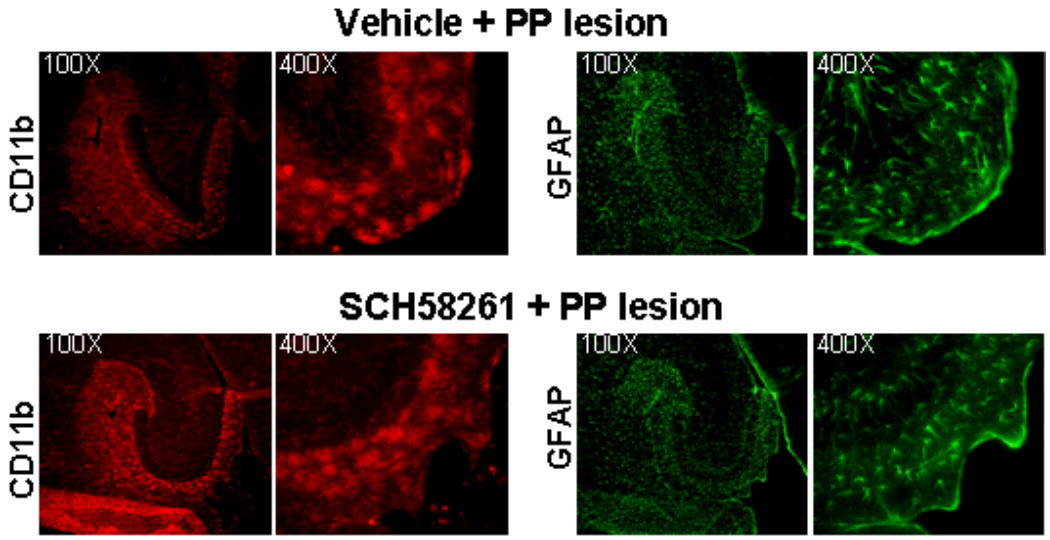


Figure 36: Blockade of $A_{2A}R$ does not prevent up-regulation of CD11b and GFAP immunoreactivity in perforant pathway lesion. Mice were treated either with vehicle (10% DMSO in 0.9% NaCl) or SCH58261 (0.05 mg/kg) 30 minutes before the perforant pathway lesion. SCH58261 was injected intraperitoneally, daily. Representative images of mouse hippocampus dentate gyrus, sacrificed 3 days after perforant pathway lesion, through a immunocytochemical labeling with a rat anti-mouse CD11b antibody (red) as microglial marker and a anti-GFAP (green) as an astrocytic marker. n=2 animals *per* experimental group (100x and 400x magnification).

Figure 36 indicates that 3 days after perforant pathway lesion, microglia cells display an activated morphology characterized by enlarged cell bodies and thicker processes, which confirmed the CD11b mRNA increase observed after 2 days-post lesioning that is sustained up to 5 days. Astrogliosis has also been described in this model, as confirmed in Figure 36 (Rose et al., 1976; Gall et al., 1979; Fagan and Gage, 1994; Jensen et al., 1994; Jensen et al., 1997). Blockade of $A_{2A}R$, through SCH58261 administration, did not prevented microgliosis and astrogliosis. In this model, where massive neuroinflammation occurs, the $A_{2A}R$ blockade was ineffective. A different neuroprotective strategy was attempted, by increasing extracellular adenosine levels through inhibition of adenosine kinase (Gouder et al., 2004). This strategy was beneficial in preventing toxicity in a kainate-induced convulsions model, in a stroke model and in an ischemia model (Kowaluk et al., 1998; Gouder et al., 2004; Pignataro et al., 2007). However, the systemic administration of 5-iodotubercidin causes severe side effects, such as sedation (Ugarkar et al., 2000; Gouder et al., 2004).

