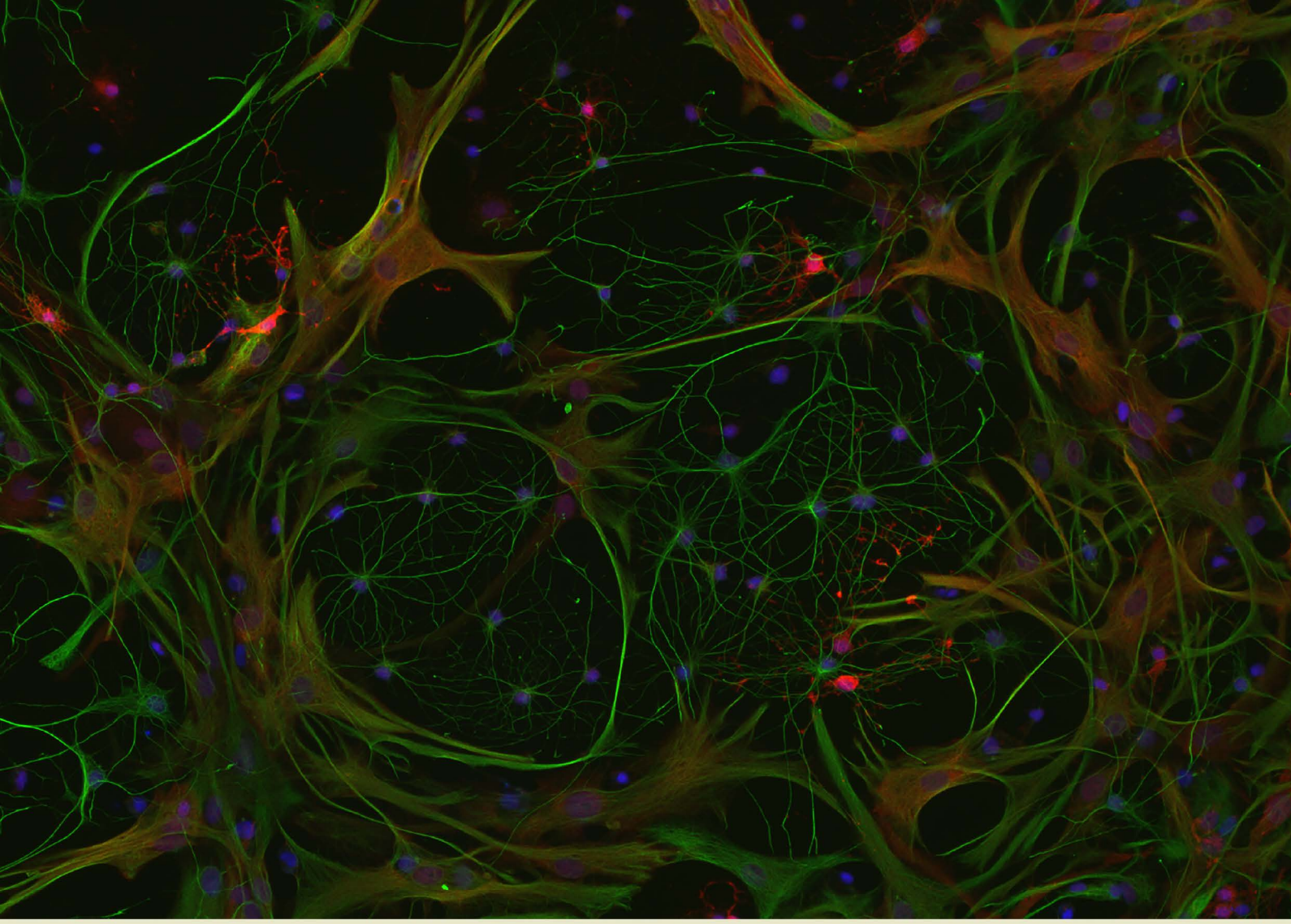

Daniela Isabel Ferreira Madeira

ATP AND GLUTAMATE RELEASE BY ASTROCYTES UNDER ALZHEIMER'S DISEASE CONDITIONS: MODULATION BY ADENOSINE A2A RECEPTORS

Dissertação de Mestrado em Investigação Biomédica.

Julho 2017

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UNIVERSIDADE DE COIMBRA

ATP and glutamate release by astrocytes under Alzheimer´s disease conditions: modulation by adenosine A_{2A} receptors

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica, realizado sob orientação da Doutora Paula Maria Garcia Agostinho (Faculdade de Medicina da Universidade de Coimbra).

Daniela Isabel Ferreira Madeira

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Front cover: Immunocytochemical analysis of the astrocytic markers glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS) in astroglial cells. Primary cultures of astrocytes obtained from the cortex of Wistar pups were labelled with antibodies against GFAP (green) or GS (red), and stained with the cell nuclei dye DAPI (blue). – Fluorescent microscopy, magnification 20x.

The present work was performed in the Purines group, at the Center of Neuroscience and Cell Biology, University of Coimbra, under scientific guidance of Doctor Paula Agostinho.

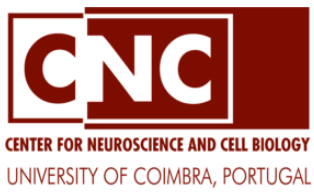

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

A_1R – Adenosine A_1 receptors

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

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

A_3R – Adenosine A_3 receptors

Aβ – Amyloid-beta

AD – Alzheimer's disease

ADK – Adenosine kinase

ADO – Adenosine

Aldh1L1 – Aldehyde dehydrogenase 1 family member L1

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APP – Amyloid precursor protein

APS – Ammonium persulphate

ATP – Adenosine triphosphate

BACE – β-secretase

BBB – Brain-Blood Barrier

BBG – Brilliant Blue G

BSA – Bovine Serum Albumin

Ca^{2+} – Calcium

cAMP – cyclic adenosine monophosphate

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

CBX – Carbenoxolone

CD11b – Cluster of differentiation 11b

CD73 – ecto-5'-nucleotidase

CHPG – *(RS)*-2-Chloro-5-hydroxyphenylglycine

CLAP – Cocktail of proteases inhibitors

CNS – Central Nervous System

Cx – Connexin

DCFS – Divalent cation free solution

DAPI – 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

DTT – Dithiothreitol

EAAT 1/2 – Excitatory amino acid transporter type 1/2

ECL – Enhanced chemiluminescence

EDTA – 2,2',2",2"'-(Ethane-1,2-diyldinitrilo) tetraacetic acid

FBS – Foetal bovine serum

FRET – Fluorescent resonance energy transfer

GFAP – Glial fibrillary acidic protein

GJC – Gap junction channels

GLAST – Glutamate-aspartate transporter

Gln – Glutamine

GLT-1 – Glutamate transporter-1

GS – Glutamine synthetase

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

K^+ – Potassium

KO – Knockout

HC – Hemichannels

IL-1β – Interleukin-1 beta

LTP – Long term potentiation

LTD – Long term depression

MAPK – Microtubule-associated protein kinases

mGluR – Metabotropic glutamate receptors

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Na^+ – Sodium

NE – Norepinephrine

NMDA – N-methyl-D-aspartate

IP_3 – Inositol triphosphate

Panx – Pannexins

PBS – Phosphate Buffered Saline

PFA – Paraformaldehyde

PI-3K – Phosphoinositide 3-kinase

PKA A/C – Protein Kinase type A/C

PLA – Proximiti Ligation Assay

PLC – Phospholipase C

PMSF – Phenylmethylsulfonyl fluoride

PS 1/2 – Presenilin 1 / 2

RPM – Rotations per Minute

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

SDS – Sodium dodecyl sulfate

SEM – Standard error of the mean

SNARE – Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

TCA – tricarboxylic acid cycle

TEMED - N, N, N′, N′-Tetramethylethylenediamine

VAMP – Vesicle associated membrane proteins

vGLUT – Vesicular glutamate transporter

vNUT – Vesicular glutamate transporter

VRAC – volume-regulated anion channels

Resumo

A doença de Alzheimer (DA) é a maior causa de demência nos idosos. Os primeiros sinais desta doença envolvem a perda de memória, que se pensa ser desencadeada pela acumulação cortical de peptídeos β-amiloide (Aβ) e, consequente, disfunção e perda sináptica. Atualmente, considera-se que os astrócitos, as células mais abundantes no cérebro, são o terceiro elemento da sinapse, tendo um papel fundamental no controlo da excitabilidade neuronal, especialmente na modulação da plasticidade sináptica, que constitui a base neurofisiológica da memória. Contudo, embora a reatividade dos astrócitos tenha sido descrita nas fases iniciais da DA, ainda permanece por esclarecer se a modificação da função dos astrócitos afeta os eventos iniciais e a progressão de DA. Os astrócitos modulam a função sináptica pela captação de neurotransmissores, como o glutamato e o GABA, da fenda sináptica e também pela libertação de gliotransmissores, principalmente glutamato e ATP. Deste modo, alterações na gliotransmissão podem contribuir para a disfunção sináptica e subsequente neurodegeneração. No entanto, ainda permanece por estabelecer a forma pela qual a libertação de ATP e glutamato pelos astrócitos é afetada em condições de DA e os mecanismos subjacentes a esta libertação. Além disso, estudos prévios do nosso grupo mostram que os astrócitos contêm recetores de adenosina A_{2A} ($A_{2A}R$), os quais têm sido implicados na patogénese da DA, uma vez que o bloqueio destes recetores para além de prevenir a sinaptotoxicidade, também previne a reatividade e disfunção dos astrócitos desencadeada por peptídeos Aβ.

No presente estudo, investigamos: i) como condições de DA afetam a libertação de ATP e glutamato, ii) os possíveis mecanismos envolvidos na libertação destes gliotransmissores, e iii) o papel dos $A_{2A}R$ no controlo da libertação de ATP e glutamato. Para atingir estes objetivos, usámos culturas primárias de astrócitos obtidas a partir do córtex de cérebros de ratos Wistar expostas agudamente (1 hora) ou cronicamente (24 horas) a peptídeos $A\beta_{1-42}$ (1μM). Os níveis de ATP e glutamato libertados no meio extracelular foram quantificados usando ensaios de bioluminescência e de colorimetria, respetivamente. Para determinar o papel dos hemicanais (HC) e dos recetores P_2X_7 na libertação destes gliotransmissores, em condições não patológicas e do tipo-DA, recorremos ao bloqueio farmacológico destes mecanismos com o inibidor carbenoxolone (CBX) e o antagonista Brilliant Blue G (BBG), respetivamente. A

potencial participação dos $A_{2A}R$ na modulação da libertação de ATP e de glutamato pelos astrócitos foi avaliada usando o antagonista seletivo destes recetores, SCH 58261.

Neste estudo observámos um aumento na libertação de ambos os gliotransmissores estudados, ATP e glutamato, por astrócitos expostos a peptídeos $A\beta_{1-42}$. Além disso, o ATP, mas não o glutamato, parece ser libertado principalmente por HC e parcialmente por recetores P_2X_7 em condições de DA. Demonstrámos também que a libertação de ATP pelos astrócitos era modulada pelos $A_{2A}R$, porém estes recetores não regulavam a libertação de glutamato nestas células. Adicionalmente, mostrámos que exposição a peptídeos $A\beta_{1-42}$ (24 horas) aumentou os níveis de conexina 43 (Cx43), que é a principal proteína constituinte dos HC nos astrócitos, e este efeito foi prevenido pelo bloqueio de $A_{2A}R$, sugerindo uma modulação dos Cx43 HC pelos $A_{2A}R$. Esta modulação foi validada pela observação de uma proximidade física entre os $A_{2A}R$ e a proteína Cx43, através de uma metodologia designada em inglês como "Proximity ligand assay (PLA)"

No seu todo, os nossos resultados mostram que os peptídeos $A\beta_{1-42}$ aumentam a libertação de ATP e de glutamato, e que a libertação destes gliotransmissores ocorre por mecanismos distintos. A libertação de ATP em condições de AD ocorreu principalmente através de HC e é modulada por $A_{2A}R$, sendo que esta modulação talvez ocorra devido aos $A_{2A}R$ estarem associados fisicamente às Cx43. Estas descobertas podem contribuir para elucidar o impacto dos astrócitos na patogénese de DA e abrir caminho para o desenvolvimento de novas estratégias terapêuticas contra esta doença neurodegenerativa.

Palavras-Chave: Doença de Alzheimer; Astrócitos; ATP; Glutamato; Recetores de adenosina A_{2A}

Abstract

Alzheimer's disease (AD) is the leading cause of dementia among the elderly. The first signs of this disease involve memory loss, which begins with cortical accumulation of amyloid-β peptides (Aβ) that is thought to underlie synaptic dysfunction and loss. Currently, it is considered that astrocytes, the most abundant cells in the brain, are the third element of synapse, having a key role in control of neuronal excitability, especially in shaping synaptic plasticity, the neurophysiological basis of memory. However, albeit reactivity of astrocytes has been described in early AD, it remains to be clarified if the modification of astrocytic function affects the onset and progression of AD. Astrocytes modulate the synaptic function by the uptake of neurotransmitters, glutamate and GABA from synaptic cleft, and also by the release of gliotransmitters, such as glutamate and ATP. Thus, alterations in gliotransmission may contribute to synaptic dysfunction and subsequent neurodegeneration. However, it remains to be established how astrocytic ATP and glutamate release are affected in AD conditions, and the mechanisms underlying their release. Furthermore, previous studies from our group showed that astrocytes have adenosine A_{2A} receptors ($A_{2A}R$), which have also been implicated in AD pathogenesis, since the blockade of these receptors prevents not only synaptotoxicity but also astrocyte reactivity and dysfunction triggered by Aβ peptides.

In this study, we aim to investigate: i) how AD conditions affect the release of ATP and glutamate by astrocytes, ii) the putative mechanisms involved in the release of these gliotransmitters, and iii) the role of astrocytic $A_{2A}R$ in controlling ATP and glutamate release**.** To achieve these goals, we used primary cultures of astrocytes from cortical brain of Wistar rats that were exposed acutely (1 hour) or chronically (24 hours) to $A\beta_{1-42}$ peptides (1μM). The levels of ATP and glutamate released in extracellular medium were quantified using bioluminescence and colorimetric assays, respectively. To determine the role of hemichannels (HC) and P_2X_7 receptors in gliotransmitters release, in non-pathological and in AD-like conditions, we resort to the pharmacological blockade of these pathways by the inhibitor carbenoxolone (CBX) and by the antagonist Brilliant Blue G (BBG), respectively. The potential participation of $A_{2A}R$ in the modulation of astrocytic ATP and glutamate release was evaluated using the selective antagonist of $A_{2A}R$, SCH 58261.

We observed an increase in both gliotransmitters release, ATP and glutamate, in astrocytes exposed to $A\beta_{1-42}$ peptide. Moreover, ATP but not glutamate, seems to be released mainly through HC and partially by P_2X_7 receptors in AD-like conditions. In addition, we also demonstrated a modulation of astrocytic ATP, but not of glutamate, release by $A_{2A}R$. Furthermore, we showed that $A\beta_{1-42}$ peptide (24 h exposure) increased the levels of connexin 43 (Cx43), which is the main component of HC in astrocytes, and that this effect was prevented by $A_{2A}R$ blockade, suggesting a modulation of Cx43 HC by $A_{2A}R$. This modulation was further validated by the finding of a close proximity between the $A_{2A}R$ and the Cx43 protein, using the proximity ligand assay (PLA).

Taken together, our results show that Aβ peptides increased astrocytic ATP and glutamate release, and that the release of these gliotransmitters occurred by distinct mechanisms. The release of ATP in AD-like conditions occurred mainly through HC, and is modulated by $A_{2A}R$, probably due to a physical interaction of this receptor with Cx43. These findings may contribute to elucidate the impact of astrocytes on AD pathogenesis, and pave the way to develop novel therapeutic strategies against this neurodegenerative disorder.

Keywords: Alzheimer's disease; Astrocytes; ATP; Glutamate; Adenosine A_{2A} receptors

CHAPTER 1| INTRODUCTION

1.1. Alzheimer´s Disease (AD)

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a progressive memory decline and is the most common form of dementia in the elderly (Selkoe, 2001), being estimated to affect over 47 million people worldwide (Rojas-gutierrez et al., 2017). AD, similarly to other neurodegenerative disorders, implicates deterioration of memory related areas, in fact the cognitive deficits characteristic of this pathology seem to be related with extensive damage in temporal lobe limbic structures, particularly in the entorhinal cortex, hippocampus and amygdala (Purves et al., 2004; Orellana et al., 2016). Nevertheless, AD also impairs comprehension, learning, orientation and judgment (Sanabria-Castro et al., 2017).

AD may be categorized in two types: i) familial form, also known as early onset AD, is mostly a consequence of genetic mutations in amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) and ii) sporadic form, also known as late-onset AD, is the most common form of AD accounting for over 90% of cases and the major risk factor is ageing (Rahman, 2009; Finsterwald et al., 2015). Nevertheless, histopathologically both forms of AD are similar. The neuropathological hallmarks of AD include the presence of neurofibrillary tangles constituted by intraneuronal cytoskeletal filaments of phosphorylated tau and extracellular neuritic plaques formed by aggregates of amyloid-beta (Aβ) peptides (Purves et al., 2004; Osborn et al., 2016). In non-pathological conditions, tau is present in axons where stabilizes microtubules and regulates axonal transport (Skaper, 2012). However, in AD conditions, the phosphorylation of tau disrupts axonal transport and leads to formation of insoluble aggregates, a process believed to disrupt neuronal functions and contribute to neuronal death (Skaper, 2012; Rojas-gutierrez et al., 2017). As mentioned above, another neuropathological hallmark of AD is the presence of neuritic plaques formed by the accumulation of Aβ peptides, which is thought to be the causative agent of AD. The deposition of Aβ peptides alters mainly the glutamatergic and cholinergic pathways and the process of synaptic plasticity (Agostinho et al., 2010; Finsterwald et al., 2015). Furthermore, the accumulation of this peptide is linked to a neuroinflammatory process, but its contribution to AD progression is still not understood. Increasing evidence suggest that during neuroinflammation there are an intense cross-talk between the peripheral immune cells, mainly lymphocytes, and the cells of Central Nervous System (CNS). However, it is though that the inflammatory process in the brain are mainly mediated by microglia (immune resident

cells) and by astrocytes, which once activated by the pathological accumulation of Aβ, release mediators and modulators of inflammation, such as chemokines, cytokines and caspases among others. (Agostinho et al., 2010; Dam et al., 2016; Guillot-Sestier et al., 2015). Accordingly, reactive astrocytes and microglia are found surrounding Aβ plaques in the brain of AD patients, a phenomenon linked to neuroinflammation which is considered by some researchers another neuropathological hallmark of AD (Fiala and Veerhuis, 2010; Osborn et al., 2016; Soreq et al., 2017).

1.1.1. Amyloid-β peptide: the causative agent of synaptic loss

For a long time, the presence of neuritic plaques formed by accumulation of Aβ peptides was thought to be the key event underling the impairment of synaptic function characteristic of AD, a principle which was widely accepted and was the main focus of the amyloid cascade hypothesis (Hardy and Allsop, 1991). However, emerging evidences have been emphasizing a central role of Aβ in the oligomeric/soluble form, instead of in aggregates forming neuritic plaques, as the main trigger for neurotoxicity in AD (Hardy and Selkoe, 2002). Supporting this hypothesis is the observation of soluble Aβ being biologically more active than the insoluble forms (Batarseh et al., 2016). Accordingly, accumulation of Aβ oligomers is a major event underling synaptic dysfunction, through the disruption of dendritic spines, leading to an impairment and eventual loss of synapses, which ultimately leads to neuronal loss (Sivanesan et al., 2013; Rudy et al., 2015). Therefore, the accumulation of oligomeric Aβ, not Aβ neuritic plaques, and subsequent hampering of synaptic function are early events in AD pathogenesis leading to memory deficits, whereas the formation of Aβ neuritic plaques is a posterior event thought to occur as a result of extracellular accumulation of Aβ and of neuronal lysis of Aβ-burden neurons (reviewed in Agostinho et al., 2010). Consistent with this modified Aβ cascade hypothesis are observations of a correlation between the early memory impairments and the synaptic loss, instead of the Aβ neuritic plaque load or neurofibrillary tangles (Hardy and Selkoe, 2002; Agostinho et al., 2010; Rudy et al., 2015).

Aβ peptide is produced through proteolytic processing of the transmembrane APP, which can occurs by two cleavage pathways: the non-amyloidogenic and the amyloidogenic (Rojas-gutierrez et al., 2017), In the non-amyloidogenic pathway, APP undergoes proteolytic cleavage

by α- and ϒ-secretase and is secreted as αAPP, averting the formation of Aβ peptides (Rojas-gutierrez et al., 2017; Takahashi et al., 2017). In the amyloidogenic pathway, Aβ monomers are formed by proteolytic cleavage of APP by the sequential action of β-secretase (BACE) and ϒ-secretase (Batarseh et al., 2016; see figure 1). The active site of ϒ-secretase is composed by PS1 and PS2 and mutations in their coding genes are implicated in the familial form of AD, favouring the processing of APP through the amyloidogenic pathway (Dam et al., 2016).

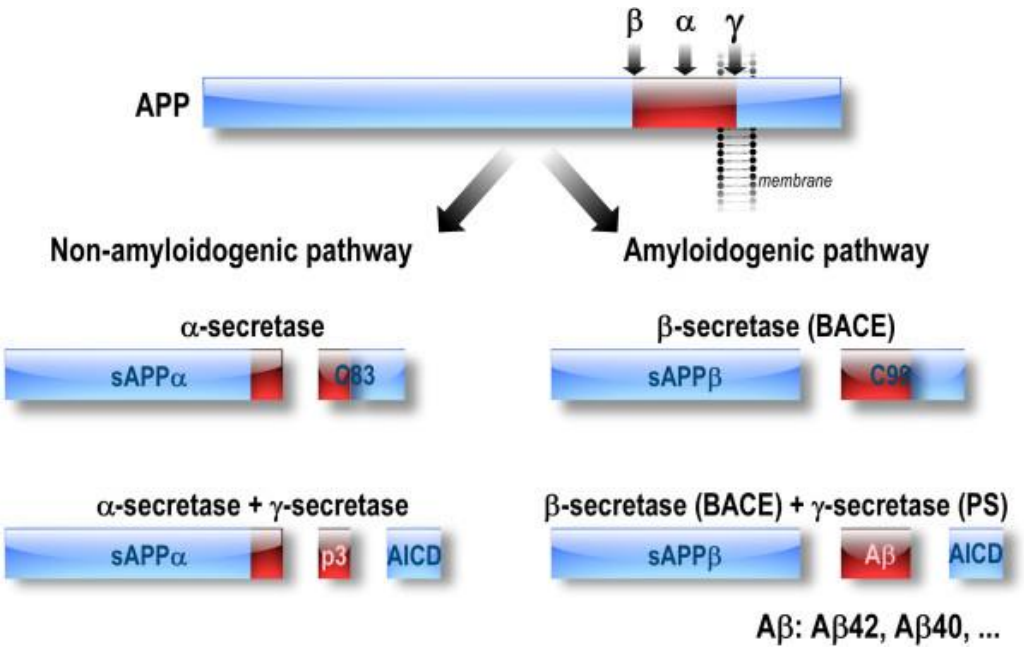

Fig. 1: Processing of APP through the amyloidogenic and non-amyloidogenic pathways: Aβ peptides are the result of APP processing through the amyloidogenic pathway, implicating the action of BACE1 and γ-secretase. APP processing through the non-amyloidogenic pathway averts Aβ production and implicates α-secretase and γ-secretase (from Prete et al., 2014).

The proteolytic cleavage of APP can occur in different sites, originating different forms of Aβ peptides, being the $A\beta_{1-40}$ and $A\beta_{1-42}$ the most common. This last form is considered to be the most toxic, since $A\beta_{1-42}$ is the most hydrophobic and the main component of Aβ neuritic plaques (Sanabria-Castro et al., 2017). In non-pathological conditions, Aβ peptides are also formed, but are maintained at low levels, and have a relevant role in processes of synaptic plasticity and memory (Palmeri et al., 2017). Since this peptide can be continually released into the extracellular milieu, it is important the existence of mechanisms for Aβ clearance, which occurs through two pathways: i) drainage to the bloodstream by amyloid-binding proteins (Sivanesan et al., 2013) or ii) by glial cells, astrocytes and microglia, which are able to internalize

and process Aβ peptides (Fiala and Veerhuis, 2010). Although microglia are the main phagocytic effector in the brain, the astrocytes are also involved in the processing of the Aβ peptide. In conditions of AD, Aβ peptides reach pathological levels possibly due to an increase in Aβ production and/or a decrease in this peptide clearance caused by the impairment of mechanisms above mentioned (Shankar and Walsh, 2009; Fiala and Veerhuis, 2010). This increase in Aβ peptides leads to their accumulation and subsequent synaptic dysfunction and loss, which underlies the memory deficits in early stages of AD.