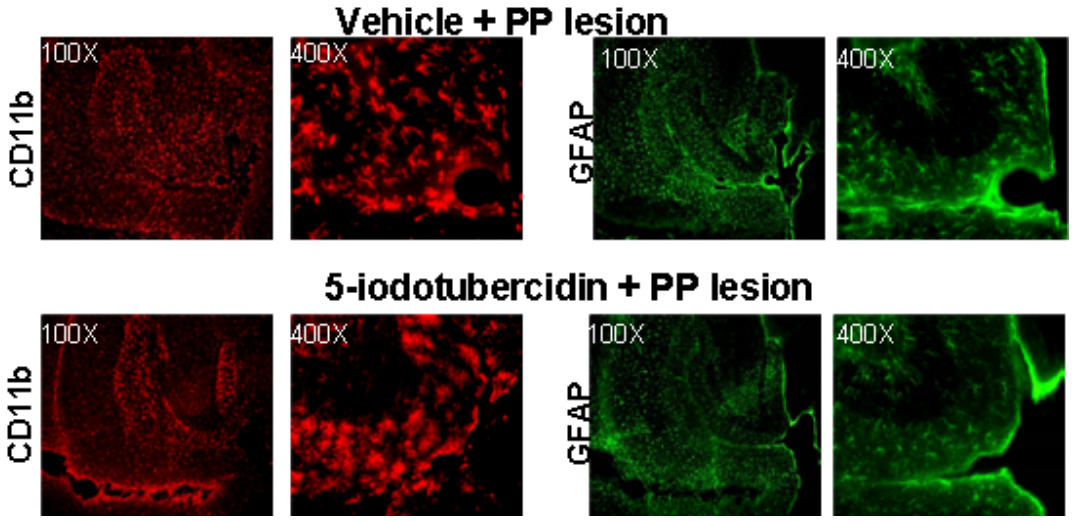


Figure 37: Inhibition of adenosine kinase does not prevent up-regulation of CD11b and GFAP immunoreactivity in perforant pathway lesion. Mice were treated either with vehicle (20% DMSO in 0.9% NaCl) or 5-iodotubercidin (3.1 mg/kg) 30 minutes before the perforant pathway lesion. 5-iodotubercidin was injected intraperitoneally, daily. Representative images of mouse hippocampus dentate gyrus, sacrificed 3 days after perforant pathway lesion, through a immunocytochemical labeling with a rat anti-mouse CD11b antibody (red) as microglial marker and a anti-GFAP (green) as an astrocytic marker. n=2 animals per experimental group (100x and 400x magnification).

As shown in Figure 37, it can be concluded that 3 days post-lesioning microglia activation and astrogliosis occur, as previously observed (Fig. 36). Inhibition of adenosine kinase did not prevent microgliosis and astrogliosis (Fig. 37). In this mechanical model, the increase of extracellular adenosine levels was not effective in preventing neuroinflammation, neither was the blockade of $A_{2A}R$.

4.6.5. KAINATE-INDUCED CONVULSION MODEL

In a model of KA-induced convulsions, neuronal death and microglial activation is observed in CA1, CA3 and hilus region of the hippocampus (Vezzani et al., 1999; Tooyama et al., 2002).

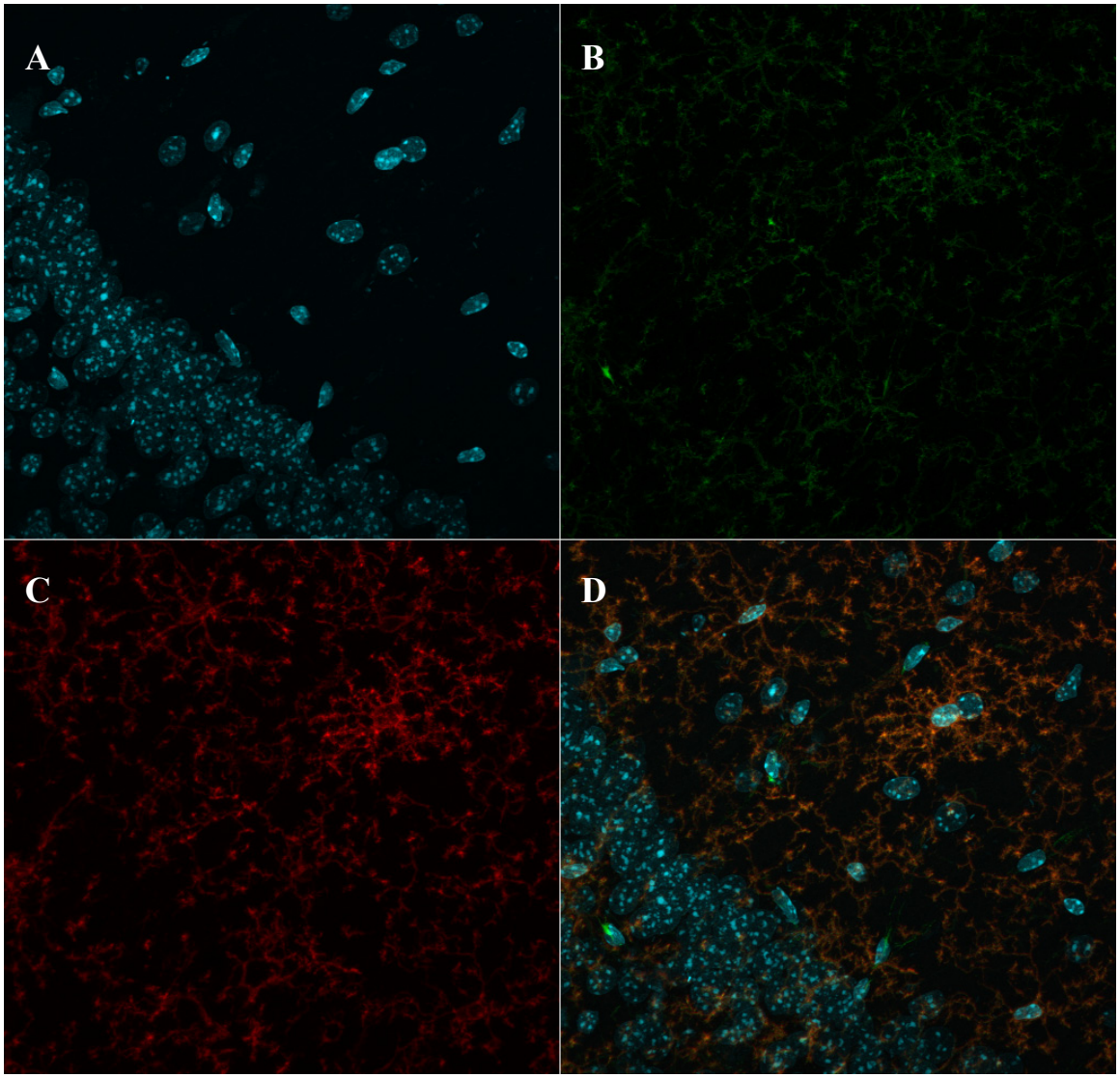


Figure 38: Co-localization of A_{2A}R with microglia cells in hippocampal slices of mice. Representative images of mouse hippocampus CA1 region, sacrificed 24 hours after injection of saline solution through a immunocytochemical labeling with a mouse anti-A_{2A}R antibody (B- **green**), rat anti-mouse CD11b antibody (C- **red**) and nuclei were stained with Hoescht (2 µg/ml) (A- **blue**) and D- merged image. These images are representative of similar results obtained in 3 independent groups of animals.

Figure 38 and 39 show that A_{2A}R are localized in hippocampal microglia cells. Kainate administration caused an up-regulation of this receptor in activated microglia cells (Fig. 39). This is similar to what has been described by Chen's group in the striatal microglial cells of MPTP-treated mice, a model of Parkinson's disease (Yu et al., 2008). In agreement with these results, there is also a preferential expression of A_{2A}R in activated microglia in humans suffering from Alzheimer's disease (Angulo et al., 2003).