1.2. Astrocytes

1.2.1. Defining astrocytes

Astrocytes are a type of cells with a complex and heterogeneous morphology, characterized by elaborated processes that give to these cells a star-like appearance (Purves et al., 2004; Wang and Bordey, 2008). In fact, the name astrocyte translates into a star-like cell, with its origin in Greek, the name astrocyte is composed by two words *astron* which means star and kytos that translates to a hollow vessel, latter a cell (Parpura and Verkhratsky, 2012).

Astrocytes are the most abundant cells in the brain and are a highly heterogenous population, differing among each other in developmental origin, morphology, gene expression profile, physiological properties and function (Hu et al., 2016). Typically, they are classified as protoplasmic in white matter and fibrous in grey matter. Protoplasmic astrocytes have a complex morphology with many branches that give rise to branching processes, which ensheath synapses and cover blood vessels; meanwhile fibrous astrocytes have a less complex morphology characterized by long thin unbranched processes that envelop nodes of Ranvier (Wang and Bordey, 2008; Sofroniew and Vinters, 2010; see figure 2).

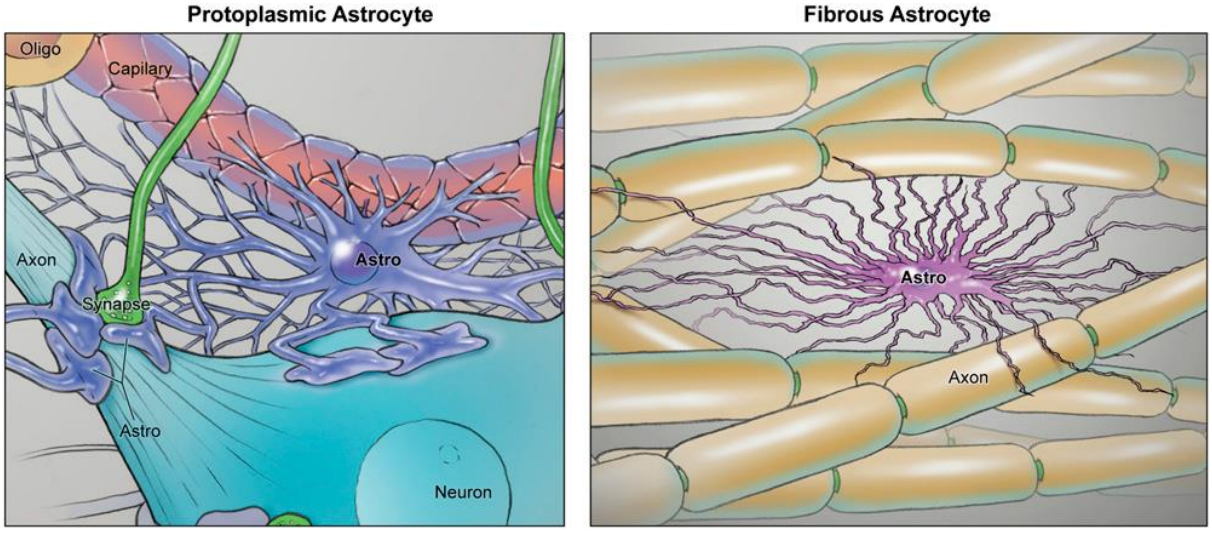

Fig. 2: Schematic representation of protoplasmic and fibrous astrocytes in the brain. A protoplasmic astrocyte (left) is shown in close connection with a neuron and a capillary, constituting the so-called "neurovascular unit" and highlighting the roles of astrocytes in developmental synaptogenesis and in modulating the brain-blood barrier. A fibrous astrocyte (right) is shown in a white matter tract, where it may interact with oligodendrocytes to promote myelination (from Molofsky et al., 2012).

In the human brain astrocytes present a more complex morphology which led to the necessity of expand this criteria of classification, existing four types of astrocytes categorized accordingly to their morphology: i) protoplasmic, ii) interlaminar, iii) polarized and iv) fibrous (Hu et al., 2016, see table 1).

The high heterogeneity of astrocytes makes it difficult to find a molecular marker (protein) able to label specifically astrocytes, and even more distinguish the different types of astrocytes. Antibodies to cell-type specific markers identifies a cell at molecular level and are more reliable than the cell morphologic identification. Several molecular markers are used to identify astrocytes, being the glial fibrillary acidic protein (GFAP) the most commonly used. Although GFAP, the major component of glial fibrils, is expressed by most astrocytes, this protein is also expressed by other CNS cell types derived from radial glial stem cells, namely the ependymal cells (Wang and Bordey, 2008) that are widely involved in the production of cerebrospinal fluid (Jiménez et al., 2014). The radial glial stem cells (radial neuroglia) are the precursors of glial cells, namely astrocytes and oligodendrocytes, which are formed during the gliogenesis (Zhang, 2001). In fact, GFAP is not expressed by all types of astrocytes (Parpura and Verkhratsky, 2012), being its expression reduced in protoplasmic astrocytes but not in mature fibrous astrocytes (Molofsky et al., 2012). Nevertheless, GFAP is still the main marker

used in astrocytes, being widely used to recognize reactive astrocytes (Sofroniew and Vinters, 2010). Another commonly used astrocytic markers are: i) the S100β, a calcium binding protein only expressed by a subtype of mature astrocytes; ii) the glutamate transporters: glutamate-aspartate transporter (GLAST; or excitatory amino acid transporter type 1 (EAAT1) in humans) and glutamate transporter 1 (GLT-1;or EAAT2 in humans), and iii) the metabolic enzymes: aldehyde dehydrogenase 1 family member L1 (Aldh1L1) and glutamine synthetase (GS; Wang and Bordey, 2008; Sofroniew and Vinters, 2010; Garwood et al., 2017).

Table 1: Different types of astrocytes in human brain (adapted from Hu et al., 2016)

SUBTYPE	LOCATION	FEATURES
Protoplasmic astrocytes	Deep layers of the cortex (layers 2-6)	❖ Majority of the processes do not overlap. ❖ Larger and more elaborate than their rodent counterparts. ❖ The processes of one protoplasmic astrocyte can cover five different blood vessels, eight neuronal cells bodies and numerous synapses. ❖ Highly coupled by gap junctions.
Interlaminar astrocytes	Layer 1 of the cortex	❖ Extend striking long, frequently unbranched processes throughout the layers of the cortex. ❖ Two types of processes: one contributes to the astrocytic network near the pial surface and the other penetrates deep layers of the cortex.
Polarized astrocytes	Deep layers of the cortex (near the white matter)	❖ Extend one or two long processes away from the white matter. ❖ The long processes are frequently unbranched or branched once.
Fibrous astrocytes	White mater	❖ Contain few primary processes. ❖ Their fibers are straighter and less branched than those of other glia.

1.2.2. Functions of astrocytes

Astrocytes are the main cellular component involved in the maintenance of brain homeostasis, taking on several vital house-keeping functions for normal neuronal activity, such

as provide metabolic support for neurons, buffer potassium excess and remove neurotransmitters from the synaptic cleft, namely glutamate and GABA (Wang and Bordey, 2008; Parpura et al., 2012, see figure 3). Astrocytes also have other functions namely the maintenance of the brain-blood barrier (BBB), the regulation of the cerebral blood flow (Hu et al., 2016) and the control of sleep and waking states (Parpura et al., 2012).

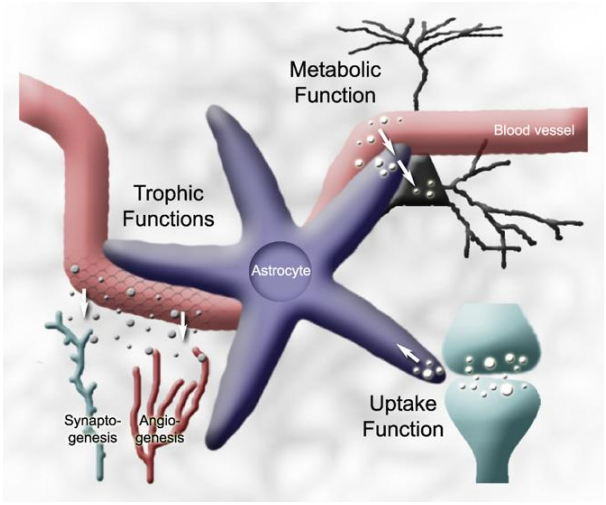

Fig. 3: Functions of astrocytes in maintaining neuronal homeostasis. Astrocytes provide metabolic support for neurons and remove potassium and neurotransmitters from the synaptic cleft. Furthermore astrocytes also are involved in synaptogenesis, angiogenesis and brain-blood barrier (BBB) maintenance (from Wang and Bordey, 2008).

Astrocytes can also modulate synaptic transmission and plasticity by releasing bioactive molecules designated gliotransmitters, namely adenosine triphosphate (ATP), adenosine, glutamate and D-serine (Rodriguez-Arellano et al., 2016), and peptides trophic factors, such as the brain-derived neurotrophic factor (BDNF). The synaptic transmission occurs within the synapse, a structure that include the pre- and post-synaptic terminals of neurons and the existing space between the two, designated synaptic cleft (Fig. 4). In the pre-synaptic terminal, the synaptic vesicles containing bioactive molecules, termed neurotransmitters, are released as a response to an action potential, which opens voltage-gated Ca^{2+} channels allowing a rapid influx of this cation into the pre-synaptic terminal. The increase of Ca^{2+} in pre-synaptic nerve terminals promotes the fusion of synaptic vesicles with the plasma membrane and the subsequent release of neurotransmitters in synaptic cleft (Purves et al., 2004). Neurotransmitters diffuse into the synaptic cleft and activate specific receptors in the post-synaptic terminal, triggering the opening or closing of ionic channels and therefore increasing

or decreasing the probability of the neuron fire an action potential, a mechanism underling the transmission of information between neurons (Purves et al., 2004).

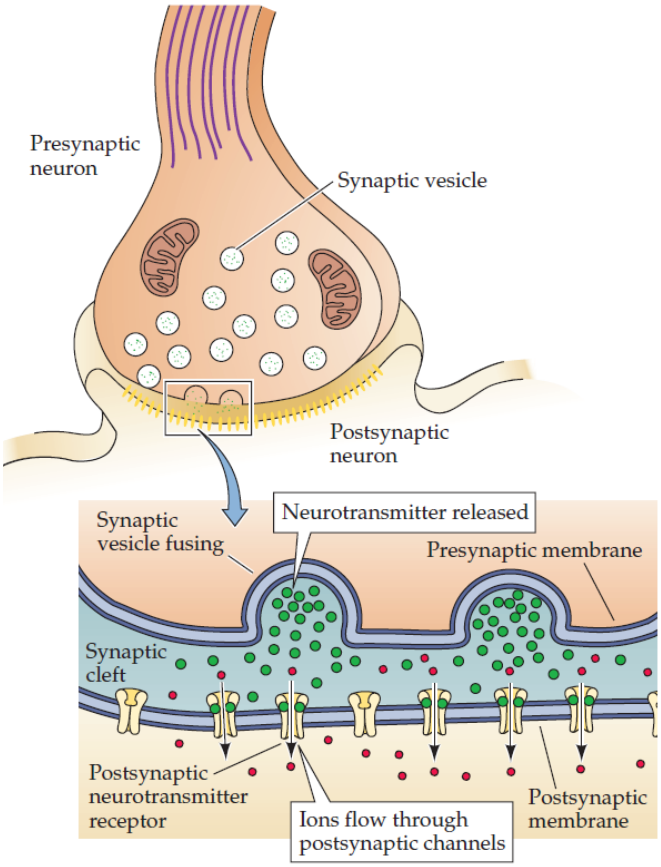

Fig. 4: Synaptic transmission. An action potential triggers the fusion of synaptic vesicles with the plasma membrane of the pre-synaptic terminal leading to neurotransmitters release into the synaptic cleft. Neurotransmitters diffuse into the post-synaptic terminal where activate specific receptors and can generate an action potential (from Purves et al., 2004)

The ability of astrocytes to modulate synaptic transmission and to perform several other functions, above mentioned, is reflected in the highly complex morphology of these cells and in the organization form of their elaborated processes in structured anatomical domains, which extend to blood vessels and to surrounding neurons and ensheath synapses (Finsterwald et al., 2015) forming a structure designated as tripartite synapse (Araque et al., 1999; Perea et al., 2009).

1.2.3. Synaptic modulation

Underlying the concept of tripartite synapse is a bidirectional communication between neurons and the astrocytes surrounding the synaptic buttons (or nerve terminals), in which astrocytes respond to neuronal activity by increasing intracellular Ca^{2+} levels and the release gliotransmitters, such as ATP, adenosine, glutamate and D-serine, which act on pre- and post-synaptic terminals and modulate synaptic transmission and plasticity (Stobart and Anderson, 2013; Rudy et al., 2015; Orellana et al., 2016, see figure 5). The proposed mechanism for this bidirectional communication involving astrocytes in the modulation synaptic transmission implicates the stimulation of astrocytes, through the neurotransmitters released into the synaptic cleft during synaptic transmission. The neurotransmitters, such as glutamate and ATP, are recognized by astrocytic receptors leading to the activation of phospholipase C (PLC) and the production of inositol triphosphate (IP_3), which trigger the release of Ca^{2+} from intracellular stores in the endoplasmic reticulum and induce Ca^{2+}-dependent gliotransmitters release (Orellana et al., 2014). In turn, the release of gliotransmitters affects differentially pre- and post-synaptic terminals providing to astrocytes a pathway to differentially modulate individual synaptic inputs (Garwood et al., 2017).

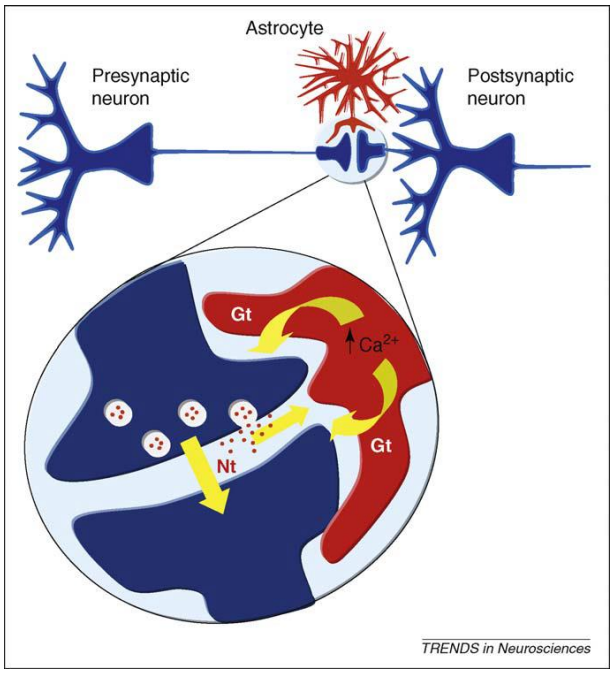

Fig. 5: The tripartite synapse. The tripartite synapse includes the pre- and post-synaptic terminals plus the astrocytes processes englobing the synapse. The image also shows a bidirectional communication between astrocytes and neurons in the tripartite synapse. Neurons release neurotransmitters during synaptic activity to which astrocytes respond with Ca^{2+} elevations and release of gliotransmitters, which in turn control neuronal excitability and synaptic transmission (from Perea et al., 2009).

Another important astrocyte signalling pathway is the generation of Ca^{2+} waves, which represent a form of astrocytic excitability (Sofroniew and Vinters, 2010), occurring in response to neuronal activity and/or spontaneously (Parpura et al., 2012). The generation of Ca^{2+} waves can be involved in the modulation of synaptic activity through the exocytotic (Ca^{2+}-dependent) release of gliotransmitters and /or BDNF (Scemes and Giaume, 2006). In astrocytes, the Ca^{2+} waves are characterized by an increase in cytosolic Ca^{2+} concentration, which triggers similar events in the surrounding astrocytes in a wave like-manner (Scemes and Giaume, 2006). The mechanism underlying the generation and propagation of Ca^{2+} waves in astrocytes is triggered by the activation of PLC followed by the production of IP_3 and subsequent Ca^{2+} release from the endoplasmic reticulum, generating a Ca^{2+} signal within the cell that propagates by triggering similar events in neighbour astrocytes (Scemes and Giaume, 2006; Orellana et al., 2014). The propagation of Ca^{2+} waves is proposed to occur through two different pathways involving: i) the Ca^{2+}-dependent release of ATP and glutamate and sequential activation of purinergic or glutamate receptors in neighbour astrocytes and/or ii) the mobilization of Ca^{2+} and second messengers through gap junctions channels (GJC; Stout et al., 2002; Orellana et al., 2013,

2014). These mechanisms of Ca^{2+} wave propagation are not necessarily mutually exclusive, they may occur in parallel in order to contribute to coordinated activity of astrocytes (Scemes and Giaume, 2006) and embody a long-range signalling pathway in the brain (Parpura et al., 2017).

Moreover, astrocytes are also responsible for the uptake of neurotransmitters, glutamate and GABA, from the synaptic cleft (Dallérac and Rouach, 2016), and the speed of these bioactive molecules clearance can affect the levels and duration of post-synaptic activation (Garwood et al., 2017). Therefore, astrocytes have a critical role in the modulation of synaptic transmission either by releasing gliotransmitters or by taken up neurotransmitters from synaptic cleft.

1.2.4. Gliotransmitters: glutamate and ATP

1.2.4.1. Glutamate

Glutamate is the major excitatory neurotransmitter in the CNS, nonetheless also plays an important role as a gliotransmitter (Harada et al., 2016). Glutamate is involved in the regulation of synaptic activity and plasticity and in cognitive processes, such as learning and memory (Byrnes et al., 2009).

Glutamate-mediated neurotransmission occurs through glutamate receptors, which are divided into two categories: ionotropic and metabotropic receptors. Ionotropic receptors are classified in N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate receptors (Purves et al., 2004). This type of receptors are nonselective cation channels, which allow the efflux of Na^+ and K^+, and in some cases small amounts of Ca^{2+} (Purves et al., 2004). AMPA receptors are involved in fast excitatory neurotransmission; meanwhile NMDA receptors are mostly activated under certain conditions, specifically when there is induction of synaptic plasticity (Danysz and Parsons, 2012). Metabotropic glutamate receptors (mGluRs) belong to the family of G-protein coupled and are divided into three groups (Group I, II, and III). This type of receptors modulate postsynaptic ionic channels indirectly, causing slower postsynaptic response that may endure

longer (Purves et al., 2004; Byrnes et al., 2009). The activation of metabotropic receptors is involved in the regulation of several cellular functions, namely gene expression, metabolic activity and gliotransmitters release (Parpura et al., 2012). Activation of mGluR from group I is linked to neuronal excitability increase and glutamatergic/excitatory signalling potentiation (Byrnes et al., 2009). This group of receptors, comprise mGluR 1 and mGluR 5, and are coupled to G_q and its activation is linked to activation of PLC, oscillations of intracellular Ca^{2+} and gliotransmitters release (Loane et al., 2012). By contrast, the groups mGluR II (includes mGluR 2 and 3) and mGluR III (includes mGluR 4, 6, 7 and 8) inhibit adenylate cyclase, and thus their activation reduce glutamatergic signalling, through the inhibition of Ca^{2+} entry in the cells, and restrain gliotransmitters release (Byrnes et al., 2009).

Although, glutamate is an important neurotransmitter/gliotransmitter in synaptic transmission, when it is present in high concentrations extracellular can become a potent neurotoxin (Rimmele and Rosenberg, 2016). In fact, when in excess in the synaptic cleft, glutamate can overactivate glutamate receptors, which cause neuronal toxicity, leading to cellular damage or death, a process commonly referred as excitotoxicity (Choi, 1988). Therefore, to maintain glutamate homeostasis and avoid excessive activation of glutamate receptors (excitotoxicity), it is important the existence of efficient mechanisms for glutamate clearance from the synaptic cleft. As mentioned in section 1.2.2, one of the functions attributed to astrocytes is the uptake of neurotransmitters, particularly glutamate. The removal of glutamate from the synapse occurs through Na^+-dependent glutamate transporters present in the membranes of astrocytes, namely GLAST or EAAT1 and GLT-1 or EAAT2 (Robinson and Jackson, 2016). The levels of these glutamate transporters are particularly high in the membranes of astrocytes adjacent to synapses, where they are of great importance to control synaptic transmission and avoid excitotoxicity (Steele and Robinson, 2012; Rimmele and Rosenberg, 2016). Although both glutamate transporters participate in glutamate clearance, GLT-1 is the main transporter involved in this process (Matos et al., 2012b; Robinson and Jackson, 2016), which is driven by a Na^+ gradient created through the Na^+/K^+ ATPase, being the glutamate co-transported with three Na^+ ions into astrocytes (Parpura et al., 2017). This mechanism confers to astrocytes, not only the ability to protect neurons from glutamate excitotoxicity, but also the ability to modulate glutamatergic transmission by controlling the concentration of glutamate in the synaptic cleft and, therefore, regulating the extent of activation of glutamate receptors.

Once glutamate is taken up through glutamate transporters, it is converted into glutamine in the cytoplasm of astrocytes in an ATP dependent reaction catalysed by GS, an enzyme specifically expressed in astrocytes (Parpura et al., 2017). Subsequently, glutamine is released by astrocytes, taken up into neurons through glutamate transporters, and converted back to glutamate by the phosphate-activated glutaminase. This sequence of events working between neurons and astrocytes is known as glutamate-glutamine cycle (Stobart and Anderson, 2013). In neurons, glutamate synthetized *de novo* is re-packed into vesicles by the vesicular glutamate transporter (vGluT) for aftermost synaptic release (Niciu et al., 2012). Since glutamate is not able to cross the BBB, it must be synthesized from local precursors (Purves et al., 2004). There are two sources of glutamate: i) recycling through the glutamate-glutamine cycle (Rudy et al., 2015) and ii) Krebs/tricarboxylic acid cycle (TCA) where glutamate is synthesized by transamination of 2-oxoglutarate, an intermediate of the TCA cycle (Purves et al., 2004).

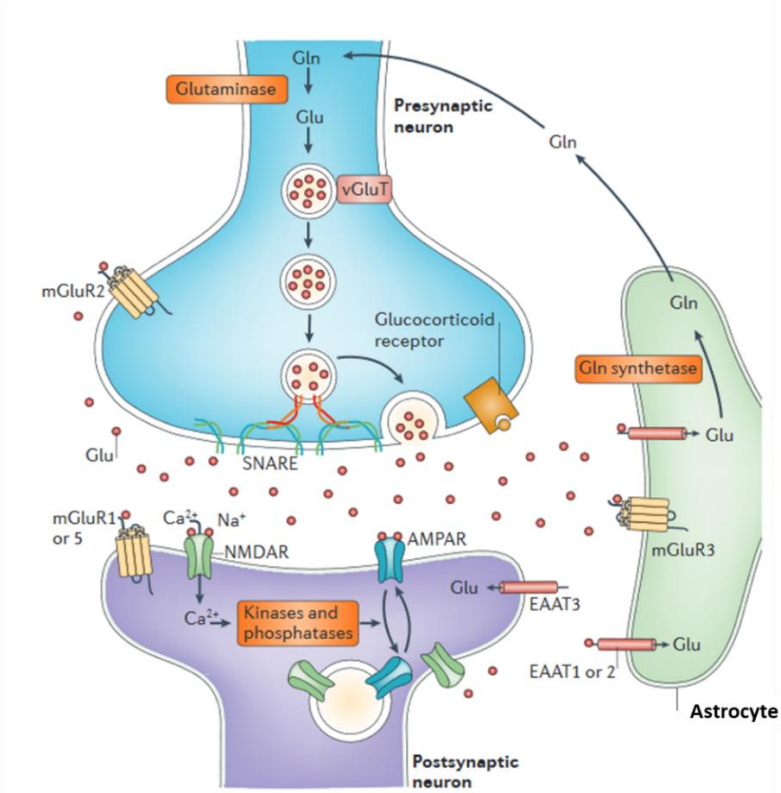

Fig. 6: Glutamatergic transmission. Glutamate released during neuronal activity into the synaptic cleft activates glutamate receptors in post-synaptic neurons and in astrocytes. The glutamate is then taken up by astrocytes and converted to glutamine by the glutamine synthetase (Gln Synthetase), which is transported to neurons where is converted back to glutamate, a reaction catalysed by glutaminase, transported into synaptic vesicles through vGluT and released to the synaptic cleft in a voltage- and Ca^{2+}-dependent manner (adapted from Popoli et al., 2011).

1.2.4.2. ATP

ATP is the main source of energy in the cellular metabolism (Harada et al., 2016) and also enrols an important role in modulation of synaptic transmission, being involved not only in conveying information between neurons, but also in neuron-glia communication (Puerto et al., 2013). Furthermore, this transmitter can also be involved in modulation of brain inflammation, acting as a danger signal (Rodrigues et al., 2015).