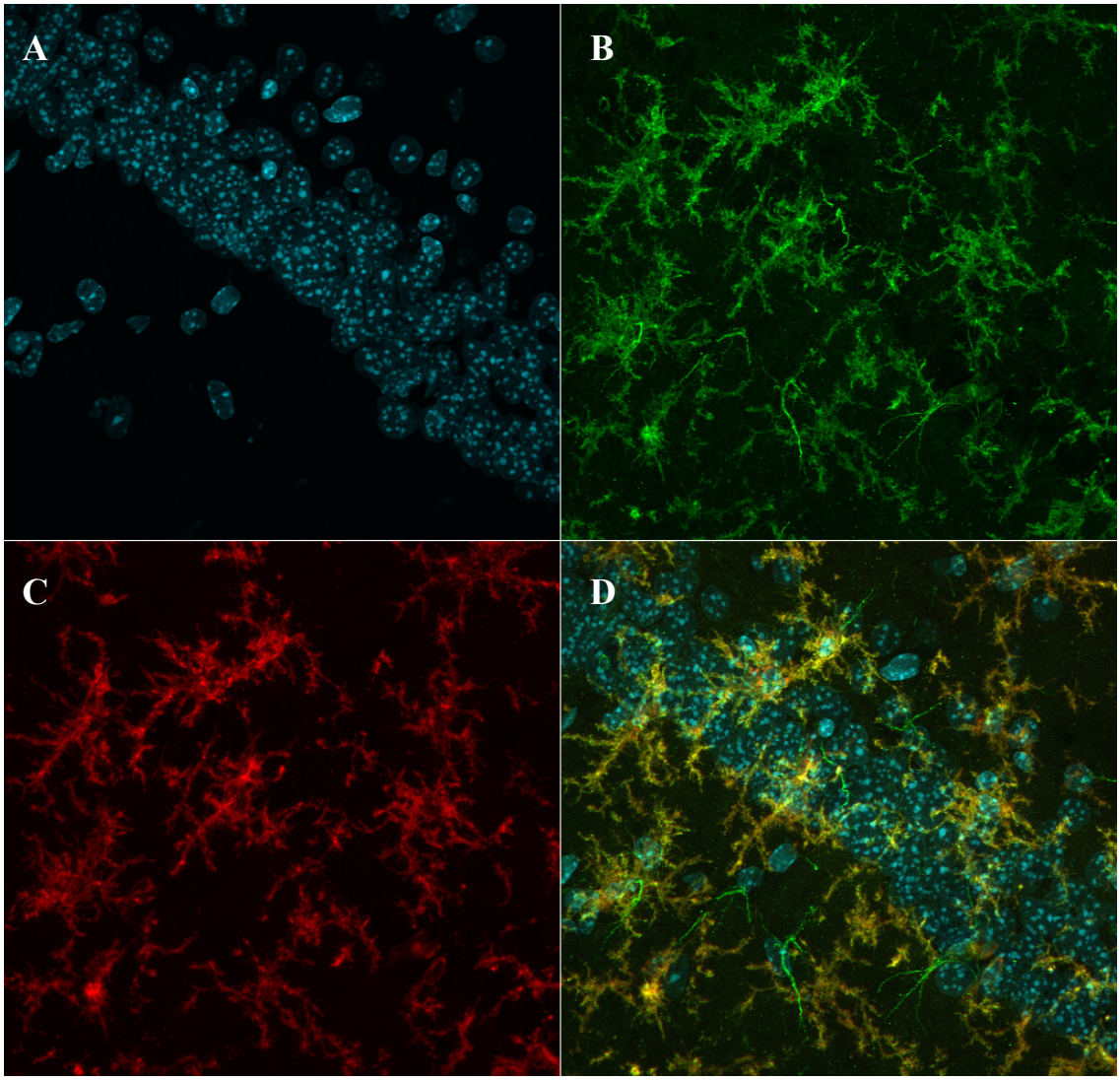


Figure 39: Up-regulation of $A_{2A}R$ in activated microglia cells of hippocampal slices of kainate-treated mice Representative images of mouse hippocampus CA1 region, sacrificed 24 hours after kainate injection subcutaneously (25-35 mg/kg), through a immunocytochemical labeling with a mouse anti-$A_{2A}R$ antibody (B- **green**), rat anti-mouse CD11b antibody (C- **red**) and nuclei were stained with Hoescht (2 µg/ml) (A- **blue**) and D- merged image. The images are representative of similar results obtained in 3 independent groups of animals.

To confirm the role of $A_{2A}R$ in this model of KA-induced neuroinflammation and neurodegeneration, genetic inactivation of $A_{2A}R$ was used as a tentative neuroprotective strategy.

Kainate administration caused neuronal death in CA3 region (Schauwecker and Steward, 1997; Benkovic et al., 2004). In parallel with this putative loss of neurons, the treatment with kainate triggered an apparent astrogliosis and microgliosis, which were also observed in CA3 regions (Fig. 40).

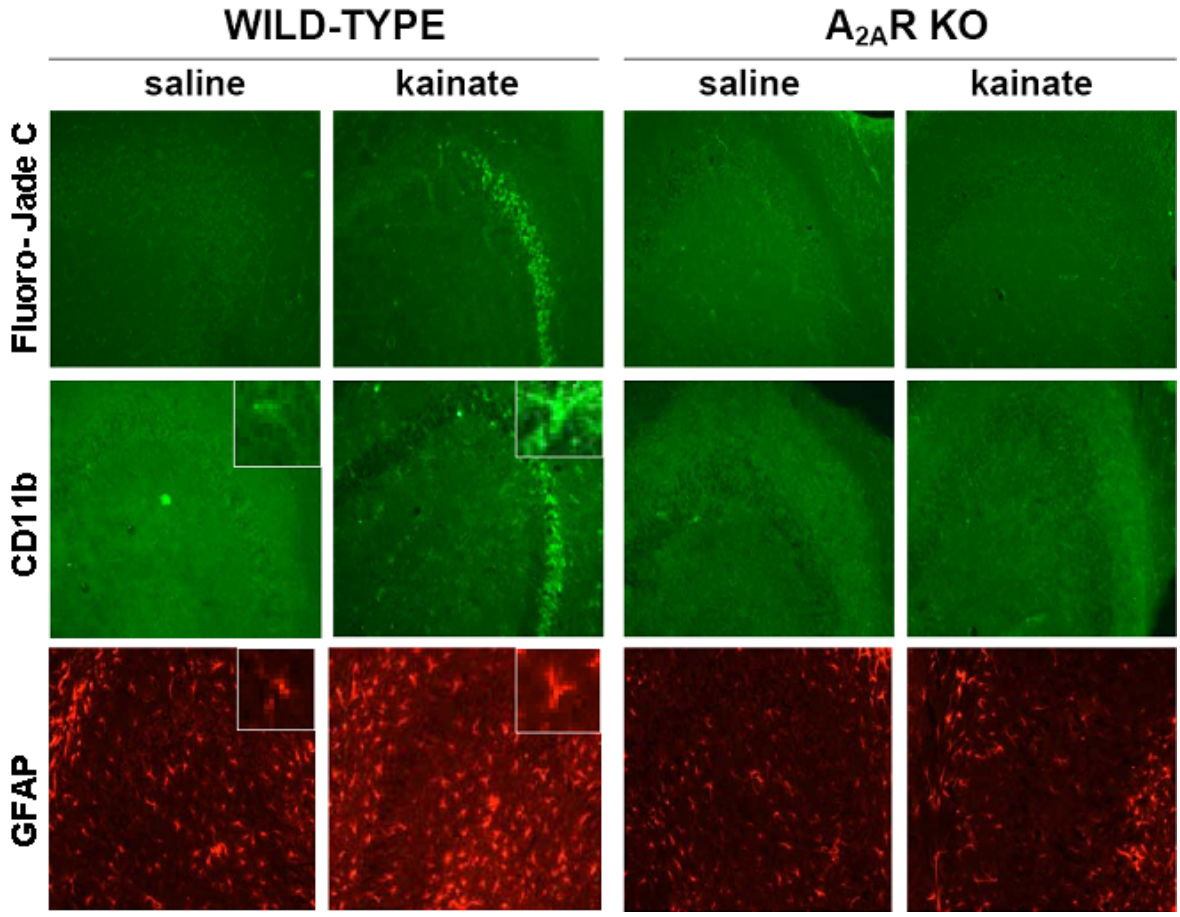


Figure 40: The genetic inactivation of adenosine A_{2A}Rs abrogates hippocampal damage caused by kainate administration. Either wild type of A_{2A}R knockout mice were either injected with saline (control) or kainate (25-35 mg/kg, sc), sacrificed after 24 hours and the brains processed to evaluate neuronal damage (FluoroJade-C staining) or astrogliosis (GFAP immunoreactivity) or microgliosis (CD11b immunoreactivity). The photographs are representative of 5 sets of mice (one wild type and one A_{2A}R knockout mice) processed in parallel.

In fact, at 24 hours post-treatment with kainate, there was an increased number of GFAP-stained profiles (2.5-fold increase in the number of GFAP-stained profiles, n=5) and each labeled astrocytes also displayed a more intense GFAP staining compared to saline controls (Fig. 40). Likewise, there were an increased number of profiles labeled with CD11b and, again, each labeled profile also displayed a more intense CD11b staining compared to saline controls (Fig. 40), in accordance with the expected behavior of activated microglia (Jensen et al., 1997; Kloss et al., 2001). The genetic deletion of A_{2A}R had a profound impact on kainate-induced morphological changes in the hippocampus. In fact, the kainate-induced appearance of FluoroJade-C stained pyramidal cells, astrogliosis or microgliosis were not observed in A_{2A}R knockout mice (Fig. 40). Again, the photographs presented in Figure 40, clearly illustrate this full protection afforded by the genetic deletion of A_{2A}R.