ATP is a known signalling molecule, acting mainly through activation of purinergic receptors (Harada et al., 2016). Purinergic signalling constitutes a mechanism of intercellular communication mediated by ATP and its breakdown products, namely ADP, AMP and adenosine (Inoue et al., 2010). Each breakdown product of ATP has different affinities for different purinergic receptors; nevertheless, in general they act through two families of receptors: i) the P1 receptors, also known as adenosine receptors, which encompasses 4 subtypes of G-protein-coupled receptors: A_1, A_{2A}, A_{2B} and A_3 (explored in section 1.3), ii) and the P2 receptors, also known as ATP purinoreceptors (Puerto et al., 2013). P2 receptors are categorized into two groups: metabotropic (P_2Y) receptors and ionotropic (P_2X) receptors (Parpura et al., 2017). The P_2Y receptors are G-protein coupled receptors divided in eight subtypes: P_2Y_1, P_2Y_2, P_2Y_4, P_2Y_6, $P_2Y_{11\text{-}14}$ (Woods et al., 2016). This type of receptors is mostly involved in ATP signalling and in the generation and propagation of Ca^{2+} waves in astrocytes through the control of IP_3-induced Ca^{2+} release from endoplasmic reticulum (Parpura et al., 2017). In contrast to P_2X receptors, this type of metabotropic receptors is associated with long-lasting and tropic functions (Puerto et al., 2013). The P_2X receptors are ATP-gated cation channels involved in the regulation of several processes, namely neurotransmitter release, neuroinflammation and pain sensation (Woods et al., 2016). Moreover, ATP also conveys the modulation of synaptic transmission by the adenosine signalling pathway, being an important source of adenosine due to the catabolism of extracellular ATP into adenosine by ecto-5'-nucleotidase (CD73; Augusto et al., 2013). The purinergic system is involved in the bi-directional communication between astrocytes and neurons (see figure 7) and dysfunction of this system can be associated with several brain disorders (Rodrigues et al., 2015).

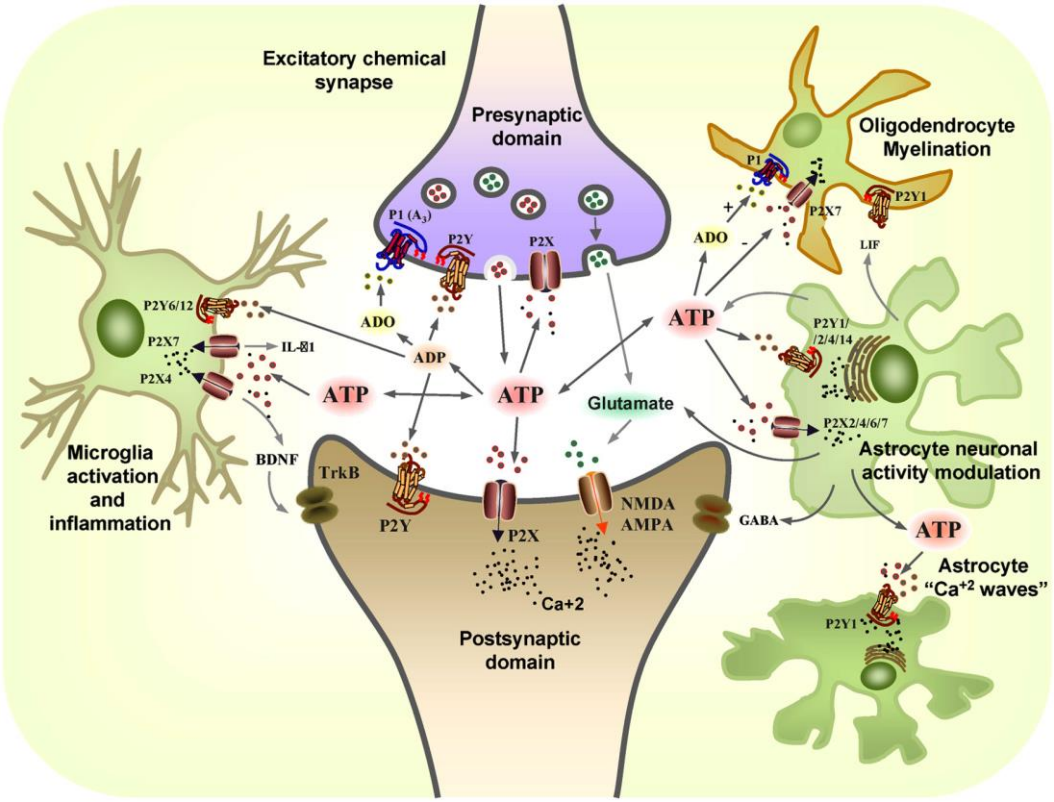

Fig 7: Integrated view of purinergic receptors and purines in the CNC. ATP and glutamate are released from the pre-synaptic terminals. ATP and its breakdown products, mainly adenosine (ADO) that can activate pre- and post-synaptic purinergic receptors, P1 and P2. These receptors exist in astrocytes, microglia and also in neurons. ATP and glutamate are also released by astrocytes and are able to modulate synaptic transmission. Glutamate activates NMDA and AMPA receptors in the post-synaptic terminal and is removed from the synaptic cleft by astrocytes (from Puerto et al., 2013).

1.2.5. Mechanisms of gliotransmitters release

Several mechanisms for gliotransmitters release in physiological conditions have already been described (Fig. 8), namely: i) vesicular release through Ca^{2+}-dependent exocytosis, ii) release through reversal activity of glutamate transporters, iii) release though anion transporter opening induced by cell swelling, iv) release via P_2X_7 receptors, and v) release by hemichannels (Harada et al., 2016).

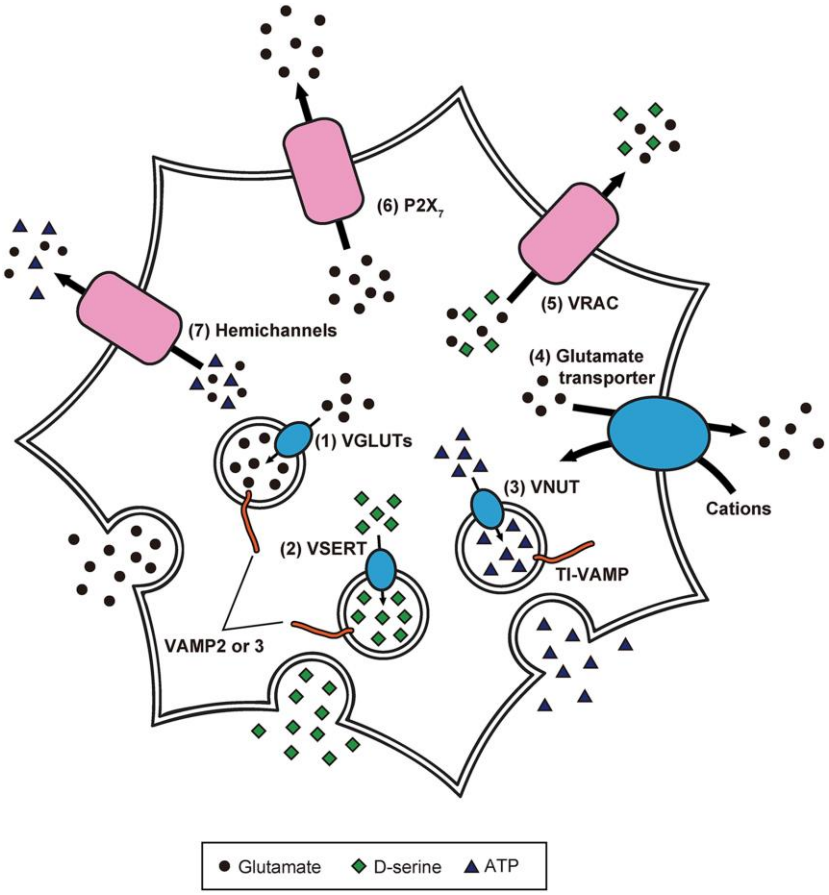

Fig. 8: Mechanisms of gliotransmitters release. (1) Glutamate and (2) D-serine are packed into synaptic-like vesicles through VGLUTs and VSERT, respectively. The synaptic-like vesicles fuse with the plasma membrane and release the gliotransmitters. (3) ATP is stored in secretory lysosomes and released through Ca^{2+}-dependent exocytosis. Furthermore, other mechanisms were described for the release of gliotransmitters: (4) reverse operation of plasma membrane glutamate transporters, (5) cell swelling-induced anion transporter (VRAC) opening, (6) release via P_2X_7 receptors, and (7) hemichannels on the cell surface of astrocytes (from Harada et al., 2016).

1.2.5.1. Ca^{2+}-dependent exocytosis

The most well described mechanism for the release gliotransmitters, namely ATP and glutamate, by astrocytes is through Ca^{2+}-dependent exocytosis. Firstly, is the storage of ATP and glutamate into different compartments (Coco et al., 2003). ATP is stored in secretory lysosomes by the vesicular nucleotide transporter (VNUT), meanwhile glutamate is stored into synaptic-like vesicles by vesicular glutamate transporters (vGLUT), a process dependent on a proton gradient created by vacuolar-type H^+-ATPases (Harada et al., 2016). The fusion

of synaptic-like vesicles with the plasma membrane is proposed to be driven by the formation of a complex with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), vesicle associated membrane proteins (VAMP), synaptosomal-associated protein (SNAP) 23 or 25 and syntaxin (Hamilton and Attwell, 2010). The fusion of this complex with the plasma membrane of astrocytes is regulated by a Ca^{2+} sensor protein, possibly synaptotagmin, which is activated by the entry of Ca^{2+} in the cells though voltage-gated Ca^{2+} channels (Hamilton and Attwell, 2010). The Ca^{2+}binding to Ca^{2+} sensor triggers the fusion of the synaptic-like vesicles with the membrane and there is exocytosis of the gliotransmitters to the extracellular milieu. The Ca^{2+} involved in the exocytotic process does not originate exclusively from its influx (from extracellular space), it can also be released from internal astrocytic stores, such as endoplasmic reticulum and mitochondria through a mechanism described in section 1.2.3 (Parpura et al., 2012).

1.2.5.2. Reverse operation of glutamate transporters

This mechanism of glutamate release involves an alteration in the normal function of glutamate transporters, GLAST and GLT-1. In "normal" basal conditions, the glutamate transporters favour the uptake of glutamate into the astrocytes, due to a Na^+ gradient created by a Na^+/K^+ ATPase, as described in section 1.2.4.1. However, pathological alterations in ionic conditions, such as increased extracellular K^+ levels, can reverse this Na^+-dependent mechanism favouring the co-release of glutamate and Na^+ and the uptake of K^+ (Malarkey and Parpura, 2008).

1.2.5.3. Anion channel opening induced by cell swelling

This mechanism occurs mainly in hypo-osmotic conditions that are responsible for cellular swelling. In an attempt to compensate the volume increase by swelling, astrocytes open volume-regulated anion channels (VRACs), permeable to several anions and also glutamate, allowing its release. The release of glutamate through VRACs constitutes a Ca^{2+}-independent pathway for glutamate release (Malarkey and Parpura, 2008).

1.2.5.4. Glutamate exchange via the cystine-glutamate antiporter

Another mechanism described for glutamate release is through an amino acid antiporter, which mediates exchange of extracellular cystine by intracellular glutamate. This system does not seem to enrol a relevant role in glutamate release in physiological conditions (Malarkey and Parpura, 2008).

1.2.5.5. Ionotropic purinergic receptors

As mentioned in 1.2.4.1, P_2X receptors are ATP-gated ionotropic receptors, a subtype of P2 receptors. There are 7 forms of P_2X receptors (P_2X_1–P_2X_7) able to form homomeric and heteromeric structures, being the subtype P_2X_7 receptor responsible for ATP and glutamate release (Woods et al., 2016). The P_2X_7 receptor forms a pore that allows passage of molecules until 900 Da (Malarkey and Parpura, 2008). Among the P_2X receptors, the P_2X_7 receptor is the subtype with lower affinity for ATP leading to the possibility of its activation be more relevant in pathological conditions associated with increased ATP levels than in non-pathological conditions (Rodrigues et al., 2015). Accordingly, the P_2X_7 receptor was described to be involved in the regulation of brain inflammatory processes (Woods et al., 2016).

1.2.5.6. Hemichannels in cell membrane

Furthermore, gliotransmitters release can occur through hemichannels (HC) in the cell surface. HC are hexamers of connexins and/or pannexins inserted in the plasma membrane, forming pores. The HC between apposed astrocytes can form GJC. Nevertheless, HC also exist *per se* in the plasma membrane of astrocytes, where they embody a mechanism for release of small molecules, like glutamate and ATP (Hamilton and Attwell, 2010).

1.2.6. Astrocytic hemichannels and Gap Junctions channels

Gap junctions channels (GJC) are an important pathway for astrocyte-astrocyte intercommunication, forming a pore interconnecting adjacent astrocytes and allowing the efflux of small cytoplasmic molecules until about 1 kDa (Malarkey and Parpura, 2008). GJC are assembled by docking of two HC in adjacent astrocytes. In turn, HC are formed by oligomerization of six subunits (hexamer) of connexins (Cx) proteins, a structure also termed connexon (Malarkey and Parpura, 2008; Orellana et al., 2011a). Alternatively, HC can be composed by pannexins (Panx) proteins, also designated pannexons (Malarkey and Parpura, 2008; Orellana et al., 2012). Although the structure of Panx and Cx HC is similar, it is still controversial if Panx HC are able to form GJC, since overexpression of Panx seems to increase gap junctional coupling but it was not yet demonstrated the existence of GJC composed by Panx HC in physiological conditions (reviewd in Giaume et al., 2013). Nevertheless, HC purpose is beyond the formation of GJC, functional HC exist *per se* (Ye et al., 2009) allowing efflux of molecules, namely ATP and glutamate, being an important mechanism of gliotransmitters release (Hamilton and Attwell, 2010).

Cxs and panxs proteins have really similar structures, as is shown in figure 9, both proteins have four α-helical transmembrane domains linked by two extracellular loops and one cytoplasmic loop with intracellular carboxy- and amino- termini (Orellana et al., 2011b). The oligomerization of Cx and Panx proteins forms a pore which appears to remain closed in basal conditions, and is proposed to open in several pathological and non-pathological conditions (Orellana et al., 2011a). GJC as well as HC seem to be gated by Ca^{2+}; HC are directly exposed to the extracellular milieu which allows a direct regulation of HC state by several cations (Ye et al., 2003). The main cation involved in regulation of HC opening is extracellular Ca^{2+}, which is proposed to close the HC pore through the binding with a Ca^{2+} binding site in the extracellular vestibule of the HC (Giaume et al., 2013). Accordingly, in resting conditions with normal Ca^{2+} concentration in the extracellular milieu the HC are mostly closed, and removal of Ca^{2+} leads to the opening of HC resulting in gliotransmitters release (Ye et al., 2003).

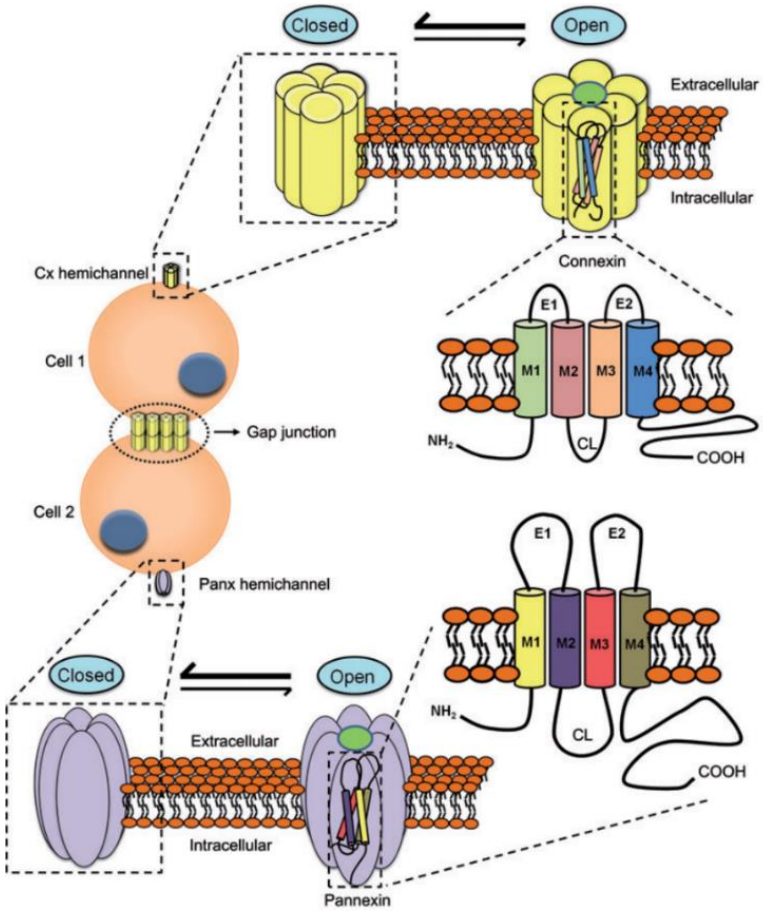

Fig. 9: Schematic representations of Cxs and Panxs protein structure and organization in the membrane of astrocytes. Cx and Panx proteins can oligomerize into HC, meanwhile GJC are only formed by the docking of two Cx HC. HC are preferentially closed but can open by several pathological and non-pathological stimuli (from Orellana et al., 2011a).

Furthermore, HC can be homomeric, when composed by only one isoform of Cx or Panx proteins, or heteromeric when composed by more the one isoform of Cx or Panx (Orellana et al., 2013). In turn, GJC can be classified as homotypic or heterotypic according to the composition of HC by one or more isoforms of Cx proteins, respectively (Orellana et al., 2013; Lapato and Tiwari-woodruff, 2017).

1.2.6.1. Connexins in astrocytes

Cx proteins are involved in several cellular processes, such as development, morphogenesis, differentiation and cell death (Lin et al., 2008; Orellana et al., 2013). Several isoforms of Cx proteins are expressed in astrocytes, the main are Cx43, Cx30 and Cx26 (Orellana et al., 2016). The Cx43 protein is the most expressed Cx in astrocytes either *in vivo*

or *in vitro,* and also astrocytes are the cells in the brain with higher levels of Cx43 expression (Orellana et al., 2016). Cx43 proteins are translated in rough endoplasmic reticulum and form the HC structure by oligomerization of six subunits of Cx43, the HC is trafficked through the Golgi compartment (Lin et al., 2008), and further transported into the astrocytic membrane through microtubules where remains as HC or dock with a HC of a apposed astrocyte forming a GJC (Jiang et al., 2011). Cx43 protein has a short half-life between 1.5 and 5 hours relatively to most proteins, which have half-lives between 20 and 80 hours (Lin et al., 2008). Cx43 HC targeted for degradation are internalized in annular junctions, a double membrane structure, (Xie et al., 2017) and degraded by lysosomes and proteasome (Lin et al., 2008).

1.2.7. Astrocytes: role in cognition / memory

The cognitive processes of learning and memory involves a series of events ranging from alterations at the molecular level to the remodulation of neuronal network, which includes formation and restructuring, strengthening and stabilization of synaptic connections that are associated with synaptic plasticity (Adamsky and Goshen, 2017; Zorec et al., 2017). The main forms of synaptic plasticity are through: i) long-term potentiation (LTP) a mechanism mediated by NMDA receptors involving the insertion of AMPA receptors in the post-synaptic membrane and ii) long-term depression (LTD), also mediated by NMDA receptors but linked to AMPA receptors removal from the post-synaptic membrane (Parpura et al., 2012). In LTP, activation of NMDA receptors leads to an influx of Ca^{2+} thought to activate signalling cascades, which prompt to changes in synaptic efficacy and plasticity (Prickaerts et al., 2017). Several animal models of AD show an impairment in LTP, which is thought to be instigated by alterations mediated by NMDA receptors in glutamatergic synapses, characterized by their high plasticity and ability of express LTP (Danysz and Parsons, 2012). In fact, high levels of Aβ oligomers were shown to alter glutamatergic transmission in *in vitro* and *in vivo* studies possibly by favouring LTD, through the blockade of NMDA receptors or by hampering glutamate uptake (Mucke and Selkoe, 2012). This is a possible mechanism underlying synaptic dysfunction and subsequent memory deficits in AD. In fact, the severity of memory deficits in AD is closely associated with the levels of soluble Aβ oligomers in hippocampus and cortex (Tu et al., 2014). The hippocampus, one of the brain regions severely affected in AD and highly involved in

learning and memory processes (Danysz and Parsons, 2012) contains about 57% synapses associated with astrocytes (Harada et al., 2016). This tight coupling of neurons and astrocytes in the tripartite synapse seems to be important in the control of synaptic plasticity and memory formation (Finsterwald et al., 2015). The role of astrocytes in these processes is mainly attributed to their importance in the maintenance of neuronal homeostasis, providing metabolic support for neuronal activity and glutamate recycling, which simultaneously is a source of "new" glutamate formation and a pathway for avoiding excitotoxicity. Therefore, the role of astrocytes in cognitive processes is through the regulation of mechanisms involved in the management of information and memories formation such as synaptic formation, synaptic transmission and plasticity. The regulation of these mechanisms by astrocytes occurs namely through the release of gliotransmitters and uptake of glutamate and GABA (Dallérac and Rouach, 2016; Adamsky and Goshen, 2017). For instance, glutamate is not only vital for basal synaptic transmission but is also an active and crucial participant the formation of new neuronal networks required for synaptic plasticity, mainly LTP, upon activation of NMDA and AMPA receptors (Esposito et al., 2013; Moraga-Amaro et al., 2014). Moreover, astrocytes also release GABA which is the major inhibitory transmitter in the CNS, having a repressive role in synaptic transmission and plasticity (Luján et al., 2005; Moraga-Amaro et al., 2014).

Considering the relevance of gliotransmitters in the processes of synaptic transmission and plasticity, it was postulated a possible role of HC in memory, which is logical considering the relevance of HC in gliotransmitters release. In fact, gliotransmitters release through Cx43 HC seems to have an important role in memory, since the blockade of Cx43 HC in basolateral amygdala impairs memory consolidation, an effect prevented by a cocktail of bioactive molecules, glutamate, D-serine and ATP among others (Stehberg et al., 2012).

Furthermore, the retraction of astroglial processes is believed to be crucial for memory consolidation (Dallérac and Rouach, 2016), through the modulation of bioactive molecules in the synaptic cleft and stability of dendritic spine. Underlying the mechanism of astrocytic processes retraction is the ability of astrocytes to sense neuronal activity and respond to the neuronal input by rapidly modifying their actin filaments by extending or retracting their processes and, consequently, alter dendritic spine coverage, a mechanism related with dendritic spine stability (Adamsky and Goshen, 2017). The mobility of astrocytes processes is regulated through the activation of astrocytic mGluR by glutamate released during synaptic activity, which can promote further gliotransmitters release (Adamsky and Goshen, 2017). This point to an important role of neuro- and glio-transmitters in processes of memory.

1.2.8. Astrocytes in AD

The exposure to Aβ peptides triggers a series of structural and morphological changes in the astrocytes, a process commonly referred as reactive astrogliosis (Pekny and Nilsson, 2005). This process occurs as a response to pathological insults and involves multiple biochemical and immunological changes in addition to hypertrophy and proliferation of astrocytes, reflected in an up-regulation of GFAP, surrounding the damaged area, which can lead to glial scars formation in an attempt to isolate the damage area and allow regeneration (Rodríguez *et al.*, 2009; Rodríguez-Arellano *et al.*, 2016; Garwood *et al.*, 2017), these alterations can result in a gain and/or loss of functions in astrocytes (Sofroniew and Vinters, 2010). In AD astrogliosis seems to contribute to synapses weakening and consequently, to memory impairment (Zorec et al., 2017). Underlying this synaptic dysfunction can be glutamate excitotoxicity (see section 1.2.4.1), a major event in AD pathophysiology characterized by pathological high levels of glutamate in the synaptic cleft, which can be a consequence of an increase in glutamate release and/or to an impairment in glutamate uptake by astrocytes (Rudy et al., 2015). In fact, reports already provided a demonstration of an impairment in glutamate uptake by astrocytes due to a downregulation of glutamate transporters, GLT-1 and GLAST, triggered by Aβ peptides (Matos et al., 2008; Matos et al., 2012a). Moreover, in an animal model of AD, Aβ peptide accumulation was shown to decrease glutamine synthetase levels, the astrocytic enzyme responsible for conversion of glutamate in glutamine, which is further transported to neurons for glutamatergic transmission (Kulijewicz-Nawrot et al., 2013). Thus, glutamate excitotoxicity involves also an impairment in the glutamate-glutamine cycle, highlighting the importance of astrocytes in AD pathogenesis.