4.6.6. DISCUSSION

The present study provides an insight about one of the possible mechanisms that can explain the neuroprotective role of the blockade of $A_{2A}R$, which is controlling neuroinflammation processes. For that purpose, it was studied the influence of $A_{2A}R$ in microglia cells, which are one of major players in neuroinflammation (Kreutzberg, 1996; Streit et al., 1999). First it was studied if the $A_{2A}R$ protein was present in cultured microglia cells, since only the expression of $A_{2A}R$ mRNA had been reported to occur in microglial cells (Moreau and Huber, 1999) and in LPS-activated microglial cells (Wittendorp et al., 2004). Cultured microglia, in which the presence of $A_{2A}R$ was tested, was previously exposed to LPS (4 hours). These microglia cells became activated, as assessed by an up-regulation of the two activation markers CD11b and ED1. Similarly to the results by Wittendorp and collaborators, there was an up-regulation of $A_{2A}R$ immunoreactivity staining in LPS-activated cells (Wittendorp et al., 2004). This provides the first demonstration of an increase of $A_{2A}R$ protein density in microglial cultures upon LPS-stimulation.

Using an LPS systemic injection model, it was studied if the blockade of $A_{2A}R$ was neuroprotective in a classical model of neuroinflammation (e.g. Gehrmann et al., 1995; Kreutzberg, 1996; Tomas-Camardiel et al., 2004; Qin et al., 2007). An $A_{2A}R$ antagonist (SCH58261) attenuated the LPS-induced activated morphology and CD11b immunoreactivity increased in microglia cells. Thus, in the LPS-model, $A_{2A}R$ controlled the activation state of microglial cells. Similar to what was reported for that model, blockade of $A_{2A}R$ (either by a selective antagonist $A_{2A}R$ or by genetic inactivation) conferred neuroprotection in a Parkinson's disease model (MPTP administration), where nigral and striatal dopaminergic toxicity occurs (Chen et al., 2001). It was also reported by Chen's group that the $A_{2A}R$ in brain cells other than forebrain neurons (likely glial cells) are the most important components for protection against acute MPTP toxicity (Yu et al., 2008). Intracerebroventricular administration of KW6002 into the forebrain of $A_{2A}R$ knock-out mice reinstated protection against acute MPTP-induced dopaminergic neurotoxicity and attenuated MPTP-induced striatal microglial and astroglial activation (Yu et al., 2008).

The perforant pathway lesion was another model chosen to study the role of $A_{2A}R$ in the control of neuroinflammation. This mechanical model is suitable for the study of microglia population dynamics (Ladeby et al., 2005). In this model, CD11b and $A_{2A}R$ exhibit the same

pattern of modification of mRNA levels through time, strongly suggesting an interaction between $A_{2A}R$ and activated microglia cells. It was found an increase of mRNA of both genes 2 days after lesioning, sustained until 5 days post-lesioning, with a decrease after 10 days the perforant pathway lesion. To confirm the interaction between the 2 genes, the blockade of $A_{2A}R$ was used as a neuroprotective strategy. However, SCH58261 did not prevent microgliosis or astrogliosis induced by perforant pathway lesion. It can be concluded that, in a model with massive neuroinflammation processes associated with a profound degeneration of nerve terminals, $A_{2A}R$ blockade does not confer neuroprotection. Another neuroprotective strategy reported in neurodegenerative conditions, such as in a kainate induced-convulsions and in an ischemia model (see review Boison, 2008), was increasing extracellular levels of adenosine, by inhibiting adenosine kinase. It was tested if this neuroprotective strategy was effective in this neuroinflammation model. However, the inhibition of adenosine kinase did not prevent microgliosis or astrogliosis induced by perforant pathway lesion. The 2 strategies were ineffective in this model. The absence of protection can be explained by the severe microglial activation that occurs in the perforant pathway model. Maybe a conjugation of both neuroprotective strategies would be adequate to refrain the neuroinflammation process.

In the kainate-induced convulsion model, the genetic deletion of $A_{2A}Rs$ also abrogated the ability of kainate to trigger neuronal damage (Schwob et al., 1980; Sperk et al., 1983), astrogliosis (Khurgel and Ivy, 1996) or microgliosis (Vezzani et al., 1999). This ability of inactivation of $A_{2A}Rs$ to simultaneously suppress all 3 readouts of kainate toxicity in mice provides strong evidence that the tonic activation of $A_{2A}Rs$ by endogenous adenosine is a crucial step in the expression of toxic features following kainate-induced convulsions. Particular mechanisms by which $A_{2A}Rs$ would control kainate-induced brain damage could be controlling neuroinflammation (since there is an up-regulation in activated microglia), or controlling glutamate outflow from cortical tissue under noxious conditions (Lopes et al., 2002; Dall'Igna et al., 2003) and/or controlling NMDA responses in cortical tissue (Tebano et al., 2005; Rebola et al., 2008).

In these studies it was monitored the role of $A_{2A}R$ in controlling activated microglial cells; however, it can not be ruled out the involvement of other participants in the development of neuroinflamatory processes, such as astrocytes (e.g. Hanisch, 2002), neurons (e.g. De Simone et al., 2004), as well as the infiltrating myeloid cells (see Jensen et al., 1997; Akiyama et al., 2000; Lyons et al., 2000), all of which possess $A_{2A}R$. In fact, it has been reported that there is an

infiltration of polymorphonuclear leukocytes (Bolton and Perry, 1998; Benkovic et al., 2004) in brain tissue in animal models of epilepsy, upon partial disruption of the blood brain barrier (Zucker et al., 1983; Bolton and Perry, 1998). Chen's group also reported that in a model of focal ischemia, brain resident $A_{2A}R$ play a minor role in controlling neurodegeneration, whereas the neuroprotection seem to result from the blockade of $A_{2A}R$ located in infiltrating bone marrow-derived cells (Yu et al., 2004).

As a main conclusion, this chapter provides further information supporting a possible mechanism by which $A_{2A}R$ confers neuroprotection. $A_{2A}R$ can control neuroinflammation, thus contributing to the control of neurodegeneration. However, it does not rule out the possibility that other mechanisms may be involved at the same time, underlying the neuroprotection achieved by $A_{2A}R$ blockade.

5. DISCUSSION

The main goal of this thesis was to study the role of $A_{2A}R$ in aging, and in a model of Alzheimer's disease, where mnemonic deficit and synaptotoxicity occur, and which mechanisms underlie the neuroprotection upon blockade of $A_{2A}R$.

Since modulatory systems can change with aging, it was first studied how the $A_{2A}R/A_1R$ density changed from the young adult animal to an aged animal. In aging, there is an increase of $A_{2A}R$ density and a decrease of A_1R. This imbalance favoring facilitatory *versus* inhibitory system may represent an attempt to counteract the age-induced reduction in neurotransmitter release (e.g. Giovannelli et al., 1988). Similarly, chronic noxious stimulus triggers an up-regulation of $A_{2A}Rs$, alongside a down-regulation of A_1Rs that contributes to hamper the neuroprotective effectiveness of the A_1R system in chronic noxious brain conditions (Cunha, 2005). The previous results reinforce the idea that the blockade of $A_{2A}R$ is an important strategy to consider for the treatment of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease, since these are prevalent in elderly.

In a retrospective study, caffeine consumption decreased the incidence of Alzheimer's disease in humans (Maia and de Mendonça, 2002). To provide a mechanistic insight for this observation, it was developed an animal model of AD based in the administration of $A\beta_{1-42,}$ a possible causative factor of the disease. In this model, mnemonic deficit and synaptototoxicity were detected, therefore providing the necessary end-points to study of the role of $A_{2A}R$ antagonists. It was found that synaptic $A_{2A}Rs$ have a pathophysiological role in this model, which was clearly demonstrated by abrogation of $A_{2A}Rs$ either by pharmacological blockade or genetic inactivation. Likewise, synaptic $A_{2A}Rs$ prevent synaptotoxicity in other neurodegenerative conditions such as restraint stress model (Cunha et al., 2006) and staurosporine model (Silva et al., 2007). The attempt to unravel the mechanisms underlying this neuroprotection revealed that this neuroprotection is mediated through p38 MAPK signaling pathway and is independent of cAMP/PKA pathway.

Moreover, it was tested whether $A_{2A}R$ blockade was a general mechanism for prevention of different cognitive models. This involved the use of 2 models where an acute and transient mnemonic deficit in the animals has been described, without occurrence of synaptotoxicity. $A_{2A}R$ antagonists did not prevent an acute mnemonic deficit. This leads to the conclusion that, while $A_{2A}Rs$ do not affect general processes of memory impairment, they play a significant role restricted to neurodegenerative conditions where synaptic deterioration is implicated.