In early stages of AD, astrocytes activation has a neuroprotective role since these cells are able to uptake and degrade Aβ peptides (Finsterwald et al., 2015; Osborn et al., 2016), thus controlling the levels of these peptides in the brain. However, the uptake of Aβ severely alters the metabolism of astrocytes (Agostinho et al., 2010) and when an overload of this peptide is reached astrocytes can suffer lysis, which further contributes to exacerbate AD neurodegeneration, through the release of intracellular Aβ that contribute to the burden of neuritic plaques (Batarseh et al., 2016). Accordingly, in the cortex and hippocampus of AD patients, reactive astrocytes are found in the vicinities of Aβ accumulations with processes infiltrating the plaques.

Astrocytic proteins Cx43 and Cx30, which compose HC/GJC, were also shown to be associated with Aβ plaques in regions lacking viable neurons (Mei et al., 2010), suggesting a role of these astrocytic channels in AD. In fact, dysregulation of Cx and Panx HC has been associated with the progression of neurodegenerative diseases; however, the mechanisms involving HC in neurodegeneration remain to clarified (Orellana et al., 2016). Nevertheless, it was proposed that in the early stages of neurodegenerative disorders, such as AD, the associated neuroinflammatory process underling astrocyte and microglia activation and the subsequent release of ATP and glutamate are associated with HC opening and reduced astrocytic neuroprotective functions (Orellana, et al., 2012b). The increased release of this gliotransmitters can activate neuronal P_2X_7 receptors and NMDA receptors that in turn are thought to activate neuronal Panx1 HC (Orellana et al., 2012), leading to a large efflux of Ca^{2+}, and ultimately neuronal death (Orellana et al., 2011b). Interestingly, astroglial HC seem to be activated by the Aβ peptide, meanwhile the effect of this peptide on GJC is still controversial, since intercellular communication through this pathway seems to decrease in cultured astrocytes exposed to the Aβ peptide, a finding that is not supported by studies using an AD animal model (Cruz et al., 2010).

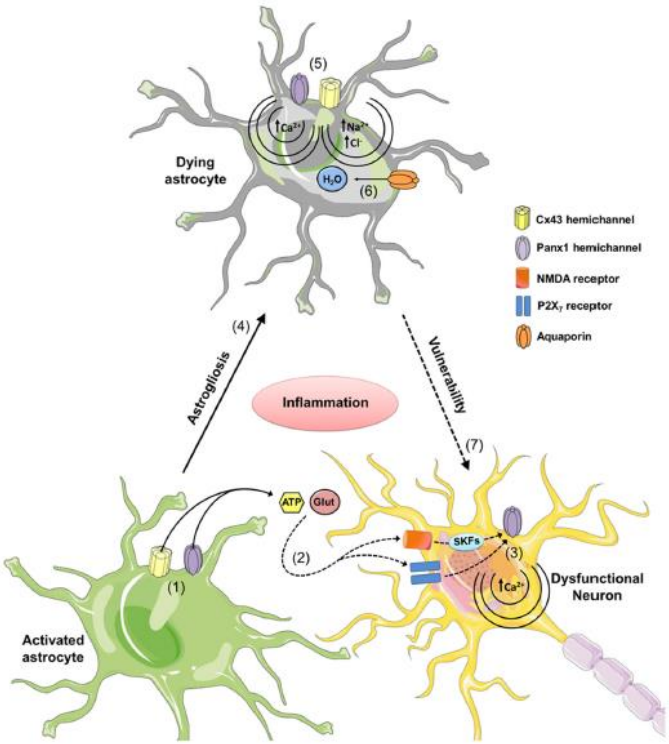

Fig. 10: The role of astrocytic Cx and Panx HC in neurodegeneration. During the early stages of various neurodegenerative diseases, the associated inflammatory process promotes the opening of astrocytic HC (1). This results in the release of the gliotransmitters ATP and glutamate, and increases activation of neuronal NMDA and P_2X_7 receptors (2). It is hypothesized that NMDA and P_2X_7 receptor activation increases the opening of neuronal Panx1 channels through phosphorylation of Panx1 (3), which could affect intracellular Ca^{2+} homeostasis resulting in cell death. Uncontrolled activation of astrocytes (reactive astrogliosis) may further contribute to cell death by a mechanism related to HC opening (4). In particular, dysregulated opening of Cx43 and Panx1 channels could elicit cellular damage by the entry of Ca^{2+} (5), as well as Na^+ and Cl^-, triggering cellular swelling due to an increased influx of H_2O via aquaporins (6). Finally, given that astrocytes provide support to neurons, astroglial cell damage associated with HC opening could indirectly increase neuronal susceptibility and vulnerability, due to the homeostatic imbalance occurring during neurodegeneration (from Orellana et al., 2016).

1.3. Adenosine receptors

Adenosine exists both in neurons and glia cells, being intensively released following insults, which makes adenosine as a sort of 'regulator' of synaptic communication that lead to the homeostatic coordination of brain function (Ribeiro, 1999). This purine is a relevant player in neuro-glia communication, and can affect the release and the action of neurotransmitters and

gliotransmitters (Ribeiro and Sebastião, 2010). Adenosine is present in the synaptic cleft by two pathways: i) through the release by the equilibrative nucleoside transporters (Na^+-dependent) that are present in astrocytes and neurons or ii) by metabolism of extracellular ATP by ectonucleotidases (Ke et al., 2009; Wall and Dale, 2013). In the adult brain, the intracellular adenosine levels are largely controlled by adenosine kinase (ADK) that metabolizes adenosine into AMP. This enzyme specific of astrocytes is considered to modulate the astrocyte-based adenosine cycle that controls the uptake or release of adenosine through the equilibrative nucleoside transporters (Boison et al., 2010).

Adenosine acts mainly through the P1 receptors which comprise 4 subtypes of G-protein-coupled adenosine receptors: A_1, A_{2A}, A_{2B} and A_3 (Puerto et al., 2013). Adenosine receptors interact with other receptors as well as with transporters as part of its attempt to fine-tune synaptic transmission (Ribeiro and Sebastião, 2010). Adenosine A_{2A} receptor ($A_{2A}R$) and adenosine A_{2B} receptor ($A_{2B}R$) are mostly stimulatory being involved in adenylate cyclase activation and in the increase cyclic adenosine monophosphate (cAMP) production, on the contrary, adenosine A_1 receptor (A_1R) and adenosine A_3 receptor (A_3R) are mostly inhibitory, inhibit adenylate cyclase and decrease the levels of cAMP (Woods et al., 2016). Since adenosine is not stored into synaptic vesicles, its metabolism is highly regulated by intracellular metabolism of AMP (Wei et al., 2011).

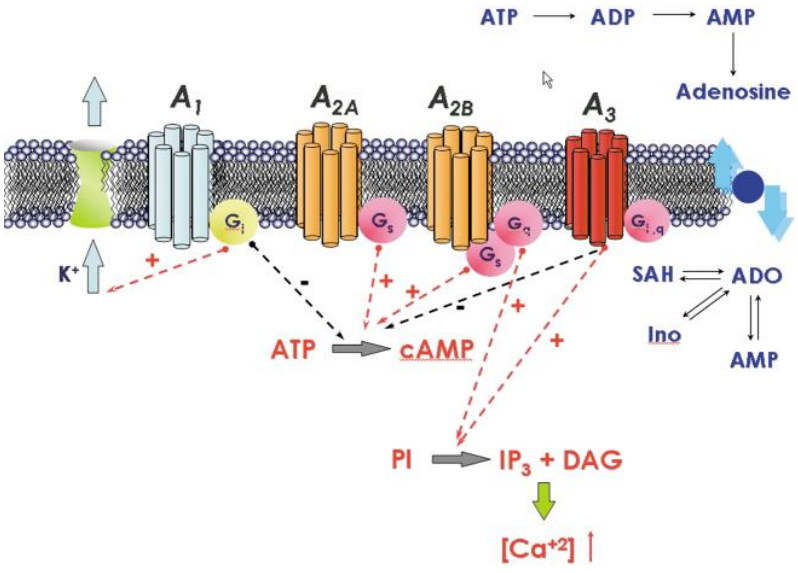

Fig. 11: Adenosine receptor signalling. The A_1, A_{2A}, A_{2B}, and A_3 are G-protein coupled receptors, which activation increases or decreases cAMP production that is thought to be linked to the modulation of ion-channel activity (from Moro et al., 2003)

Synaptic transmission is mainly modulated by A_1R and $A_{2A}R$ signalling, A_1R has been linked to depression of excitatory synaptic transmission being able to limit the response to a excitatory stimuli, meanwhile $A_{2A}R$ is facilitatory and can induce neuronal glutamate release, which contradicts the inhibition by A_1R (Gomes et al., 2011; Cunha et al., 2016). Therefore, adenosine can have both inhibitory and excitatory functions in the synaptic transmission. The $A_{2A}R$ are also involved in processes of synaptic plasticity (Gomes et al., 2011). The $A_{2A}R$ signalling through cAMP induced activation of protein kinase A (PKA) is well established, however, it can also be involved in the activation of other signalling pathways, namely in microtubule-associated protein kinases (MAPK), phosphoinositide 3-kinase (PI-3K) and protein kinase C (PKC; Cunha et al., 2016). Furthermore, the $A_{2A}R$ are also found in astrocytes and were shown to control astrocytic glutamate uptake and the production of pro-inflammatory cytokines, which modulate synaptic plasticity (Cunha et al., 2016), and the activation of this receptor is also linked to astrogliosis (see section 1.2.8; Ke et al., 2009; Matos et al., 2012a).

1.3.1. Adenosine Receptors and AD

Aging itself, a major risk for AD, are related with alterations in the adenosine signalling. Aging is related with a parallel decrease in A_1R density and an increase in $A_{2A}R$ density, which are thought to augment the susceptibility to develop brain disorders (Cunha et al., 2016). Alterations in the density of these receptors can also be found in brains of human patients with AD, for instance meanwhile in the frontal cortex the A_1R and $A_{2A}R$ seem to be increased, in the dentate gyrus of the hippocampus the expression of A_1R is decreased (Puerto et al., 2013; Woods et al., 2016).

The $A_{2A}R$ were proposed to be involved in modulation of cognitive processes, such as learning and memory (Rahman, 2009). Accordingly, the selective deletion of astrocytic $A_{2A}R$ in mice seems to enhance memory (Orr et al., 2015). Furthermore, the activation of $A_{2A}R$ seems to be a mechanism underling the memory impairment in models of AD and their blockade averts memory deficits (Cunha et al., 2016). For instance, the selective blockade of $A_{2A}R$ was shown to prevent synaptic loss and neuronal death triggered by the Aβ peptide

(Canas et al., 2009) and was also shown to forestall glutamate excitotoxicity by preventing the decline in glutamate uptake by astrocytes exposed to $A\beta_{1-42}$ peptides (Matos et al., 2008, 2012a). Moreover, the activation of astrocytic $A_{2A}R$ was reported to decrease glutamate uptake by these cells (Matos et al., 2012b) suggesting a relevant role of $A_{2A}R$ in glutamate excitotoxicity, which may be a mechanism contributing to synaptotoxicity. Additionally, several studies reported a protective role of caffeine, a known antagonist of the adenosine receptors, in cognitive function (Santos et al., 2010; Chu et al., 2012; Duarte et al., 2012; Ghoneim et al., 2015). Particularly, an epidemiologic study with a Portuguese cohort also showed that the consumption of caffeine is inversely associated with the risk of cognitive decline in both men and women (Santos et al., 2010). Although, several studies already showed a neuroprotective role of caffeine against the memory decline, which is seems to be mediated by $A_{2A}R$ blockade (Dall'Igna et al., 2003; Cunha et al., 2016), the participation of astrocytic $A_{2A}R$ in AD pathophysiology remains to be clarified, but increasing evidences suggest that these receptors might be a valuable therapeutic target for AD and other neurodegenerative disorders, where the activity of astrocytes are impaired.

CHAPTER 2| OBJECTIVES

Early events in AD pathogenesis include synaptic dysfunction and loss triggered by the accumulation of Aβ oligomers, which is thought to underlie the memory deficits in AD (Sivanesan et al., 2013). Astrocyte cells, which make part of tripartite synapse, have a crucial role in synaptic transmission and are also vulnerable to Aβ peptides accumulation. Indeed, several studies have reported a condition of reactive astrogliosis in the brain of AD patients and animal models. The reactive astrogliosis is characterized by evident alterations in astrocytes morphology; however, these cells also become dysfunctional in pathological conditions, contributing to exacerbate neuronal damage. Accordingly, our group already showed that $A\beta_{1-42}$ peptides impair the astrocytic glutamate uptake function (Matos et al., 2008, 2012a). Besides this crucial function of neurotransmitters uptake from synaptic cleft, the astrocytes are also able to release gliotransmitters, such as glutamate and ATP, with impact synaptic transmission. Although, there are some evidences showing that the release of gliotransmitters are affected in AD conditions, it remains to be clarified the mechanisms underlying the Aβ-induced gliotransmission changes (Giaume et al., 2013). One candidate responsible for disrupting astrocytic function is the $A_{2A}R$. This stems from our observations that $A_{2A}R$ are located in astrocytes, where they critically control the glutamate uptake and also the Na^+/K^+-ATPase activity (Matos et al., 2012b, 2013) the main energizing system to sustain membrane-dependent processes in astrocytes. Moreover, astrocytic $A_{2A}R$ were shown to regulate memory functions (Orr et al., 2015), and it was also reported that the selective blockade of $A_{2A}R$ prevents synaptotoxicity in an AD animal model (Canas et al., 2009) and the reduced astrocytic glutamate uptake triggered by $A\beta_{1-42}$ peptide (Matos et al., 2012a). Therefore, the knowledge about astrocyte responses to Aβ exposure is imperative for understanding the AD pathogenesis and the astrocytic $A_{2A}R$ can provide a valid therapeutic target.

The present study aims: 1) to establish how AD-like conditions affect the release of gliotransmitters by astrocytes, 2) to investigate the mechanisms involved in the astrocytic release of ATP and glutamate in non-pathological and in AD-like conditions, and 3) to determine the role of $A_{2A}R$ in the modulation of gliotransmitters release in the same conditions. To address these goals, we used primary cultures of astrocytes, which were incubated with the synthetic peptide $A\beta_{1-42}$ to mimic AD-like conditions. Although, this *in vitro*

cellular model does not fully mimic the complex events occurring *in vivo*, the cultures of astrocytes are an invaluable tool for studying molecular mechanisms of astrocytes activity in non-pathological and in pathological conditions (Lange et al., 2012). The three aims, and the associated tasks, are detailed below:

Aim 1| Establish the effect of AD-like conditions in the release of gliotransmitters, ATP and glutamate, by astrocytes.

The initial task of this project was to determine the impact of AD-like conditions on gliotransmitters (ATP and glutamate) release. Thus, it was assessed the effect of $A\beta_{1-42}$ peptides on ATP and glutamate release, having been tested acute and chronical times of peptide exposure.

Aim 2| Investigate the mechanisms involved in the release of gliotransmitters in AD and in non-pathological conditions.

This task was designed to study the mechanisms involved in the gliotransmitters release in both non-pathological and AD-like conditions, focusing on the release of ATP and glutamate through HC or P_2X_7 receptors. To this purpose, the primary cultures of astrocytes were treated with a HC blocker (carbenoxolone) or with an antagonist of P_2X_7 receptors (Brilliant Blue G) before the cells were challenged with $A\beta_{1-42}$ peptides.

Aim 3| Determine the role of $A_{2A}R$ in the modulation of gliotransmitters release in AD and in non-pathological conditions

In this task, it was studied whether $A_{2A}R$ control the astrocytic release of ATP and glutamate. Thus, cultured astrocytes were exposed to the selective antagonist of $A_{2A}R$, SCH 58261, before acute or chronic exposure to the peptide $A\beta_{1-42}$. With this task, we intended to determine if the blockade of $A_{2A}R$ prevents the alterations in gliotransmitters release triggered by $A\beta_{1-42}$ exposure. Moreover, it was explored whether the $A_{2A}R$ and the Cx43, the main protein of astrocytic HC, have a physical interaction.

CHAPTER 3| MATERIALS & METHODS

3.1. Materials

3.1.1. Reagents

Table 2: Reagents used

REAGENT	SUPPLIER
2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid (**EDTA**)	Sigma Aldrich
2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid **(HEPES)**	Sigma Aldrich
3-(Cyclohexylamino)-1-propanesulfonic acid (**CAPS)**	Sigma Aldrich
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide **(MTT)**	Sigma Aldrich
4',6-diamidino-2-phenylindole **(DAPI)**	Invitrogen
30% Acrylamide/Bis-acrylamide solution	Bio-Rad
Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit	Sigma Aldrich
Ammonium persulphate **(APS)**	Sigma Aldrich
BCA protein assay kit	Thermo Scientific
Bicine	Sigma Aldrich
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad
Boric Acid	Sigma Aldrich
Bovine Serum Albumin **(BSA)**	Sigma Aldrich
Bromophenol blue	Sigma Aldrich
Calcium chloride **($CaCl_2$)**	Fluka Analytical
CLAP (cocktail of proteases inhibitors)	Sigma Aldrich
Dako mounting medium	Agilent technologies
Dimethyl sulfoxide **(DMSO)**	Sigma Aldrich
Dithiothreitol **(DTT)**	Sigma Aldrich
DNase I	Sigma Aldrich
Dulbecco Modified Eagle Medium **(DMEM 5648)**	Sigma Aldrich
Duolink® In Situ Red Starter Kit Mouse/Rabbit	Olink Bioscience
Enhanced Chemiluminescence **(ECL)**	Thermo Scientific
Fetal Bovine Serum **(FBS)**	Gibco by Life technologies
Glutamate assay kit	Abcam
Glucose	Sigma Aldrich
Glycerol	Sigma Aldrich

Abcam – Cambridge, UK; Agilent Technologies - California, USA; Bio-Rad – California, USA; Gibco by Life Technologies – California, USA; Invitrogen / Thermo Scientific – Massachusetts, USA; Olink Bioscience – Uppsala, Sweden; Fluka Analytical / Sigma Aldrich – Missouri, USA

Horse serum	Gibco by Life technologies
Hydrocloric acid **(HCL)**	Sigma Aldrich
Magnesium chloride	Fluka Analytical
Magnesium sulfate **($MgSO_4$)**	Fluka Analytical
Methanol	Sigma Aldrich
Paraformaldehyde **(PFA)**	Sigma Aldrich
Penicillin Streptomycin	Gibco by Life technologies
Percoll	GE Healthcare
Phenylmethylsulfonyl fluoride **(PMSF)**	Sigma Aldrich
Poly-D-Lysine	Sigma Aldrich
Ponceau S	Sigma Aldrich
Potassium chloride **(KCl)**	Sigma Aldrich
Potassium dihydrogen phosphate **(KH_2PO_4)**	Sigma Aldrich
Sodium bicarbonate **($NaHCO_3$)**	Sigma Aldrich
Sodium chloride **(NaCl)**	Sigma Aldrich
Sodium Dodecyl Sulfate **(SDS)**	Bio-Rad
Sodium hydroxide **(NaOH)**	Sigma Aldrich
Sodium phosphate dibasic heptahydrate **($Na_2HPO_4 \cdot 7H_2O$)**	Sigma Aldrich
Sucrose $C_{12}H_{22}O_{11}$	Sigma Aldrich
N,N,N′,N′-Tetramethylethylenediamine **(TEMED)**	Sigma Aldrich
Triton X-100	Sigma Aldrich
Trizma	Sigma Aldrich
Trypan blue	Sigma Aldrich
TrypLE™ Express	Gibco by Life technologies
Trypsin	Sigma Aldrich
Tween 20	Fluka Analytical

Bio-Rad – California, USA; GE Healthcare – Illinois, USA; Gibco by Life Technologies – California, USA; Fluka Analytical / Sigma Aldrich – Missouri, USA

3.1.2. Drugs

Table 3: Drugs used

DRUG	SUPPLIER	STOCK	FINAL [] USED
2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-e][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine **(SCH 58261)**	Tocris	50 µM	50 nM
β-amyloid$_{1-42}$ **(Aβ$_{1-42}$)**	Bachem	221.5 µM	1 µM
Brilliant Blue G **(BBG)**	Sigma Aldrich	25 µM	100 nM
Carbenoxolone disodium salt **(CBX)**	Sigma Aldrich	15 mM	50 µM
D-Noradrenaline hydrogen L-tartrate **(NE)**	Fluka Analytical	20 mM	30 µM
(*RS*)-2-Chloro-5-hydroxyphenylglycine **(CHPG)**	Tocris	50 mM	500 µM

3.1.3. Antibodies

Table 4: Primary and secondary antibodies for Western blot

ANTIBODY	SUPPLIER	HOST	TYPE	DILUTION
Connexin 43	Sigma-Aldrich: C6219	Rabbit	Polyclonal	1:8000
β-actin	Sigma Aldrich: A5316	Mouse	Monoclonal	1: 20 000
Anti-rabbit peroxidase conjugated	Thermo Scientific: 31462	Goat	Polyclonal IgG (H+L)	1:10000
Anti-mouse peroxidase conjugated	Thermo Scientific: 31432	Goat	Polyclonal IgG (H+L)	1:10000

Bachem – Bubendorf, Switzerland; Fluka Analytical / Sigma Aldrich – Missouri, USA; Thermo Scientific – Massachusetts, USA; Tocris – United Kingdom

Table 5: Primary and secondary antibodies for immunocytochemistry and proximity ligand assay

ANTIBODY	SUPPLIER	HOST	TYPE	DILUTION
$A_{2A}R$	Santa cruz: SC-32261	Mouse	Monoclonal	1:500
Connexin 43	Sigma-Aldrich: C6219	Rabbit	Polyclonal	1:600
CD11b	Serotec: MCA 275R	Mouse	Monoclonal	1:200
GFAP	Santa cruz: Sc-6170	Goat	Polyclonal	1:200
Anti-goat 488	Invitrogen: A11055	Donkey	Polyclonal IgG (H+L)	1:500
Anti-rabbit 594	Invitrogen: A21207	Donkey	Polyclonal IgG (H+L)	1:500

3.1.4. Animals

Wistar and C57Bl/6 pups (4–5 day postnatal) were obtained from a colony of these animals in CNC animal facilities. All efforts were made to minimize the number of animals used in the experiments. The studies were conducted in agreement with standard procedures to reduce animal suffering, in accordance with approved animal welfare guidelines and European legislation (ORBEA 128_2015/04122015) and the certification of Direcção Geral de Alimentação e Veternária (DGAV; 0421/000/000/2016 Ref 014420).

3.2. Methods

3.2.1. Cortical Astrocyte Primary Cultures

Astrocyte cell culture systems can be used, as an *in vitro* experimental model, to study the biological functions of these glial cells in detail. Thus, there is an increasing number of studies

Invitrogen – Massachusetts, USA; Sigma Aldrich – Missouri, USA; Santa Cruz – Texas, USA; Serotec - California, USA;

using primary cultures of astrocytes to help unravel the continuing enigma of the properties and functions of astrocytes in the brain (Matos et al., 2008, 2012b; Lange et al., 2012) .

Primary astrocyte cultures were prepared from cerebral cortices of 4-5 day postnatal pups accordingly to previously described procedures, with some modifications (Matos et al., 2012a). After the pups were sacrificed, under anaesthesia, by cervical dislocation, the cortex was removed and placed on TrypLE Express. The tissue was chopped up with a 10 blade and incubated at 37°C in tryple with DNAse I (10 mg/mL in 150 mM NaCl) for enzymatic digestion for 30 minutes. The enzymatic digestion was stopped by addition of astrocyte culture medium, Dulbecco's Modified Eagle Medium (DMEM) - high glucose supplemented with 10% foetal bovine serum (FBS), penicillin-streptomycin (10 mL/L), HEPES (6g/L) and sodium bicarbonate (0.84 g/L). Afterwards, the cell suspension was centrifuged for 2 minutes at 115 x *g* (800 rpm). The obtained pellet was resuspended in astrocyte culture medium and the number of cells in suspension counted in a hemocytometer using trypan blue dye. Then, the cells were plated onto poly-D-lysine (0.1 mg/mL in borate buffer, pH 8.2)-coated 75-cm^2 culture flasks, at a density of $1x10^5$ cells/cm^2 and maintained at 37°C in a 5% of CO_2 and 95% room-air humidified incubator. The culture medium was replaced every 2-3 days. The mixed-glial cultures typically reached confluence after 12-15 days in culture (Fig. 12).

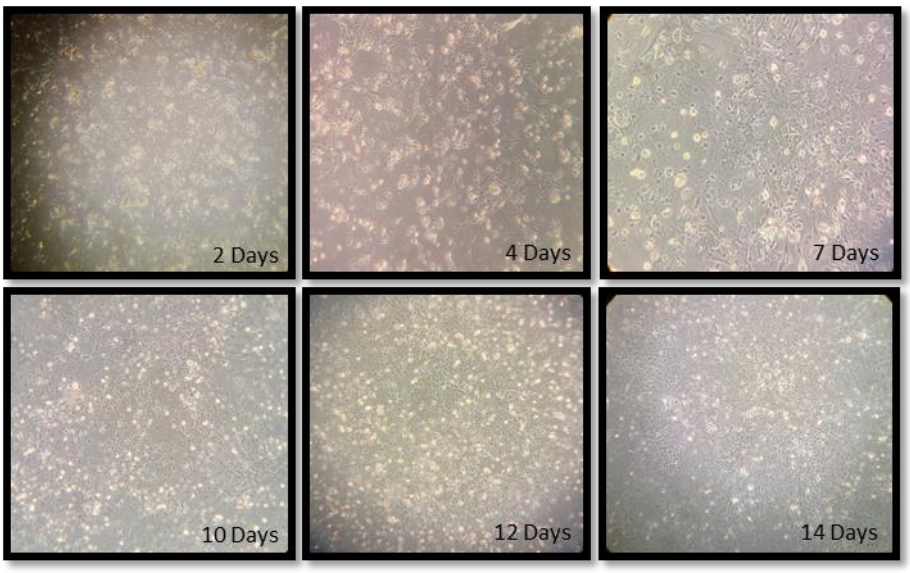

Fig. 12: Representative images of astrocytes development in culture. The images were obtained through an optical microscope (amplification 10x).

After the cells reached confluence and in order to remove microglial cells from the astrocyte monolayer, the cells were shaken in an orbital shaker at 180 rpm at 37°C for 2 hours. Then, the medium was replaced to remove microglia in suspension, and the cells (mainly astrocytes) that remained attached to the flasks were left to re-establish from a minimum of 3 hours to overnight. Afterwards, a subculture of astrocytes was performed, thus the astrocytes cultured in flasks were mildly trypsinizated and further plated in poly-D-Lysine coated plates or coverslips. In brief, the cells were rinsed with PBS (in mM: 137 NaCl, 2.7 KCl, 10 $Na_2HPO_4 \cdot 7H_2O$ and 1.9 KH_2PO_4, pH 7.4) containing EDTA (1mM) and then incubated PBS containing 0.05% trypsin and EDTA, for 5-10 minutes at 37°C. After this incubation, some strokes were given to flasks to help cell detachment, and as soon as the cells were in suspension (visualization in optic microscope), it was added astrocyte culture medium, which contained FBS; to inactivate trypsin. The cell suspension was then centrifuged for 5 minutes at 180 x *g* (1000 rpm) and the pellet washed and resuspended with astrocyte culture medium; and number of cells in suspension counted in a hemocytometer using trypan blue dye. The cells were plated at different densities according with the assay where they are used, and remained in culture for 1-2 days, in an incubator at 37°C humidified and with 5% of CO_2 and 95% room-air, before the experiments beginning.

3.2.2. Astrocytes treatments

Astrocytes were exposed to $A\beta_{1-42}$ acutely (5 minutes or 1 hour) or chronically (24 hours). Drugs used in pharmacological modulation of $A\beta_{1-42}$ mediated effects (SCH 58261, CBX or BBG) were added to the cells 30 min before the incubation with the peptide. NE was used as positive control and the incubations were always of 5 minutes (Fig. 13).

β-amyloid$_{1-42}$ ($A\beta_{1-42}$) peptide was reconstituted in type I sterile water accordingly to manufacturer's instructions to obtain the concentration of 1mg/mL (221.5 μM); the final concentration of $A\beta_{1-42}$ added to the cells was 1 μM. The SCH 58261 was used at a final concentration of 50 nM (stock of SCH 58261 50 μM dissolved in DMSO). Carbenoxolone disodium salt (CBX), brilliant blue G (BBG) and norepinephrine (NE), were reconstituted in type I sterile water, and the final concentration used in the cells were, 50 μM, 100 nM and 30

µM, respectively. CHPG was reconstituted in a NaOH solution (1%) and the final concentration used in the astrocyte cultures was 500 µM.

Fig. 13: Schematic representation of incubations with different time points: (A) 5 minutes, (B) 1 hour and (C) 24 hours.

Incubations of 5 minutes and 1 hour were performed in Krebs buffer (in mM 132 NaCl, 4 KCl, 1.2 Na_2HPO_4, 1.4 $MgCl_2$, 6 glucose, 10 HEPES, 1 $CaCl_2$, pH 7.4) and 24 hours incubations were performed with astrocyte culture medium (see 3.2.1). After incubations, the medium of each condition was collected and stored at -80°C until analysis. The cells were rinsed with PBS and lysed with Tris 150 mM with proteases inhibitors DTT (1mM), PMSF (1mM) and CLAP (0.001%) to further quantify the protein and/or to obtain cell extracts for Western-blot assays.

3.2.3. ATP Quantification

ATP release quantification was performed using a high sensitivity luciferin-luciferase bioluminescent assay kit (Sigma Aldrich, FLAA). In the presence of ATP, luciferin is catalysed by luciferase in a reaction that emits light, which is proportional to the amount of ATP present (Fig. 14).

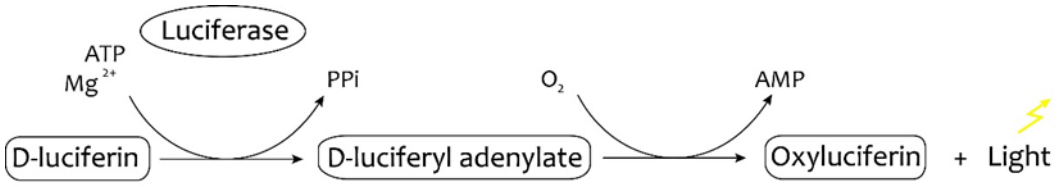

Fig. 14: Schematic representation of PLA technique. In this reaction luciferase catalyses the oxidation of D-luciferin, which is converted into oxyluciferin in a reaction that emits energy as light. Therefore, the intensity of bioluminescence is proportional to the amount of ATP present.

This assay was performed as previously described by our group (George et al., 2015). In brief, a standard curve was done with an ATP standard solution in culture medium with concentrations ranging from 0 to 1×10^{-5}M. The measurements were performed by automatic injection of 40 µL of ATP assay mix (provided by the kit that among other components has luciferase and luciferin) to 80 µL of standards/samples in a white 96-well plate. Luminescence was measured in a VICTOR^3 multilabel plate reader (Perkin Elmer) with Wallac 1420 software (5 seconds of acquisition). Empty wells were left between each measurement to avoid signal contamination.

ATP concentration, in the samples of medium collected after astrocytes incubations, was quantified by extrapolation of the standard curve and normalized by protein concentration of astrocytes lysates.

3.2.4. Glutamate Quantification

Glutamate quantification was performed using a colorimetric assay kit (Abcam, ab83389), which measures the levels of free glutamate and not the glutamic acid found in the backbone of peptides and proteins. The glutamate enzyme mix (provide by the kit) recognizes the glutamate as a specific substrate developing a colour proportional to the amount of glutamate. The assay was performed accordingly to manufacturer's instructions. Thus, a standard curve with known amounts of glutamate (0, 0.5, 1, 2, 4 and 6 nmols) was done using the provided standard solution. In a 96 multi-well were added the standards (50µL) and samples with assay buffer (40µL + 10µL). The reaction mix (100µL for each well), prepared with assay buffer, glutamate developer and glutamate enzyme mix, was added to each standard and sample and

incubated at 37°C for 30 minutes (protected from light). The absorbance was read at 450 nm in a multi-well plate spectrophotometer. The glutamate concentration of samples was extrapolated from the standard curve. The levels of glutamate were normalized by protein concentration in astrocytes lysates of each condition.

3.2.5. Protein quantification (Bio-Rad method)

Protein determination of astrocytes lysates was performed using the Bio-Rad protein assay reagent kit. A standard curve was drawn with known concentrations of BSA (0.0025; 0.005; 0.01; 0.02 and 0.025 µg/µL). Sample buffer was added in each standard. Samples were added to the 96 multi-well in a proportion of 1:40. Bio-Rad reagent was diluted 1:3 with type 1 water accordingly to manufacturer's instructions and added to each well. After 15 minutes of incubation at room temperature (protected from light), the absorbance was read at 600 nm in a spectrophotometer. The standard curve was used to calculate the protein concentration of the samples in µg/µL.

3.2.6. Viability Assay (MTT Test)

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a colorimetric assay commonly used for assessing cell metabolic activity, and thus infer about the viability of cells. This assay is based on the principal that dehydrogenase enzymes, of metabolic active (viable) cells are able taken up and metabolize MTT (yellow) to insoluble formazan (purple), meanwhile unviable cells lose that ability (Cookson et al., 1995).

After the incubation of the cultured astrocytes with the drug, MTT (0.5 mg/ml in PBS) was added to culture medium and incubated for 2 hours at 37°C, protected from light. After the cell incubation with the MTT, the culture medium was completely removed and it was added DMSO to dissolve the salt precipitate formed within the cells (formazan), which was further transferred to 96 multi-well. The absorbance of reduced MTT was read in the

spectrophotometer at 570 nm. Cell viability was determined in percentage in comparison to the control cells (non-treated).

3.2.7. Immunocytochemistry

Immunocytochemistry is a technique widely used to visualize the localization of a specific protein or antigen in cultured cells, using a specific primary antibody that binds to it. The primary antibody allows the protein visualization, under fluorescence microscope, when it is bound by as secondary antibody conjugated with a fluorophore.

Astrocytes seeded at density of 25,000 cells/cm^2 on coverslips remained in culture for 2-3 days until incubation and/or fixation. The cells were fixed with a solution of 0.4% PFA (pH 7.4) in PBS for 15 minutes, and washed 3 times with PBS before storage at 4°C. Fixed astrocytes were permeabilized through incubation with a solution of PBS with 0.2% triton X-100, for 10 minutes. After washing 3 times with PBS, it was performed an incubation with PBS containing 0.3% BSA and 5% horse serum to block the nonspecific binding of antibodies, for 1 hour. Subsequently, and after removing and washing the blocking solution, the cells in coverslips were incubated with primary antibodies (goat anti-GFAP (1:200) and rabbit anti-Cx43 (1:600)) dissolved in PBS 3% BSA for 2 hours at room temperature. Afterwards, the cells were rinsed with PBS 3% BSA 3 times and incubated with the secondary antibodies (donkey anti-goat 488 (1:500) and donkey anti-rabbit 594 (1:500)) for 1 hour at room temperature, protected from light. Negative controls of the assay were performed by labelling in the absence of primary antibodies to check for non-specific labelling of the secondary antibodies or cross-reactivity between secondary anti-bodies. Then, cells were washed with PBS, stained with the nuclei dye DAPI in PBS (1:5000) and further washed with PBS. Coverslips were mounted with Dako mounting medium and visualized by fluorescence microscopy (Zeiss, model imager Z2 with Axiovision software 4.8).

3.2.8. Proximity Ligation Assay (PLA)

PLA is an antibody-based method in which the proteins of interest are first immunolabeled with primary antibodies and then with secondary antibodies conjugated to complementary oligonucleotides (PLA probes), which can only ligate and are amplified if the immunolabeled proteins stand in close proximity (below 16 nm). In this case, it is generated a signal that is visualized as fluorescent puncta.

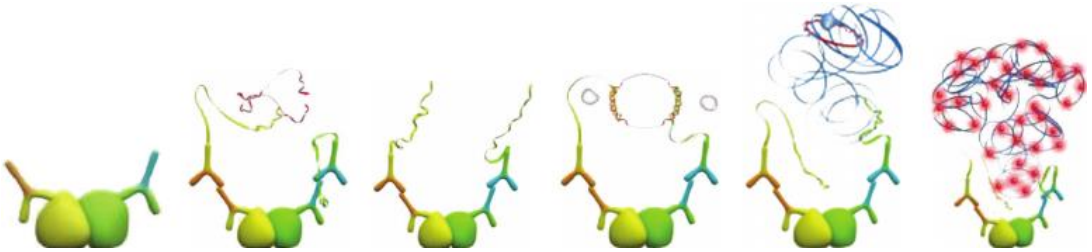

Fig. 15: Schematic representation of PLA technique. PLA probes with complementary oligonucleotides bond to the primary antibodies and when the distance is bellow 16 nm, they ligate and are amplified. The amplification products between DNA probes linked to primary antibodies generate fluorescent signals.

PLA was performed using a Duolink® Assay Kit from Sigma-Aldrich, accordingly with the manufacturer's instruction and with slight modifications (see Matos et al., 2013). In brief, astrocytes cultured on coverslips, 25,000 cells/cm^2, previously fixed with PFA as described in 3.2.7; were rinsed with PBS and permeabilized with 0.2% triton X-100 in PBS solution, for 10 minutes. Then, the cells were incubated with PBS containing 0.3% BSA and 5% horse serum for 1 hour at room temperature to block the unspecific binding. Afterwards, the cells were incubated with the primary antibodies: mouse anti-$A_{2A}R$ (1:200) and rabbit anti-Cx43 (1:600) in PBS plus 5% FBS, for 2 hours at room temperature. After washing the coverslips using PBS solution, the cells were incubated with the PLA secondary probes anti-rabbit PLUS and anti-mouse MINUS (diluted 5x in 0.1M Tris and 0.9% w/v NaCl, pH 7.4), during 1 hour at 37°C. The detection step was further initiated by washing twice the samples with Duolink II Wash Buffer A followed by an incubation with ligation-ligase solution, for 30 minutes at 37°C in a humidified chamber. The cells were washed again with Duolink II Wash Buffer A and incubated with DNA polymerase (1:40) in the amplification solution (Olink Bioscience) for 100 minutes at 37°C, protected from light. After several washes with Duolink II Wash Buffer B, the coverslips with the cells are mounted with Duolink In Situ mounting medium that contains

DAPI, and further analysed in a fluorescence microscope (Zeiss, model imager Z2 with Axiovision software 4.8).

3.2.9. Western blot

The Western blot assay (also named as immunoblot) allow identifying and making a relative quantification of specific proteins from a complex mixture of proteins extracted from cells. The technique consists of 3 major steps: (i) separation of proteins by size, (ii) transfer of proteins to a solid support, and (iii) marking the target protein using a specific primary antibody that is further bound by a secondary antibody to visualize (Lee, 2007).

After determining the amount of protein in the samples (astrocytes lysates) using the Bio-Rad method, the samples were normalized for a desired amount of protein and denatured in sample buffer (500 mM Tris, 600 mM DTT, 10.3% SDS, 30% glycerol and 0.012% bromophenol) at 70°C for 20 minutes. Protein samples were electrophoretically separated by SDS-PAGE using a 10% polyacrylamide resolving gel with 4% polyacrylamide stacking gel (see table 6) with running buffer (24 mM Tris, 192 mM bicine and 0.1% SDS, pH 8.3). The samples were separated initially with 80 V, and after they reached the resolving gel, a voltage of 100 V was used for the rest of the electrophoresis. Then, the proteins were electrotransferred to nitrocellulose membranes for 2 hours at 1 A, using a wet electroblotting system (Bio-Rad) and CAPS solution (10 mM CAPS, pH 11, 10% methanol). To ensure that the proteins were transferred, membranes were stained with ponceau S (5 minutes) and then washed with water. Afterwards, to block unspecific binding, the membranes were incubated in TBS-T (20 mM Tris, 137 mM NaCl and 0.1% tween 20, pH 7.6) with 5% low-fat milk for 1 hour. Then, the membranes were incubated with primary antibodies (see table 4, in section 3.1.3.) diluted in TBS-T with 1% low-fat milk, overnight, at 4°C. Subsequently, the membranes were rinsed 3 times with TBS-T and incubated with the secondary antibodies diluted in TBS-T with 1% milk for 2 hours at room temperature. After washing the membranes with TBS-T, their proteins were revealed using the enhanced chemiluminescence (ECL) solution, and the luminescence was visualized in a Chemidoc imaging system. Densities of blots bands were calculated in the Image Lab 5.2.1 software (Bio-Rad).

The reprobing of the membranes was performed by washing the membrane twice in TBS-T for 5-10 minutes, submerge the membrane twice in stripping solution (200 mM glicine, 0.1% SDS and 1% tween 20, pH 2.2) for 15 minutes and wash the membrane in TBS-T for 5-10 minutes. Afterwards, the membranes were blocked, incubated with the primary antibodies and secondary antibodies, until the visualization and quantification of proteins of interest, similarly to the procedures described above.

Table 6: Gel Formulation

Gel Formulation (2 gels)	10% - Resolving Gel	4% - Stacking Gel
Acrylamide	3.3 mL	1.3 mL
H_2O	4.1 mL	6.1 mL
Tris-HCl, 1.5M, pH 8.8	2.5 mL	-
Tris-HCl, 0.5M, pH 6.8	-	2.5 mL
TEMED	5 µL	20 µL
APS (20%)	50 µL	200 µL

3.2.10. Statistical Analysis

Data were expressed as percentage in comparison to control cells (control = 100%) and presented as mean ± SEM of the number of independent experiments specified in figure captions. Statistical significance was determined using the one sample *t* test comparing with the hypothetical value of 100 (control) and two-way ANOVA followed by a post-hoc Tukey's multiple comparisons test for mean comparisons with the control group or between multiple groups, respectively. The confidence interval was set as 95% so that the difference between means was considered significant at p values of less than 5% (0.05), 1% (0.01) and 0.1% (0.001) of significance level (α). All data were analysed using GraphPad Prism software (Version 6.0).

CHAPTER 4| RESULTS

4.1. Characterization of astrocytic primary cultures

The results that we will present below were obtained using primary cultures of astrocytes. This type of cell cultures obtained from either brain rats or brain mice was previously used by our group and shown to be enriched in astrocytes, since most of the cells are immunolabelled for GFAP, an astrocytic marker (Matos et al., 2008; Matos et al., 2012a). However, since these cultures were no longer used for some time in our group we start by verifying the purity of our cell cultures, assessing the number of astrocytes and microglia, another type of glial cells that can be present in the cultures. Thus, we performed immunocytochemistry using antibodies against GFAP and CD11b, a microglia marker (Lee et al., 2011) in cultured astrocytes obtained from brain of Wistar pups (P4-P5). As can be observed in figure 16, the number of CD11b was very reduced comparatively to the number of astrocytes immunolabelled with GFAP. Thus, we had ensured that our cultures are enriched in astrocytes, having only a negligible number of microglia (<1%).

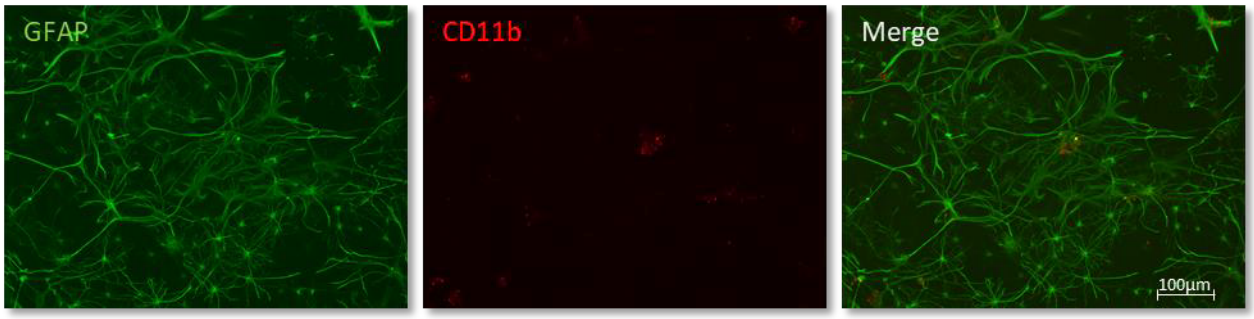

Fig. 16: Characterization of astrocyte primary cultures Representative images of immunocytochemical labelling of cultured astrocytes with GFAP (first column, green), CD11b (second column, red) and merged images (third column). The images were taken in a fluorescence microscope with a magnification of 20x. Scale bar - 100 µm.

4.2. Effect of $A\beta_{1-42}$ exposure on astrocytic ATP release

ATP release has been shown to play crucial functions in diverse brain processes, such as in synaptic transmission, cell differentiation and proliferation (Kang et al., 2008). Moreover, a

sustained increase of extracellular ATP acts as danger signal in the brain (Rodrigues et al., 2015).

In a first step, we performed studies to investigate how AD-like conditions affect ATP release by astrocytes. To this purpose, primary cultures of astrocytes were exposed to $A\beta_{1-42}$ 1 μM (Matos et al., 2012a), the putative causative agent of AD, for different time periods ranging from 5 minutes to 24 hours. As can be seen in figure 17 A, $A\beta_{1-42}$ significantly increased ATP release by astrocytes for all time points tested. The heighten in ATP release was more robust at 1 hour of acute peptide exposure (120% higher than control; 220.2 ± 31.8%), whereas for 24 hours (chronic exposure) the increase was not so high (53%; 153.2 ± 21.1%). This effect by prolonged exposure to $A\beta_{1-42}$ peptides on astrocytes was likely due to the metabolism of ATP (as discussed ahead). Curiously, also 5 minutes of $A\beta_{1-42}$ exposure caused a significant increase (40% above control, 142.6 ± 16.4%, $p<0.05$) in the amount of ATP released by astrocytes.

It should be referred that initially we performed studies using cultured astrocytes obtained from brains of C57BL/6 mice (Fig. 17 B). In these experiments, the ATP release by astrocytes acutely (204.1 ± 79.5%) and chronically (140.6 ± 0.6%) exposed to $A\beta_{1-42}$ was also increased relatively to non-treated control cells (100%). Although, the number of experiments performed with these mice astrocytes are low we can infer that the effect of $A\beta_{1-42}$ on ATP released by astrocytes was comparable in cells obtained from brain mice and from brain rats. Thus, in the further studies, we used cultured astrocytes obtained from brain Wistar rats, since they allow to obtain great number of cells, allowing thus minimize the use of animals.

Taken together, the data indicate without a doubt that AD-like conditions triggered an increase in the release of ATP by astrocytes.

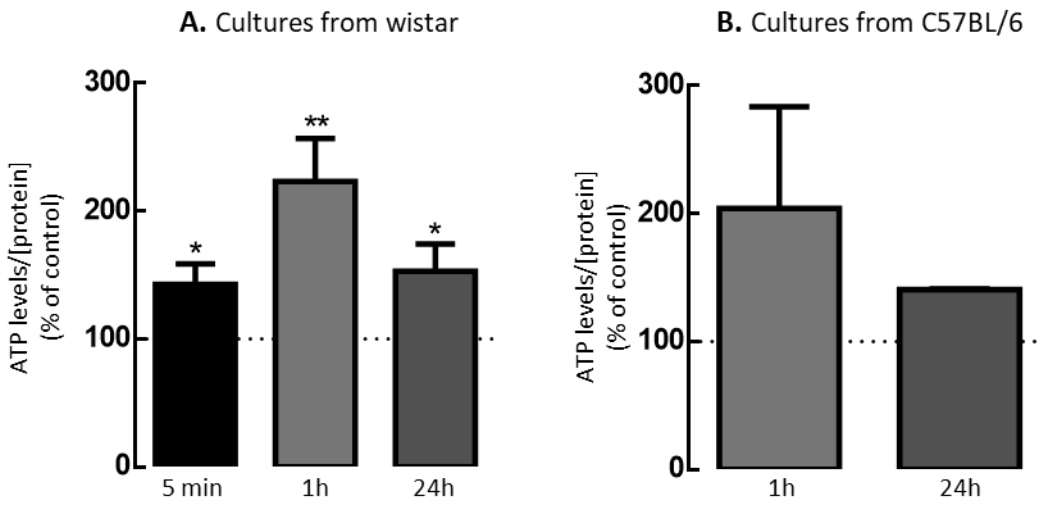

Fig. 17: ATP release by cultured astrocytes exposed to $A\beta_{1-42}$ for different time periods. Graphs show quantitative analysis of ATP release by cultured astrocytes isolated from Wistar pups exposed to $A\beta_{1-42}$ 1 µM for 5 minutes, 1 or 24 hours **(A),** and cultured astrocytes from brains of C57BL/6 mice exposed to $A\beta_{1-42}$ for 1 or 24 hours **(B)**. Bars represent the levels of extracellular ATP normalized by protein concentration of astrocyte lysates presented as percentage of control (non-treated cells, 100%). The data are mean ± SEM of 6 - 12 independent experiments in graph A; plus, data of 2 independent experiments for each time point in graph B. *$p < 0.05$, **$p < 0.01$ as compared with control cells (100%), one sample *t*-test comparing with the hypothetical value of 100.