Until this moment, several hypotheses were raised to explain $A_{2A}R$ blockade as a neuroprotective strategy. $A_{2A}R$ could control glutamate excitotoxicity, which might trigger neuronal dysfunction and could also control neuroinflammation (Cunha, 2005). It was decided to investigate one of the proposed mechanisms to explain $A_{2A}R$ protective role in stressful situations: control of neuroinflammation. For that purpose, different neuroinflammation models were used. It was concluded that there is an up-regulation of $A_{2A}R$ in LPS stimulated cultured microglia cells, similarly to what had been described by Wittendorp and collaborators (Wittendorp et al., 2004) and by Angulo and collaborators, whereas $A_{2A}R$ are also present in human activated microglia in the hippocampus and cortex of AD patients (Angulo et al., 2003). Blockade of $A_{2A}R$ controlled microglia activation in an *in vivo* LPS-injected model. These evidences suggest an $A_{2A}R$ role in hampering neuroinflammation. In a KA-induced convulsions model, blockade of $A_{2A}R$ prevented neuronal loss, microgliosis and astrogliosis. In this model, and for other neurodegenerative conditions where neuronal death and associated neuroinflammation occur, it cannot rule out the participation of synaptic $A_{2A}R$, $A_{2A}R$ from glial cells and $A_{2A}R$ from peripheral immune cells. Synaptic $A_{2A}R$ can control glutamate release in cortical regions (Lopes et al., 2002), which in turn can trigger neuronal dysfunction of brain circuits and metabolic imbalance (Cunha et al., 2008). Glial $A_{2A}R$ can control the main system of toxicity amplification that is neuroinflammation (Block and Hong, 2005; Klegeris et al., 2007; Rogers et al., 2007; Yu et al., 2008; Cunha et al., 2008). In models where blood-brain barrier breakdown takes place, inflammatory cells with $A_{2A}R$ can invade the brain parenchyma and play a more pronounced role (Yu et al., 2004; Cunha et al., 2008). The contribution of $A_{2A}R$ from different cell types might depend on the type and extent of brain insult.

In conclusion, albeit there is a low extra-striatal $A_{2A}R$ density, there is an increase in $A_{2A}R$ density upon chronic insults, which leads to a gain in $A_{2A}R$ function that explains successful strategy of blocking these receptors in neurodegenerative conditions. The neuroprotection attained by $A_{2A}R$ blockade may involve mechanisms different from the ones discussed, and more than one at the same time, regarding diverse neurodegenerative disorders. $A_{2A}R$ is a possible molecular target and $A_{2A}R$ antagonists could be promising candidates for Alzheimer's disease clinical trials, as has been described for Parkinson's disease.

6. FUTURE PROSPECTS

Sub-chapter 4.1 – Supplementary studies will be necessary to confirm if the blockade of $A_{2A}R$ is an effective neuroprotective strategy in an AD model, in which studies should be performed in aged animals to better mimic neurodegenerative conditions.

Sub-chapter 4.2 – It is possible to repeat the study using the forebrain knockout for $A_{2A}Rs$ or a viral-induced deletion of $A_{2A}R$ in different regions of the brain, to understand which cell types containing $A_{2A}R$ fulfill a role in achieving neuroprotection. Further studies are essential to comprehend the role of $A_{2A}R$ upon $A\beta_{1-42}$-impaired long term potentiation, a model for memory at the cellular/synaptic level (Lynch, 2004), and in general to evaluate $A_{2A}R$ participation in memory formation.

Sub-chapter 4.3 – Complementary studies are necessary to understand how $A_{2A}R$ can prevent an asymmetric loss of nerve terminals in Aβ-model or transgenic AD animal models, i.e. whether the surviving new terminals are the ones with the lowest density of $A_{2A}Rs$.

Sub-chapter 4.4 – Currently it is not known the role of $A_{2A}R$ in mitochondrial trafficking. Additional studies are required to understand their role, first in a physiological situation, and afterwards in pathological situations.

Sub-chapter 4.5 – The mechanism by which hyperlocomotion is prevented by the blockade of $A_{2A}R$ is at present unknown. To answer to this question it is possible to perform a study focused in striatum, evaluating the glutamatergic and dopaminergic systems. These systems have been described to be controlled by adenosine (Svenningsson et al., 1999).

Sub-chapter 4.6 – Supplementary studies are required to comprehend by which mechanisms $A_{2A}Rs$ control microglia activation in microglia culture, and confirm the data later on with *in vivo* studies. For the perforant pathway lesion, it is possible to use two neuroprotective strategies at the same time: blockade of $A_{2A}R$ and increase of adenosine levels. Furthermore, it is possible to repeat the *in vivo* studies using microglial $A_{2A}R$ KO mice (transgenic mice with targeted deletion of $A_{2A}R$ in myeloid-lineage cells – monocytes and microglial cells) generated by collaboration between Chen's and Linden's groups.

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