4.2.1. Controls of ATP experiments

Throughout the first experiments of ATP quantification in the extracellular medium (release) we observed a great variability and inconsistency in the results, which led to the necessity of a positive control to ensure the accuracy of the obtained data. For this control, we used norepinephrine (NE) that was already shown to exponentially increase ATP release in glial cells after only 5 minutes of exposure (Gordon et al., 2005). To test if this compound would be a good control, we did a series of incubations with NE 30 µM and the selective agonist of metabotropic glutamate receptor 5 (mGluR5), CHPG 500 µM, since the mGluR5 activation was also shown to increase ATP release in cultured astrocytes (Shrivastava et al., 2013). As expected, the data obtained showed that NE led to a marked increase in ATP release (346% above control; 446.5 ± 90.2%) by astrocytes when compared to control cells, (100%; $p<0.01$). We also observed that CHPG increased ATP release by 396% (496.6 ± 241.8%)

relatively to control cells (Fig. 18 A). However, in same preparations of astrocytes cultures it was found that when CHPG did not led to an obvious upregulation of ATP release, NE also did not lead to a pronounced increase in the release of this gliotransmitter (Fig. 18 B). To corroborate this observation, we tested if there was a correlation between ATP release by astrocytes exposed to NE or exposed to CHPG, and we found a strong positive correlation, $r = 0.9599$, $p = < 0.05$, with a $R^2 = 0.9214$ (Fig. 18 C). Considering these results, we established NE as a positive control of our ATP release experiments.

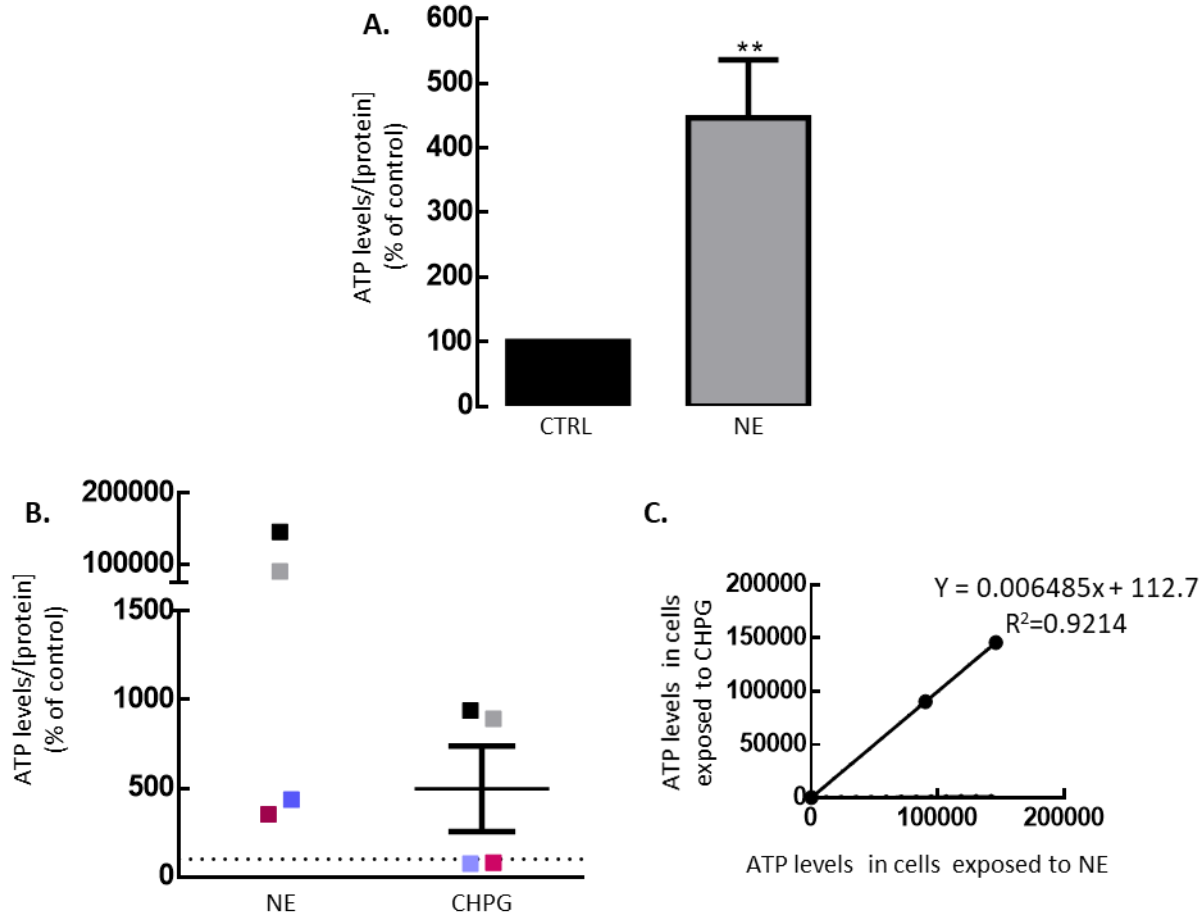

Fig. 18: Effect of NE and CHPG exposure on ATP release by cultured astrocytes. Panel **A** shows the quantitative analysis of ATP release by astrocytes exposed to NE 30 μM for 5 minutes and non-treated cells (control). Data are presented as the means ± SEM of 14 independent experiments and are expressed as percentage of control. **p < 0.01 as compared with control cells (100%), one sample *t*-test comparing with the hypothetical value of 100. Panel **B** shows the quantification of ATP release by astrocytes exposed to NE 30 μM for 5 minutes and to CHPG 500 μM for 1 hour, and panel **C** shows the correlation between the two stimuli. Data are means ± SEM of 4 independent experiments. Bars/points in the graphs represent the levels of extracellular ATP normalized by protein concentration of cell lysates.

4.3. Effect of $A\beta_{1-42}$ exposure on glutamate release

Given the important role that glutamate has in synaptic transmission and excitotoxic degeneration in AD (Esposito et al., 2013), we also investigated how AD-like conditions affect astrocytic glutamate release. To this purpose, primary cultures of astrocytes were exposed to $A\beta_{1-42}$ 1 μM for 1 hour, and the levels of glutamate in the extracellular medium were quantified (Fig. 19). The data showed that acute exposure (1 hour) to $A\beta_{1-42}$ significantly ($p < 0.05$) increased glutamate release by 40% (140.8 ± 11.8%) relatively to non-treated cells (control, 100%).

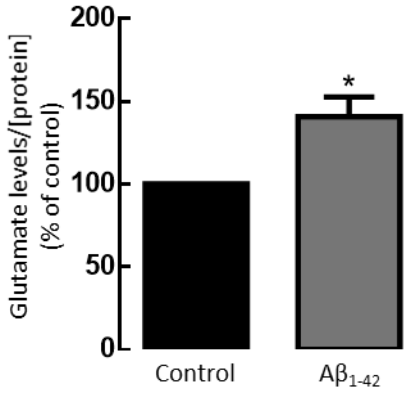

Fig. 19: Effect of acute exposure to $A\beta_{1-42}$ on glutamate release by cultured astrocytes. Astrocytes were incubated with $A\beta_{1-42}$ 1 μM for 1 hour and then the levels of glutamate in the extracellular medium was measured. Bars represent the levels of extracellular glutamate normalized by protein concentration of astrocyte lysates and are expressed as percentage of control (non-treated cells). Data are the means ± SEM of 7 independent experiments. *$p < 0.05$ as compared with control cells (100%), one sample *t*-test comparing with the hypothetical value of 100.

4.4. Mechanisms involved in gliotransmitters release triggered by $A\beta_{1-42}$ exposure

After establishing how AD-like conditions affect the ATP and glutamate release, we inquired about the possible mechanisms involved in the release of these gliotransmitters and

whether they are affected by $A\beta_{1-42}$ exposure. As already described (see section 1.2.5), gliotransmitters release can occur through several different mechanisms, namely through hemichannels (HC) and P_2X_7 receptors among others (Hamilton and Attwell, 2010).

4.4.1. Hemichannels (HC)

Astrocyte HC are composed by six proteins subunits that can be of two distinct families, the connexins (Cx) and the pannexins (Panx; Karpuk et al., 2011; Orellana et al., 2013). Connexins 43 (Cx43) are mainly expressed in astrocytes forming either HC or gap junctions channels (GJC), having a crucial role in controlling the astrocytic function (Orellana et al., 2013; Orellana et al., 2012). These Cx had been shown to be associated with reactive astrocytes at sites of Aβ plaques in AD (Nagy et al., 1996). However, little is known about the role of HC in gliotransmission under pathological conditions. So, in an attempt to better understand how AD-like conditions affect astrocytic HC and gliotransmitters release, we resort to pharmacological blockade of HC. For this purpose, we used the Cx/Panx HC blocker carbenoxolone (CBX; Nodin et al., 2005).

4.4.1.1. Effect of acute exposure to CBX on astrocytic viability

In order to define the concentrations of CBX to use in cultured astrocytes, and having in account some hints from literature, we performed a MTT assay to evaluate if the CBX at concentration used in other studies and biological models, affects the metabolic state (redox-capacity) and consequently the viability of cultured astrocytes. Therefore, the astrocytes were incubated with different concentrations of CBX (25, 50 and 100 μM) for 90 minutes and 24 hours, before performing the MTT assay (Fig. 20). The obtained data showed that acute exposure (90 minutes) to the different concentrations of CBX tested did not affect the viability of astrocytes. No statically significant decrease in MTT reduction was observed in cells incubated with 25 μM (104.9 ± 5.0%; p=0.4); 50 μM (113.6 ± 14.0%; p=0.4) or 100 μM (94.3 ± 18.0%; p=0.8) in comparison to non-treated control cells (100%). However, a more prolonged exposure to CBX (24 hours) markedly decreased the reduction MTT to formazan

crystals showing an impairment in the metabolic activity, which reflects a decrease in astrocytes viability. Even the lowest concentration of CBX, 25 µM, lead to an obvious and more importantly significant decrease in MTT reduction (67.5 ± 2.1%, $p < 0.001$) relatively to the non-treated cells. This effect seemed to be exacerbated with higher concentrations of CBX, indeed, astrocytes incubated with 50 µM (59.2 ± 1.8%, $p < 0.001$) or 100 µM (59.0 ± 2.4%, $p < 0.001$) was observed a higher decrease of MTT reduction in comparison to control (100%).

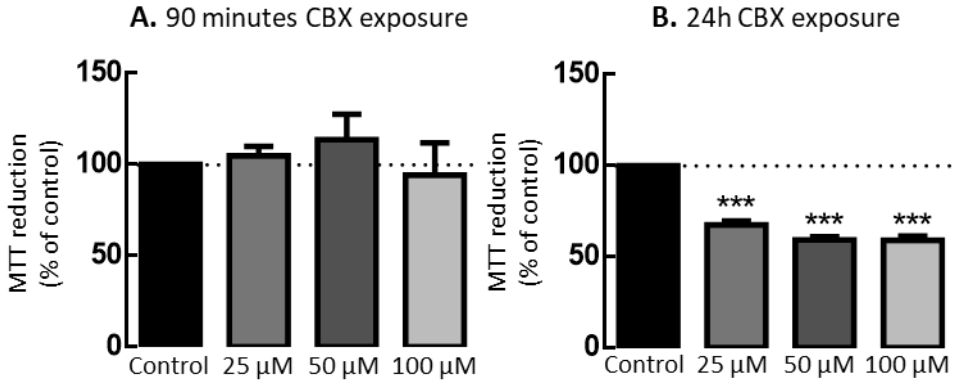

Fig. 20: Effect of CBX exposure on the astrocytes viability. Cultured astrocytes were treated or not (control) with different concentrations of CBX (25, 50 and 100 µM) for 90 minutes (A) and 24 hours (B). The reduction of MTT to formazam crystals was expressed in percentage relatively to the absorbance values of the control cells. Data are the means ± SEM of 4 independent experiments. ***$p < 0.001$ as compared with control cells (100%), one sample *t*-test comparing with the hypothetical value of 100.

4.4.1.2. ATP release through HC in non-pathological and in AD conditions

Cultured astrocytes were incubated with the HC blocker, CBX 50 µM, which was added to the cells 30 minutes before exposure to $A\beta_{1-42}$ 1 µM for 1 hour (Fig. 21). Our results showed that in conditions of acute exposure to $A\beta_{1-42}$, CBX significantly ($p<0.01$) decreased ATP release by about 110% in relation with cells solely treated with $A\beta_{1-42}$ (161.4 ± 19.5%). The levels of ATP released by astrocytes challenged with $A\beta_{1-42}$ in the presence of CBX (47.0 ± 11.2%) was even lower than the levels observed in control cells (100%; $p > 0.05$). Interestingly, we also found that cells exposed to CBX *per se* released about 53% less ATP (47.5 ± 18.2%)

than control cells. No significant differences were found in ATP release between cells treated with CBX *per se* and cells incubated with CBX plus $A\beta_{1-42}$ ($p > 0.05$). It should be refered that we also intended to test the effect of CBX on ATP release in astrocytes chronically exposed to $A\beta_{1-42}$ (24 hours); however, this did not make sense because MTT assay showed that chronic exposure to CBX affected the viability of astrocytes.

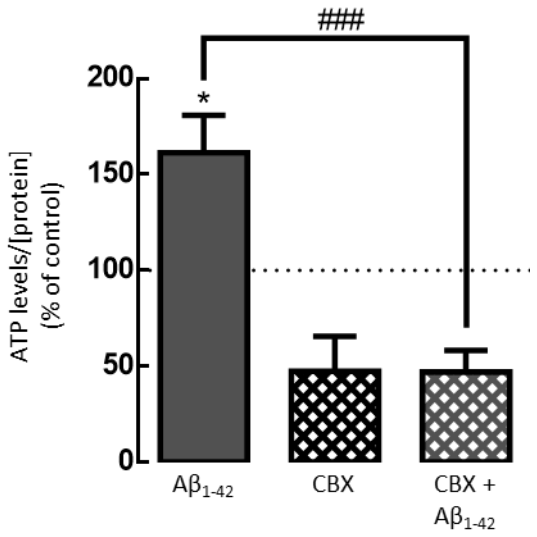

Fig. 21: Effect of HC blockade on ATP release by astrocytes acutely exposed to $A\beta_{1-42}$. The cells were treated with the inhibitor of HC, CBX 50 μM, 30 minutes before exposure to $A\beta_{1-42}$ 1 μM for 1 hour. Bars represent the levels of extracellular ATP normalized by protein concentration of astrocyte lysates and are presented as percentage of control (non-treated cells). The data are the mean ± SEM of 5 independent. * $p < 0.05$ as compared with control cells (100%). ### $p < 0.001$ as compared to $A\beta_{1-42}$-treated cells, two-way ANOVA, post-hoc Tukey's test.

To further complement this study, we also investigate the effect of HC blockade on NE-induced increase in ATP release. Astrocytes were treated with CBX 50 μM 30 minutes before the challenge with NE 30 μM, for 5 minutes (Fig. 22). A similar pattern in ATP release was observed when the cells where incubated with NE instead of $A\beta_{1-42}$. As expected NE led to an increase in ATP release (446.8%), meanwhile CBX *per se* led to a marked decrease in the release of ATP (30.2%) about 70% less than the control astrocytes. Moreover, in astrocytes incubated with CBX plus NE, CBX visibly prevented the increase in ATP release triggered by NE, decreasing the release of this gliotransmitter to levels similar to non-treated control cells, being the amount of ATP released in astrocytes exposed to CBX plus NE (for 5 minutes) of 90% relatively to the control (100%).

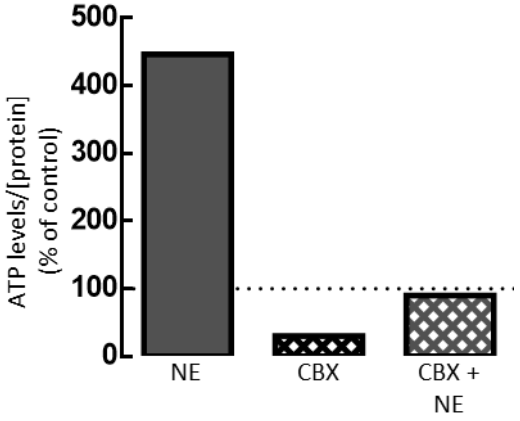

Fig. 22: Effect of HC blockade on ATP release by astrocytes exposed to NE. The cells were treated with inhibitor of HC, CBX 50 μM, 30 minutes before exposure to NE 30 μM for 5 minutes. Bars represent the levels of extracellular ATP normalized by protein concentration of astrocyte lysates and are presented as percentage of control (non-treated cells). The presented data are of 1 experiment.

Taken together the presented data suggest that the increased ATP release triggered by $A\beta_{1-42}$, and also by NE, occurred in a large part through Cx/Panx HC; and these channels seemed also to contribute to the tonic ATP release by astrocytes in non-pathological conditions.

4.4.1.3. Glutamate release through HC in non-pathological and in AD conditions

Furthermore, we also studied the effect of HC inhibitor on glutamate release triggered by acute exposure (1 hour) to $A\beta_{1-42}$ (Fig. 23). Interestingly, we found that in astrocytes solely exposed to CBX there was an increase in glutamate release of about 265% (365.0 ± 49.0%) relatively to non-treated cells, control (100%; $p < 0.01$). Similarly, we found that in astrocytes treated with CBX and further challenged with $A\beta_{1-42}$, the release of glutamate increased even more (526.1 ± 131.1%), and was statistically different from the $A\beta_{1-42}$-treated cells (154.8 ± 23.8%; $p < 0.01$) and also from the non-treated control cells ($p < 0.001$). Although, the obtained data suggests the existence of differences between glutamate released by astrocytes treated with CBX *per se* (365.0 ± 49.0%) and astrocytes treated with CBX and $A\beta_{1-42}$ (526.1 ±

131.1%), statistical analysis failed to show significant differences between the two conditions ($p > 0.05$).

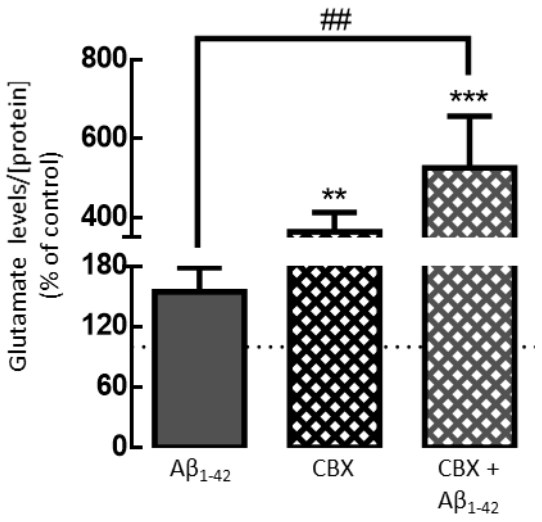

Fig. 23: Effect of HC blockade on glutamate release by astrocytes acutely exposed to $A\beta_{1-42}$. The cells were treated with inhibitor of HC, CBX 50 μM, 30 minutes before exposure to $A\beta_{1-42}$ 1 μM for 1 hour. Bars represent the levels of extracellular glutamate normalized by protein concentration of astrocyte lysates and are presented as percentage of control (non-treated cells). The data are the mean ± SEM of 3 independent experiments. ** $p < 0.01$; *** $p < 0.001$ as compared with control cells (100%). ## $p < 0.01$ as compared to $A\beta_{1-42}$-treated cells, two-way ANOVA, post-hoc Tukey's test.

These unexpected findings might indicate that the blockade of HC, and consequent the reduction of ATP release, promote the release of glutamate by astrocytes both in non-pathological and in AD-like conditions. With this set of experiments, using CBX, we demonstrated that the release of ATP by astrocytes triggered by $A\beta_{1-42}$ was mediated mainly through HC, which seem also to impact on astrocytic glutamate release in a different manner. However, it would be interesting to use blockers more specific for the Cxs and Panxs that form the HC.

4.4.2. P_2X_7 receptors

The ionotropic purinergic receptors P_2X_7 (homomeric) possess a pore that allows the passage of relatively large molecules (900 Da). This type of receptors is abundant in astrocytes and may be up-regulated after injury (Malarkey and Parpura, 2008). The release of ATP and glutamate through P_2X_7 receptors has already been described (Harada et al., 2016), however little is known about alterations in this pathway in AD conditions. Thus, in order to fill this gap, we investigated whether the astrocytic P_2X_7 receptors are mediating the increased release of ATP and glutamate under AD-like conditions. To this purpose, it was used the selective antagonist of P_2X_7 receptors, brilliant blue G 100 nM (BBG, Carmo et al., 2014), which was added to the astrocytes 30 minutes before incubation with $A\beta_{1-42}$ for 1 hour.

4.4.2.1. ATP release through P_2X_7 receptors in non-pathological and in AD conditions

Despite the number of experiments gathered so far are scanty, the data showed the antagonist of P_2X_7 receptor BBG, *per se* increased by about 150% the ATP release (258.6 ± 127.1%) in comparison to control cells (100%). However, it appears that there was a decrease of about 46% in ATP release in cells exposed to BBG plus $A\beta_{1-42}$ (174.4 ± 53.3%) in comparison to $A\beta_{1-42}$-treated astrocytes (220.2 ± 107.8%), suggesting that the blockade of P_2X_7 receptors slightly prevented the increased astrocytic ATP release triggered by $A\beta_{1-42}$ exposure. To complement this study, we also investigate the effect of P_2X_7 receptors blockade on the NE-induced increase in ATP release. Figure 24 B shows a similar pattern in the effect of BBG on ATP release by astrocytes exposed to NE. BBG also slightly prevented the increased ATP release triggered by NE (reduction of about 40%; 203.0 ± 113.4% relatively to 243.4 ± 141.8% in astrocytes treated solely with NE). BBG *per se* enhanced the ATP release (258.6 ± 127.1%) above the control. These data are alike to those obtained when the astrocytes were challenged with $A\beta_{1-42}$ in the presence of BBG.

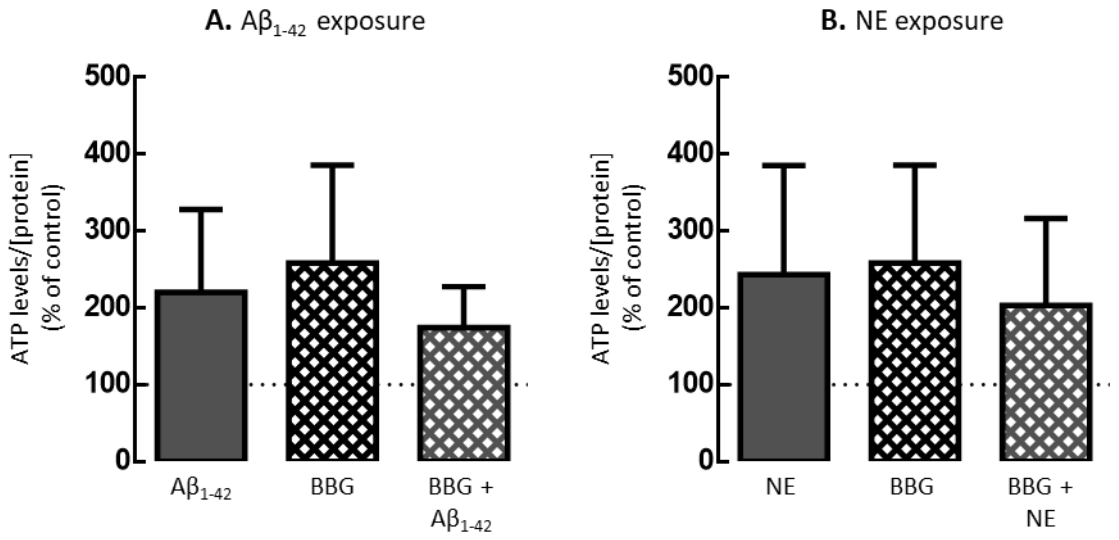

Fig. 24: Effect of P_2X_7 receptor blockade on ATP release by astrocytes acutely exposed to $A\beta_{1-42}$ and NE. The cells were treated with the selective antagonist of P_2X_7 receptors, BBG 100 nM, 30 minutes before exposure to $A\beta_{1-42}$ 1 µM for 1 hour **(A)** or NE 30 µM for 5 minutes **(B)**. Bars represent the levels of extracellular ATP normalized by protein concentration of astrocyte lysates and are presented as percentage of control (non-treated cells). The data are the mean ± SEM of 2 independent experiments.

4.4.2.2. Glutamate release through P_2X_7 receptors in non-pathological and in AD conditions

We also studied the effect of P_2X_7 receptors antagonism on the release of glutamate by astrocytes acutely exposed (1 hour) to $A\beta_{1-42}$ (Fig. 25). The selective antagonist of P_2X_7 receptor, BBG, seemed to increase the amount of glutamate released by astrocytes (287.8 ± 156.9%) in comparison to control cells (100%) and to astrocytes solely treated with $A\beta_{1-42}$ (127.9 ± 15.1%). This effect appeared to be further exacerbated by exposure to $A\beta_{1-42}$ (302.3 ± 133.5%). Therefore, no valid conclusion can be inferred from these studies because of the low number of experiments and high variance of results (large SEM), thereby more experiments should be done with the antagonist of P_2X_7 receptors.

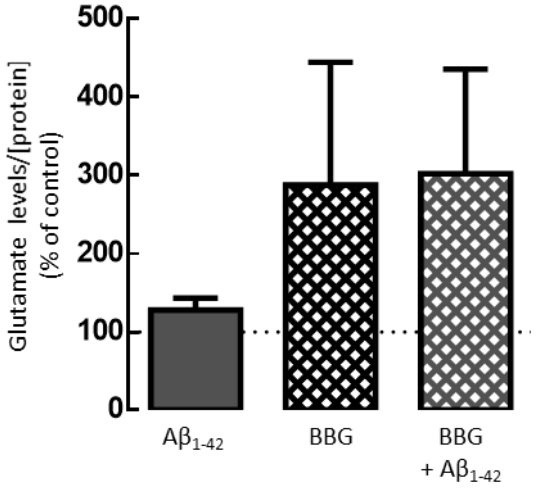

Fig. 25: Effect of P_2X_7 receptors blockade on glutamate release by astrocytes acutely exposed to $A\beta_{1-42}$. The cells were treated with the selective antagonist of P_2X_7 receptors, BBG 100 nM, 30 minutes before exposure to $A\beta_{1-42}$ 1 μM for 1 hour. Bars represent the levels of extracellular glutamate normalized by protein concentration of astrocyte lysates and are presented as percentage in comparison to control (non-treated cells). The data are the mean ± SEM of 2 independent experiments.

Regarding the experiments with the antagonist of P_2X_7 receptors, BBG, no conclusions can be taken because of high variability of the obtained data (large SEM) and the small number of experiments preformed. Thus, it is imperative to further increase the number of experiments to clarify if the P_2X_7 receptors contribute to increased release of ATP in astrocytes exposed to $A\beta_{1-42}$. It should be also referred that the concentration of BBG used (100 nM) was the same previously used by our group in cultured cells (Carmo et al., 2014), and it did not caused toxicity. Moreover, although the BBG in solution has blue color (stock solution of 25 μM) we tested if at the concentration used in the cells (which is incolour at eye) there was interference with the emission (in case of ATP) or absorption of light (in case of glutamate), but we didn´t saw any effect.

4.5. Control by adenosine A_{2A} receptors ($A_{2A}R$) of gliotransmitters release triggered by $A\beta_{1-42}$ exposure

Increasing evidences, including some gathered by our group, demonstrate that $A_{2A}R$ have a critical role in controlling synaptic function (Gomes et al., 2011; Orr et al., 2015), and it was already shown the blockade of $A_{2A}R$ confer neuroprotection in AD animal models (Canas et al., 2009). Thus, we prompted to investigate whether the pharmacological blockade of $A_{2A}R$ would have a protective effect on the $A\beta_{1-42}$-induced increase in gliotransmitters, using the selective antagonist of $A_{2A}R$ SCH 58261 (Matos et al., 2012a).

4.5.1. Modulation by $A_{2A}R$ of ATP release in non-pathological and in AD conditions

To study if $A_{2A}R$ was a possible modulator of ATP release in AD-like conditions, we resort to pharmacological blockade of this receptor. For this purpose, we used the $A_{2A}R$ selective antagonist, SCH 58261, which was added to the cells 30 minutes before exposure to $A\beta_{1-42}$ 1 μM for 1 hour (Fig. 26 A) or 24 hours (Fig. 26 B).

The results obtained showed that SCH 58261 50 nM prevented the increase in ATP release triggered by acute exposure to $A\beta_{1-42}$. In fact, we found that there was a significant decrease of about 164% in ATP released by cells treated with SCH 58261 and $A\beta_{1-42}$ (89.3 ± 20.4%) relatively to cells only treated with $A\beta_{1-42}$ (253.3 ± 53.2%; $p < 0.05$). No significant differences were found between ATP released by astrocytes incubated solely with SCH 58261 (87.9 ± 16.8%) and SCH 58261 plus $A\beta_{1-42}$ (89.3 ± 20.4%; $p > 0.05$), and between these conditions and the control (100%; $p > 0.05$).

After established the effect of $A_{2A}R$ antagonism on ATP release by astrocytes acutely exposed to $A\beta_{1-42}$, we aimed to investigate if the same protective effect was observed in conditions of chronic exposure to $A\beta_{1-42}$. The obtained data showed that $A_{2A}R$ blockade seemed to modulate the effect of chronic $A\beta_{1-42}$ exposure on ATP released by astrocytes. Although, the differences were not statistically significant there was a clear decline in ATP

release in astrocytes treated with $A\beta_{1-42}$-plus SCH 58261 (99.3 ± 12.9%) in comparison to cells only treated with $A\beta_{1-42}$ (159.1 ± 23.0%). The SCH 58261 *per se* also decreased the tonic ("basal") release of ATP relatively to non-treated control cells (75.6 ± 18.2%), although without statistical significance ($p > 0.05$). These findings suggest that the blockade of $A_{2A}R$ have a protective effect on the enhancement of ATP release triggered by acute and chronic exposure to $A\beta_{1-42}$.

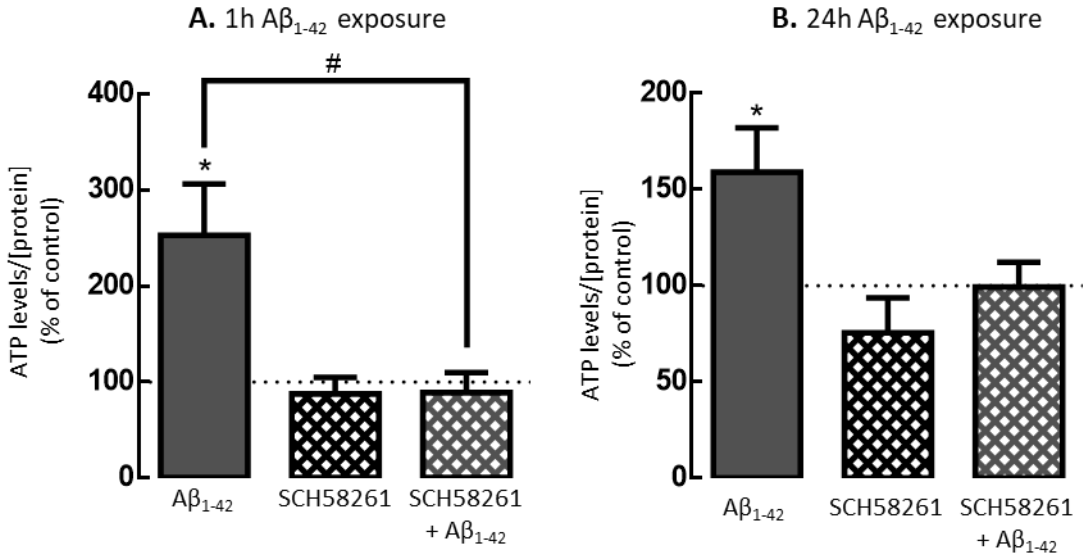

Fig. 26: Effect of $A_{2A}R$ blockade on ATP release by astrocytes acutely and chronically exposed to $A\beta_{1-42}$. The cells were treated with the selective antagonist of $A_{2A}R$, SCH 58261 50 nM, 30 minutes before exposure to $A\beta_{1-42}$ 1 μM for 1 hour (**A**) or 24 hours (**B**). Bars represent the levels of extracellular ATP normalized by protein concentration of astrocyte lysates and are presented as percentage of control (non-treated cells). The data are mean ± SEM of number of experiments 5 - 8 independent experiments. * $p < 0.05$ as compared with control cells (100%). # $p < 0.05$ as compared to $A\beta_{1-42}$-treated cells, two-way ANOVA, post-hoc Tukey's test.

To further complement this study, we also investigated the effect of $A_{2A}R$ blockade on NE-induced increase in ATP release. Astrocytes were treated with SCH 58261 50 nM 30 minutes before exposure to NE 30 μM for 5 minutes (Fig. 27). Curiously, a different pattern in ATP release was found when the cells where challenged with NE instead of $A\beta_{1-42}$. The SCH 58261 seemed to prevent only partially the NE-induced increase in ATP release (379.6 ± 195.7% relatively to 735.9 ± 180.2% in astrocytes solely exposed to NE), suggesting that $A_{2A}R$ were not controlling the release of ATP triggered by NE for 5 min.

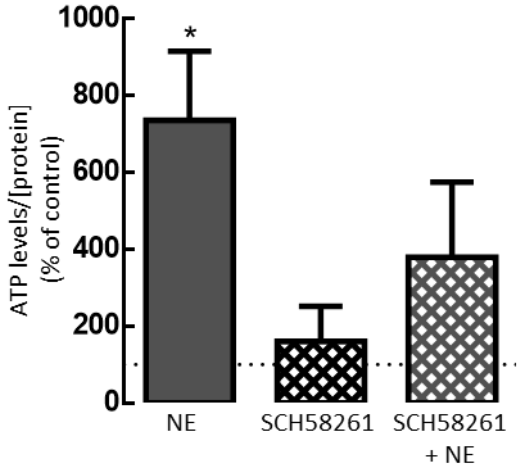

Fig. 27: Effect of $A_{2A}R$ blockade on ATP release by astrocytes exposed to NE. The cells were treated with the selective antagonist of $A_{2A}R$, SCH 58261 50 nM, 30 minutes before exposure to NE 30 μM for 5 minutes. Bars represent the levels of extracellular ATP normalized by protein concentration of astrocyte lysates and are presented as percentage of control (non-treated cells). The data of are the means ± SEM of 5 independent experiments. * $p < 0.05$ as compared with control cells (100%), two-way ANOVA, post-hoc Tukey's test.

4.5.2. Modulation by $A_{2A}R$ of glutamate release in non-pathological and in AD conditions

Furthermore, we also studied the effect of $A_{2A}R$ antagonism on glutamate release in conditions of acute exposure to $A\beta_{1-42}$. Astrocytes were treated with SCH 58261 50 nM 30 minutes before exposure to $A\beta_{1-42}$ 1 μM for 1 hour (Fig. 28). The results obtained showed that SCH 58261 50 nM had no effect on Aβ-induced alterations on glutamate release. Indeed, we did not find any differences between cells exposed to $A\beta_{1-42}$ and SCH 58261 (146.0 ± 19.4%) and cells only treated with $A\beta_{1-42}$ (138.1 ± 15.1%), the glutamate released in these two conditions was similar ($p > 0.9999$). In astrocytes exposed only to SCH 58261 the release of ATP (102.1 ± 13.6%; $p < 0.05$) was similar to that observed in control (non-treated astrocytes, 100%). These data suggest that $A_{2A}Rs$ were not controlling glutamate release neither in "non-pathological" nor in AD-like conditions.

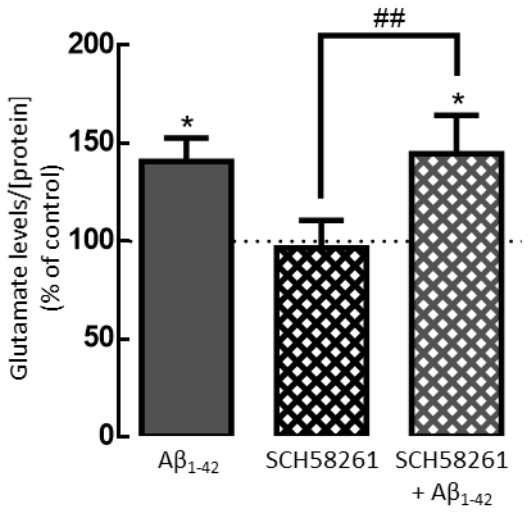

Fig. 28: Effect of $A_{2A}R$ blockade on glutamate release by astrocytes acutely exposed to $A\beta_{1-42}$. The cells were treated with the selective antagonist of $A_{2A}R$, SCH 58261 50 nM, 30 minutes before exposure to $A\beta_{1-42}$ 1 μM for 1 hour. Bars represent the levels of extracellular glutamate normalized by protein concentration of astrocyte lysates and are presented as percentage relative to control (non-treated cells). The data are mean ± SEM of 5 -7 independent experiments. * $p < 0.05$ as compared with control cells (100%). ## $p < 0.01$ as compared to the cells treated with SCH 58261 *per se*, two-way ANOVA, post-hoc Tukey's test.

4.6. Effect of $A\beta_{1-42}$ on connexin 43: protection by $A_{2A}R$ antagonism

The Cx43 is the main connexin expressed in astrocytes, where can form HC and GJC (Orellana et al., 2016). Thus, it is likely that Cx43 HC may be one of the pathways by which in our experimental conditions $A\beta_{1-42}$ exposure increases ATP and also glutamate release. Thus, in this part of the study we investigated if $A\beta_{1-42}$ exposure (acutely or chronically) affects the Cx43. Moreover, since the $A_{2A}R$ are able to modulate ATP release in AD-like conditions, we also check out whether there was an interaction between the Cx43 and $A_{2A}R$.

4.6.1. Effect of $A\beta_{1-42}$ exposure on Cx43 levels: protection by $A_{2A}R$ blockade

First, we analyzed the Cx43 "levels" in AD-like conditions and evaluated if the blockade of $A_{2A}R$ would interfere with the expression this protein by immunocytochemistry studies. Cultured astrocytes were treated with the selective antagonist of $A_{2A}R$, SCH 58261, 50 nM for 30 minutes before the incubation with $A\beta_{1-42}$ 1 μM, for 24 hours. In addition, we also assessed the immunoreactivity of glial acidic fibrillary protein (GFAP) in the astrocytes exposed or not to $A\beta_{1-42}$ in the presence or absence of SCH 58261.

As expected, by data shown previously by our group (Matos et al. 2012a) $A\beta_{1-42}$ increased the immunoreactivity of GFAP (Fig. 29 A), the increase of this astrocytic protein and alteration in the morphology of astrocytes are usually associated with a condition of reactive astrocytes. Although this is not a novel finding, it is an essential proof to verified if in our experimental conditions $A\beta_{1-42}$ caused astrogliosis. Regarding the Cx43 immunoreactivity we also observed an increase in astrocytes exposed to $A\beta_{1-42}$. Interestingly, the $A_{2A}R$ antagonist, SCH 582621, visibly prevented the increase in density Cx43 triggered by $A\beta_{1-42}$, as well as the increased GFAP immunoreactivity triggered by this peptide. As the results of the immunocytochemical analysis are essentially qualitative, we further performed Western blot analysis to strength the data obtained by immunocytochemistry (Fig. 29 B). The data showed that $A\beta_{1-42}$ enhanced the density of Cx43 (135.9 ±17.2%) about 35% relatively to non-treated control astrocytes (100%), although it didn´t reach statistical significance ($p = 0.08$). Furthermore, $A_{2A}R$ blockade seemed to prevent the increase in Cx43 levels triggered by chronic exposure to $A\beta_{1-42}$ (118.7 ± 17.9%), being the values similar to that of control cells. These findings suggest that $A_{2A}R$ control the up-regulation of Cx43 triggered by $A\beta_{1-42}$.

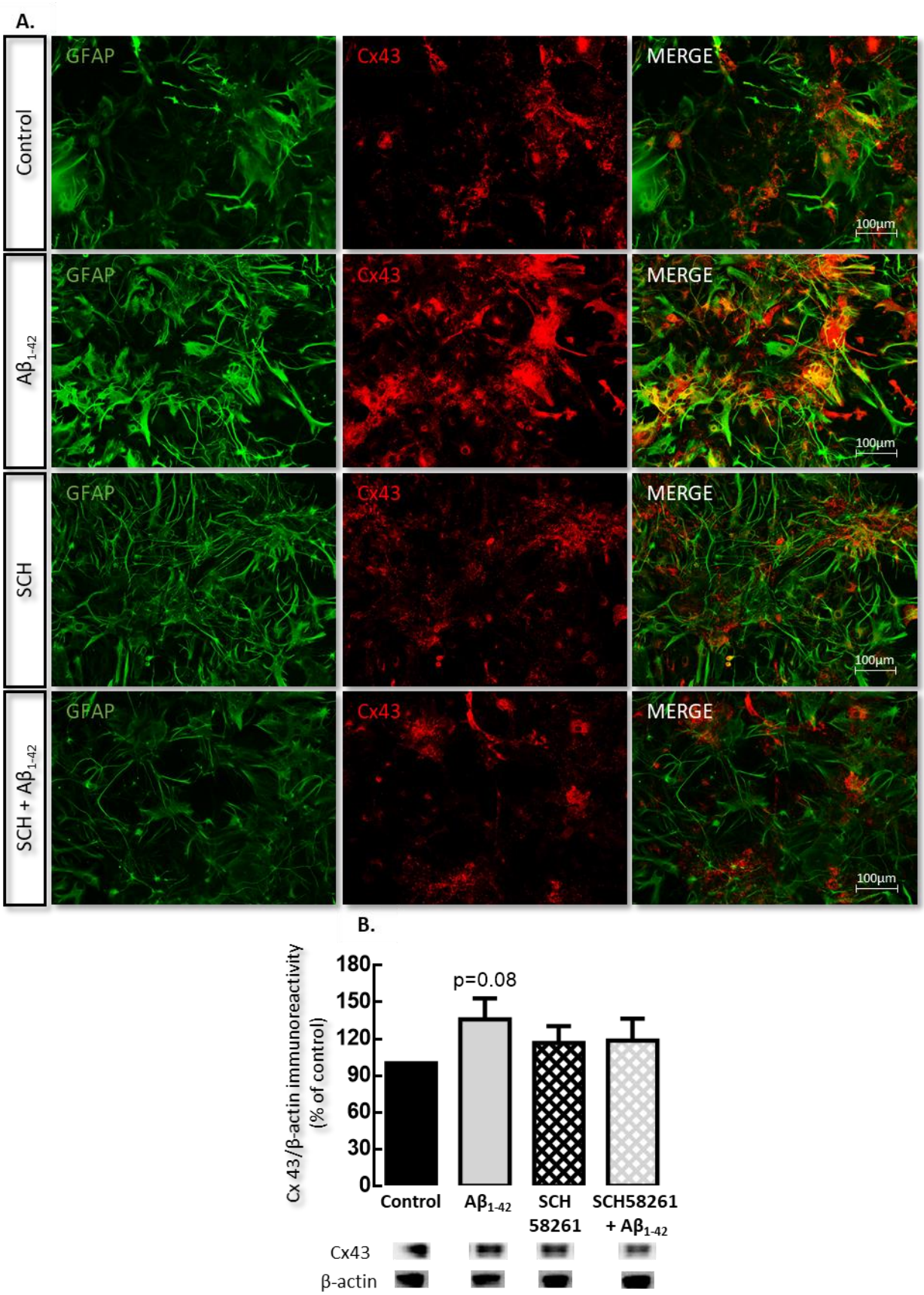
A.
Control
Aβ1-42
SCH
SCH + Aβ1-42
GFAP
Cx43
MERGE
100µm
B.
Cx 43/β-actin immunoreactivity (% of control)
180
150
120
90
60
30
0
p=0.08
Control
Aβ1-42
SCH 58261
SCH58261 + Aβ1-42
Cx43
β-actin

Fig. 29: Effect of $A_{2A}R$ blockade on $A\beta_{1-42}$-induced increase in Cx43 levels. The cells were treated with the selective antagonist of $A_{2A}R$, SCH 58261 50 nM, 30 minutes before exposure to $A\beta_{1-42}$ 1 μM for 24 hours. Panel **A** shows representative images of immunocytochemical labelling of cultured astrocytes with GFAP (first column, green), Cx43 (second column, red) and merged images (third column). The images were taken in a fluorescence microscope with a magnification of 20x. Scale bar - 100 μm. Panel **B** shows the quantitative analysis of Cx43 levels normalized by β-actin. Representative Western blots for both proteins are shown. Data are the means ± SEM of 4 - 7 independent presented as and are expressed as percentage of control (non-treated cells). One sample *t*-test comparing with the hypothetical value of 100.

4.6.2. Physical association between Cx43 and $A_{2A}R$

Since the results above showed suggest a possible interaction between the $A_{2A}R$ and Cx43, we decided to investigate whether there was a physical association between these two proteins. To this purpose, we performed a proximity ligand assay (PLA), which is a powerful technology capable of detecting protein interactions or close proximity (below 16 nm); and each detected signal is visualized as an individual fluorescent dot (see section 3.2.8).

As can be seen in figure 30, in cultured astrocytes it was detected some red fluorescent dots both in $A\beta_{1-42}$-treated in astrocytes and in control cells. Curiously, the number of dots seemed to be decreased in astrocytes exposed to $A\beta_{1-42}$ 1μM for 1 or 24 hours relatively to untreated cells (control). These data are very intriguing and we intent to perform more experiments to confirm and deepen these findings.

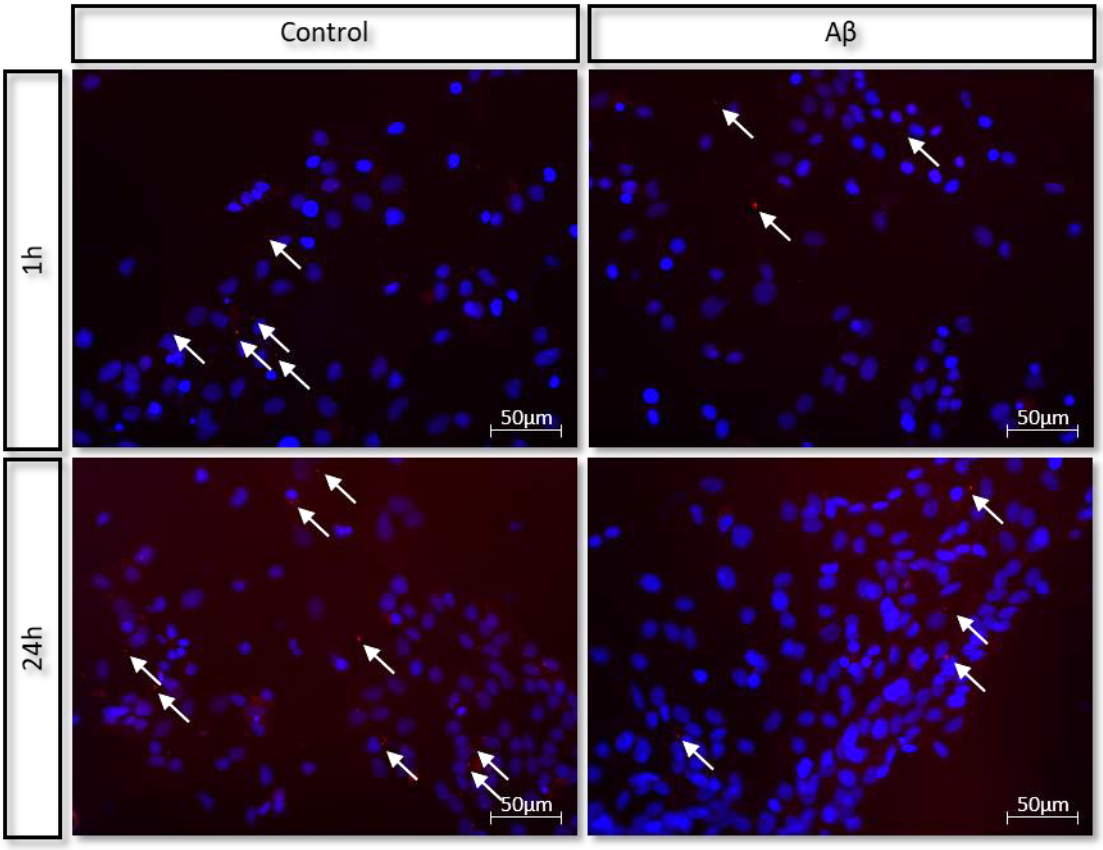

Fig. 30: $A_{2A}R$ is closely associated with Cx43. Representative images of proximity ligation assay corroborated the close proximity (below 16 nm) between $A_{2A}R$ and Cx43 in cortical astrocytes exposed to $A\beta_{1-42}$ 1 µM for 1 and 24 hours and the respective control (non-treated cells). Representative fluorescence images showing bright red spots correspond to amplification products between DNA probes linked to the anti-$A_{2A}R$ and anti-Cx43 antibodies. Two independent experiments were done. The images were taken in a fluorescence microscope with a magnification of 40x. Scale bar - 50 µm.

CHAPTER 5| DISCUSSION

5.1. Mechanisms involved in gliotransmitters (ATP and glutamate) release in AD-like conditions

Currently, it is widely accepted that astrocytes make part of the synapse, having a crucial role in the regulation of synaptic transmission through the uptake of glutamate and GABA, and also by the release of gliotransmitters, such as ATP and glutamate (Wang and Bordey, 2008; Orellana et al., 2016). The memory deficits associated to early AD phase are associated more with a synaptopathy rather than with neuronal loss (Rudy et al., 2015), thus it is likely that astrocytes might be also dysfunctional during AD conditions. In fact, increasing evidence support that $A\beta_{1-42}$ oligomers trigger reactive astrogliosis (Matos et al., 2008, 2012a), inducing phenotypes similar to those observed in plaque-associated astrocytes in human AD brain (Pike et al., 1994; Serrano-Pozo et al., 2011). These evidences are in line with our data showing that $A\beta_{1-42}$ exposure increased GFAP immunoreactivity and caused hypertrophy of astrocytes (Fig. 29), indicating that was occurring a reactive astrogliosis. These alterations in astrocytes morphology associated with reactive conditions are expected to be accompanied by alterations in astrocytic functions (Agostinho et al., 2010; Orre et al., 2014; Acosta et al., 2017). However, few studies have addressed the impact of AD conditions on astrocytic function, mainly regarding alterations in gliotransmitters release, which control synaptic function. The present study was designed to investigate the impact of AD-like conditions on the release of ATP and glutamate (two major gliotransmitters) by astrocytes and the mechanisms underlying their release. Thus, we used primary cultures of astrocytes (enriched in GFAP positive cells) exposed to $A\beta_{1-42}$ oligomers to mimic early AD-like conditions. Moreover, it was used pharmacological tools to investigate the pathways underlying the release of these gliotransmitters and the involvement of adenosine $A_{2A}R$ in controlling the astrocytic ATP and glutamate release.

It is important to refer that for describing our experimental conditions, we use the term non-pathological instead of basal conditions, since this study was performed in primary cultures of astrocytes, a model that has its limitations hence the cells are somewhat activated due to the artificial conditions of the environment in culture and also, they do not completely replicate *in vivo* basal conditions due to the lack of interaction with neurons and microglia. Nevertheless, this model allows the study of gliotransmitters release solely by astrocytes and provides the means to investigate alterations in these mechanisms in AD-like conditions with

focus on changes occurring at molecular levels, allowing deepen the knowledge about the role of astrocytes in AD pathogenesis.

Our results clearly showed that acute (5 minutes and 1 hour) and chronic (24 hours) exposure to $A\beta_{1-42}$ oligomers increased the ATP released by cultured astrocytes when compared with non-treated (control) cells (Fig.17). Curiously, 5 minutes of $A\beta_{1-42}$ exposure was enough to cause an increase (about 40%) in extracellular levels of ATP in cultured astrocytes; suggesting that astrocytes are very sensitive to Aβ exposure, being able to generate a fast response to this insult. Interestingly, the increase in ATP release was more prominent when astrocytes were exposed to $A\beta_{1-42}$ for 1 hour (120%) than for 24 hours (50%). This suggests a progressive impairment in ATP release with prolonged exposure to $A\beta_{1-42}$ peptides, however this apparent decrease in the release of this gliotransmitter can also be a consequence of ATP degradation by extracellular ectonucleotidases, namely CD73, responsible for convert ATP into adenosine (Augusto et al., 2013, Cunha, 2016). In turn, adenosine can act at the levels of adenosine receptors $A_{2A}R$ and A_1R, which are present in astrocytes, controlling the activity of several transporters, receptors and channels (Gomes et al., 2011; Matos et al., 2012a, 2012b). In accord with our results from analysis of ATP release, Jung and colleagues (2012) showed that acute $A\beta_{1-42}$ exposure (4 μM, for 3 h) increases astrocytic ATP release, which was also shown to act against Aβ-induced synaptic plasticity impairment (Jung et al., 2012).

Regarding the astrocytic glutamate release triggered by exposure to $A\beta_{1-42}$ peptides a significant rise of around 40% was also observed (Fig. 19). It is also important to refer that the quantification of this gliotransmitter was performed in a saline medium collected after incubation with $A\beta_{1-42}$ peptides and no inhibitors of glutamate uptake were used. Thus, there is the possibility of the observed effect of $A\beta_{1-42}$ peptides on glutamate levels was blunted by a decrease in glutamate uptake. In fact, previous studies from our group showed that Aβ peptides exposure for 24 hours decreases glutamate uptake by astrocytes, which is due to a decrease in the levels of glutamate transporters, mainly GLT-1 (Matos et al., 2008; Matos et al., 2012a). However, in our experimental conditions the increase in extracellular glutamate levels in astrocytes exposed to $A\beta_{1-42}$ peptides was not likely to be significantly affected by alterations glutamate transporter levels, since it is improbable that 1 hour of $A\beta_{1-42}$ exposure caused substantial changes in the amount of these proteins. It should be mentioned that we also ambitioned to evaluate the impact of chronic $A\beta_{1-42}$ exposure (24 hours) on glutamate release by astrocytes, but we were not successful because the samples (culture media with

the content released by astrocytes) contain foetal bovine serum (FBS) which has glutamate and this causes a bias in the levels of ATP measured, even using the culture media that had not be in contact with cells, as a control ("blank"). Nevertheless, in agreement with our results a study using a fluorescent resonance energy transfer (FRET)-based glutamate sensor system to detect the local concentration of glutamate contiguous to astrocytes, showed that oligomeric $A\beta_{1-42}$ increases glutamate release in both rat and human astrocyte cultures. This study also gave insights that Aβ-induced glutamate release from astrocytes be responsible, at least in part, for causing synaptic loss (Talantova et al., 2013).

Although there are some evidences supporting that $A\beta_{1-42}$ exposure triggers gliotransmitters release, it still unknown the mechanisms by which occur astrocytic ATP and glutamate release under AD-like conditions. In the present study, we aimed to address this matter, and thus we used pharmacological tools to evaluate the participation of hemichannels (HC) and of P_2x_7 receptors in gliotransmitters release under "non-pathological" (control) and AD-like conditions. To investigate the role of astrocytic HC in the release of ATP and glutamate, which were increased due to $A\beta_{1-42}$ exposure, we resort to the pharmacological inhibition of Cx/Panx HC through carbenoxolone (CBX). CBX is one of the most commonly used inhibitors of HC and gap junction channels (GJC; Nodin et al., 2005); and most of GJC blockers are also effective in blocking HC (Ye et al., 2009). The proposed mechanism of HC blockade by CBX is through its insertion in the plasma membrane, and subsequent alterations in membrane properties affecting HC structure and function, which ultimately disrupts GJC and closes HC (Ye et al., 2009; Jiang et al., 2011). Furthermore, it was shown that CBX does not alter Cx43 distribution in astrocytes, the main Cx forming HC in astrocytes (Xie et al., 2017).

The data gathered showed that CBX fully prevented the increase in ATP release by astrocytes acutely (1 hour) exposed to $A\beta_{1-42}$ peptides (Fig. 21); indeed, it was observed a significant decrease in ATP release by astrocytes exposed to CBX plus $A\beta_{1-42}$ relatively to the cells treated solely with $A\beta_{1-42}$ peptides. Furthermore, in these conditions the blockade of HC (CBX *per se*) led to a decrease without significance in ATP release below the levels of control cells. Interestingly, the levels of ATP release by astrocytes exposed solely to CBX and by astrocytes exposed to CBX and $A\beta_{1-42}$, were similar amongst each other and about half of the levels of ATP released by non-treated (control) cells, indicating a major role of HC as a pathway for ATP release not only in pathological AD conditions, but also in non-pathological conditions. A similar pattern was observed when the astrocytes were exposed to

norepinephrine (NE) instead of the $A\beta_{1-42}$ peptide, the HC blockade prevented the increase in ATP release triggered by NE (Fig. 22), suggesting HC as a pathway for ATP release as a response to other stimuli rather than $A\beta_{1-42}$. Accordingly, in conditions where astrocytes were mechanically stimulated it was also observed an increase in HC opening, which leads to a subsequent increase in ATP release and Ca^{2+} waves generation (Stout et al., 2002). In agreement with our data indicating a central role of HC in ATP release by astrocytes in non-pathological conditions, it was reported that astrocytic HC are active in basal conditions and involved in hippocampal glutamatergic transmission through the release of ATP, which activates P2 receptors (Chever et al., 2014).

Interestingly, when we focused on the alterations in glutamate release triggered by acute exposure to $A\beta_{1-42}$ peptides, we found that blockade of astroglial HC with CBX significantly increased glutamate release by astrocytes, as is shown in figure 23. As HC are described as a pathway for glutamate release in astrocytes (Harada et al., 2016) we expected to find a decrease in levels of glutamate release by astrocytes with the CBX, which blocks HC and GJC. However, our data showed an increase in glutamate release of about 265% relatively to control (non-treated) astrocytes, an effect prompted only by the blocker CBX. This effect was further exacerbated by acute exposure to $A\beta_{1-42}$ peptides, astrocytes exposed to CBX and $A\beta_{1-42}$ released around 425% more glutamate than control cells. Basing on these findings it seems that the blockade of astrocytic HC promotes the glutamate release, which in agreement with a report showing exacerbation of glutamate cytotoxicity by CBX in co-cultures of astrocytes and neurons (Ozog et al., 2002). However a study preformed in an animal model of AD ($APP_{swe}/PS1_{dE9}$ mice) showed that selective deletion of Cx43, the main constituent of HC in astrocytes, decrease the release of gliotransmitters, ATP and glutamate, and alleviate neuronal damage characteristic of AD pathogenesis (Yi et al., 2016). This contradictory data leaves to speculate if our pharmacological tool, CBX is not selective to HC and is interfering with other mechanisms, which seems unlikely since other studies were able to demonstrate a HC-dependent glutamate release in divalent cation free solution (DCFS) through HC inhibition with CBX and also by the genetic silencing of Cx43 in cultured astrocytes (Ye et al., 2003, 2009). It should be referred that HC can be gated by cations, mainly Ca^{2+} (as described in section 1.2.6), and thus in absence of this cation the HC can open and release gliotransmitters. These studies from Ye and colleagues (2003, 2009) show the efficiency of CBX in HC blockade and subsequent glutamate release, which is contradictory with our data since we should have seen at least a decrease in glutamate release, not an increase, in astrocytes solely treated with

CBX. A likely explanation for the rise of glutamate when astrocytes are exposed to CBX is that HC might be interfering with glutamate transporters activity. This assumption is strengthened by a study showing that DCFS increases glutamate release due to a reduction in glutamate transporters activity, and this effect is restored by CBX (Ye et al., 2003). Other hypotheses are that: i) the astrocytes increase the release of glutamate through Ca^{2+}-dependent exocytosis (Kreft *et al.*, 2004) in an attempt of compensate the HC blockade, and/ or ii) might occur glutamate release through the reversal of glutamate transporters, whose activity depend on the Na^+ and K^+ gradient generated by Na^+/K^+-ATPase pump that can be impaired by several insults (Boscia et al., 2016). Therefore, it is imperative to perform further experiments to explore these hypotheses and clarify the mechanisms of glutamate release in AD conditions, using for instance inhibitors of exocytosis, and of glutamate transporters or even other more specific HC blockers.

Taken together our results, obtained with CBX, suggest that the increased astrocytic ATP release triggered by $A\beta_{1-42}$ peptides occurred mainly through HC, contrary to that found to glutamate release. In astrocytes, the HC are composed mainly by Cx43 (Orellana et al., 2016), and for that reason we quantified whether the $A\beta_{1-42}$ peptides affect the levels of this protein. The data showed that $A\beta_{1-42}$ (24 hours incubation) increased visibly the levels of Cx43 (Fig. 29) in astrocytes. Although, conditions of acute (1 hour) $A\beta_{1-42}$ exposure did not cause a detectable increase in CX43 levels (data not shown), it was observed a big ATP release through HC, which can be somehow related with alterations in Cx43 expression that are likely to compose HC in our astrocytes preparations. Accordingly, previous studies showed that the blockade of Cx43, through specific antibodies ($Cx43^{E2}$), Cx43 mimic peptide (Gap 26) and Cx43 genetic silencing (Cx43 KO) prevented ATP triggered by Aβ peptides (Orellana et al., 2011). Moreover, Gajardo-Gómez and colleagues in a study aimed to investigate the potential protective effect of cannabinoids in AD also showed that Aβ peptides not only trigger HC opening, but also increase Cx43 levels. This study also showed that Aβ peptides exposure decreases astrocyte uncoupling, an effect likely mediated by the closer or reduction in the permeability of GJC, since no alterations in internalization of these structures were found (Gajardo-Gómez et al., 2017). In the present study, we focused on role of HC in gliotransmitters release in non-pathological and in AD-like conditions, however the docking of two Cx HC of apposed astrocytes form GJC that are likely to exist in our astrocyte cell preparations. Aβ exposure seems to increase HC density and activity; however, the effect of AD conditions on GJC are controversial. Since Aβ was shown to decrease intercellular

communication through GJC in cultured astrocytes, but not in Alzheimer's disease transgenic mice (Cruz et al., 2010). Furthermore, uncoupling of astrocytes by blockade of GJC/Cx43 proteins was shown to be associated to a decrease in GLT-1, which even with a compensation by an increase in GLAST expression, still leads to a reduction in glutamate uptake (Figiel et al., 2007) and glutamate homeostasis impairment. Nevertheless, this study shows an interaction between HC and glutamate transporters, which favours the hypothesis that the marked increase in the levels of glutamate released by astrocytes exposed to CBX can be due to an interference with the glutamate transporters.

Another mechanism described for gliotransmitters release is through the purinergic P_2X_7 receptor (Malarkey and Parpura, 2008). Few studies focused on alterations in the role of these receptors in astroglial cells in AD pathogenesis and even less studies explored the role of P_2X_7 receptors in gliotransmitters release by astrocytes in AD conditions. Nonetheless, the P_2X_7 receptors seem to contribute to the exacerbation of AD, since the activation of this receptor has shown to have an important role in microglia activation by Aβ peptides (Sanz et al., 2009). Moreover, a high expression of this type of receptor in microglia was reported in association with Aβ plaques (Mclarnon et al., 2006). Considering the P_2X_7 receptors as a relevant pathway for gliotransmitters release, their blockade with the selective antagonist, BBG, would decrease the levels of ATP and glutamate. However, the obtained preliminary data showed that BBG *per se* caused a manifest increase in both ATP and glutamate release in astrocytes relatively to control cells, as is shown in figure 24 and 25. Although, the scarce number of experiments done, in astrocytes challenged with $A\beta_{1-42}$ or NE the blockade of the P_2X_7 receptors seemed to partially prevent the increased ATP release triggered by these insults, but this effect was not observed in glutamate release triggered by $A\beta_{1-42}$ peptides. The levels of glutamate release were similar when astrocytes were incubated with BBG and when were further challenged by $A\beta_{1-42}$ peptides. These results suggest a partial contribution of P_2X_7 receptors in ATP, but not glutamate, release in pathological AD-like conditions; however, they did not seem to play a significant role in gliotransmitters release in non-pathological conditions. In fact, among the purinergic P_2X receptors, this is the receptor with lower affinity for ATP and the one that is particularly involved in pathological conditions (Sperlágh and Illes, 2014; Rodrigues et al., 2015). Since, these data are preliminary we cannot draw conclusions, only we can speculate that the P_2X_7 receptors may be a pathway for ATP release, but not for glutamate, in AD-like conditions. Therefore, it is imperative to increase the number of experiments to reach a solid conclusion.

5.2. Modulation of gliotransmitters release by adenosine $A_{2A}R$

The adenosine A_{2A} receptors ($A_{2A}R$) have been a subject of interest in AD pathology, since this receptor has provided an attractive target for neuroprotective strategies in AD and other brain disorders (Gomes et al., 2011; Stockwell et al., 2017). The blockade of $A_{2A}R$ was already shown to have a neuroprotective role by preventing synaptotoxicity triggered by the Aβ peptide, through a mechanism involving JNK and p38 MAPK phosphorylation (Canas et al., 2009), and also by controlling the neuroinflammation, in particular gliosis in several models (Ke et al., 2009; Rebola et al., 2011). Furthermore, our group showed that $A_{2A}R$ blockade prevents Aβ-induced decrease in glutamate uptake by astrocytes (Matos et al., 2012a). The $A_{2A}R$ in astrocytes were also shown to be involved in memory processes, since the genetic ablation of astrocytic $A_{2A}R$ enhances spatial memory and prolongs contextual memory retention (Orr et al., 2015). However, little is known about the impact of $A_{2A}R$ blockade in gliotransmitters release under AD-like conditions. To fill this gap in the present study we investigated if the blockade of $A_{2A}R$, with the selective antagonist of the $A_{2A}R$ SCH 58261, affected the $A\beta_{1-42}$-induced gliotransmitters release.

Our results showed that the selective antagonism of $A_{2A}R$ prevented the increase in ATP release triggered by $A\beta_{1-42}$ peptides acute (1 hour) or chronic (24 hours) exposure (Fig. 26). Astrocytes incubated with SCH 58261 and further challenged acutely with $A\beta_{1-42}$ peptides showed levels of ATP release similar to the ones observed in non-treated control cells, but significantly lower than the levels of ATP released by $A\beta_{1-42}$-treated astrocytes; a similar pattern was observed in conditions of chronic $A\beta_{1-42}$ peptide exposure. Interestingly, the $A_{2A}R$ antagonism only partially prevented the increase in ATP released by astrocytes challenged with NE. In contrast, $A_{2A}R$ blockade had no effect on $A\beta_{1-42}$-induced increase of glutamate, being the levels of glutamate released by $A\beta_{1-42}$-treated astrocytes similar to the levels released by cells treated with SCH 58261 and $A\beta_{1-42}$ (Fig. 28).

Taken together, these data indicate that $A_{2A}R$ controlled the ATP release, but not the glutamate release, triggered by $A\beta_{1-42}$ peptides. This is in agreement with our results suggesting that ATP and glutamate are released by distinct mechanisms in astrocytes challenged with $A\beta_{1-42}$. In fact, in AD-like conditions the astrocytes released high levels of ATP release mainly

through HC, whereas we did not identify the mechanism involved in astrocytic glutamate release. Curiously, we observed that the blockade of $A_{2A}R$, besides preventing astrocytes reactivity, could prevent the overexpression of Cx43 protein triggered by $A\beta_{1-42}$ peptide exposure (Fig. 29). These findings are consistent with our conclusion that $A_{2A}R$ control ATP release by astrocytes exposed to $A\beta_{1-42}$ peptides, which occurs through HC that are known to be mainly composed by Cx43. Therefore, we can speculate that $A\beta_{1-42}$ peptides increased the astrocytic ATP release by a mechanism that involved an upregulation of Cx43 HC. This assumption is corroborated by the observation of a physical interaction between Cx43 proteins and $A_{2A}R$ in our astrocytes preparations, which seems to be slightly decreased in AD-like conditions (see figure 30). In agreement with our data showing an increase in astrocytic Cx43 levels, other studies reported an increase of this protein in reactive astrocytes at the vicinities of Aβ plaques in an animal model of AD (Mei et al., 2010) and in human brain hippocampus (Nagy et al., 1996). Furthermore, it was also reported that chronic exposure to the $A\beta_{25-35}$ peptide increases the levels of Cx43 protein in astrocytes and microglia (Orellana et al., 2011). The hypothesis that $A_{2A}R$ signalling can modulate Cx43 is not new. There are studies showing that cAMP cascade upregulates Cx43, through the activation of PKA and CREB in an astrocytoma cell line (Mostafavi et al., 2014) and that $A_{2A}R$ are involved in Cx43 distribution in adrenal cells (Wynn et al., 2009). Nevertheless, our results show, for the first time, a direct effect of $A_{2A}R$ modulation on Cx43 protein levels, and also a physical interaction (proximity) between these two proteins.

Taken together, our studies demonstrated an increase in ATP and glutamate release by astrocytes prompted by $A\beta_{1-42}$ peptides, which occurred through distinct mechanisms. The release of ATP can act as a danger signal, nevertheless the chronic increase in ATP release may contribute to impair synaptic transmission, through the activation of purinergic and adenosinergic receptors, favouring also inflammatory processes. Furthermore, the increased release of glutamate, by mechanisms which we were not able to elucidate, leads to excessive activation of glutamate receptors, namely NMDA receptors contributing to excitotoxicity and memory impairment. Moreover, our results show a modulation of ATP release by the $A_{2A}R$ in astrocytes, further highlighting the relevance of the blockade of these receptors in AD therapeutic strategies.

CHAPTER 6| CONCLUSIONS

- Exposure to $A\beta_{1-42}$ peptides to mimicked AD conditions increased gliotransmitters release (ATP and glutamate) by cultured astrocytes.
- ATP and glutamate release occurred through distinct mechanisms. ATP was released mainly through HC and partially by P_2X_7 receptors in AD conditions. Meanwhile, glutamate did not seem to be released by HC or P_2X_7 receptors in AD conditions.
- ATP, but not glutamate, release was modulated by the activation of the $A_{2A}R$ in AD conditions.
- Exposure to $A\beta_{1-42}$ peptides increased the density of astrocytic Cx43 protein, which was prevented by the selective blockade of $A_{2A}R$.
- $A_{2A}R$ were physically associated with Cx43 proteins, and this may underlie the mechanisms by which $A_{2A}R$ control ATP release through HC.

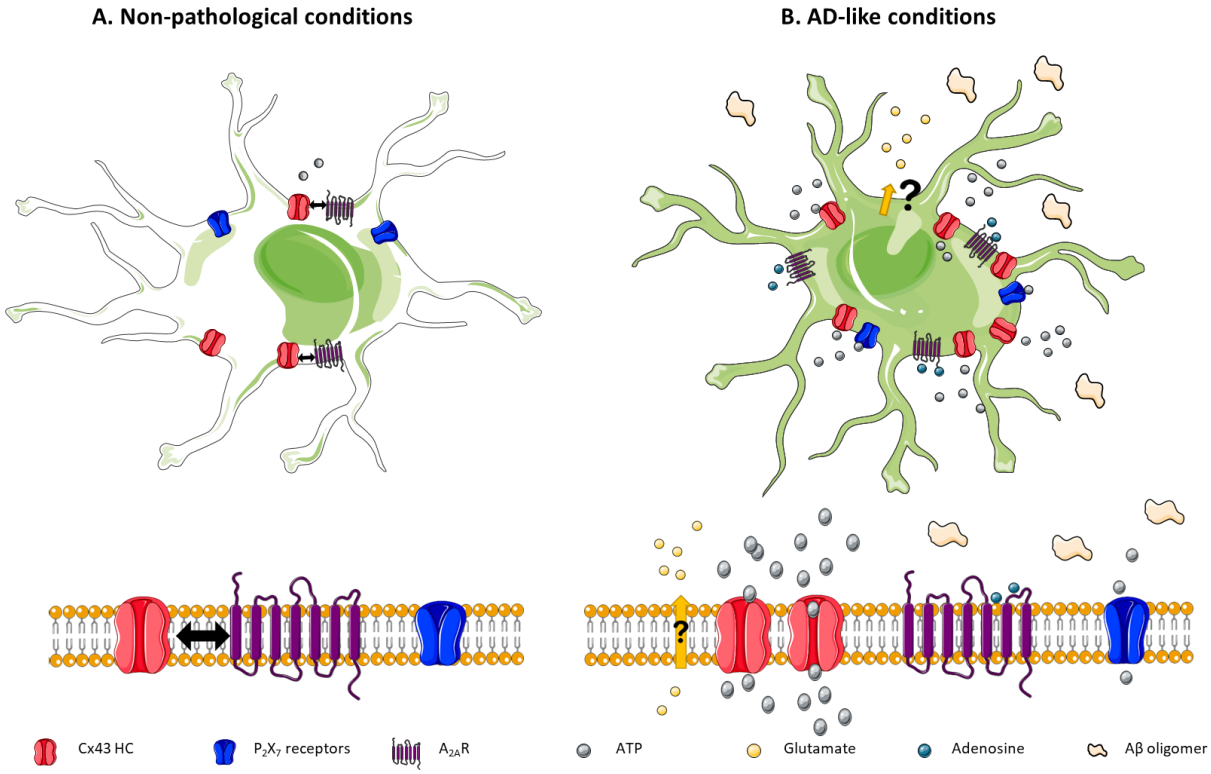

Fig. 31: Proposed mechanism for gliotransmission in non-pathological (A) and AD-like conditions (B). In AD-like conditions, exposure to $A\beta_{1-42}$ peptides trigger astrocytic reactivity (astrogliosis) and increase ATP and glutamate release. The astrocytic ATP release occurs mainly by HC, which can be composed by Cx43. The $A_{2A}R$ control the astrocytic ATP release and also the levels of Cx43 HC, probably due to a close proximity, and likely an interaction, between these two proteins. ATP release by astrocytes exposed to $A\beta_{1-42}$ peptides seems also to occur partially through the P_2X_7 receptors. Furthermore, the glutamate release also increases through mechanism (s) which we were not able to elucidate.

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