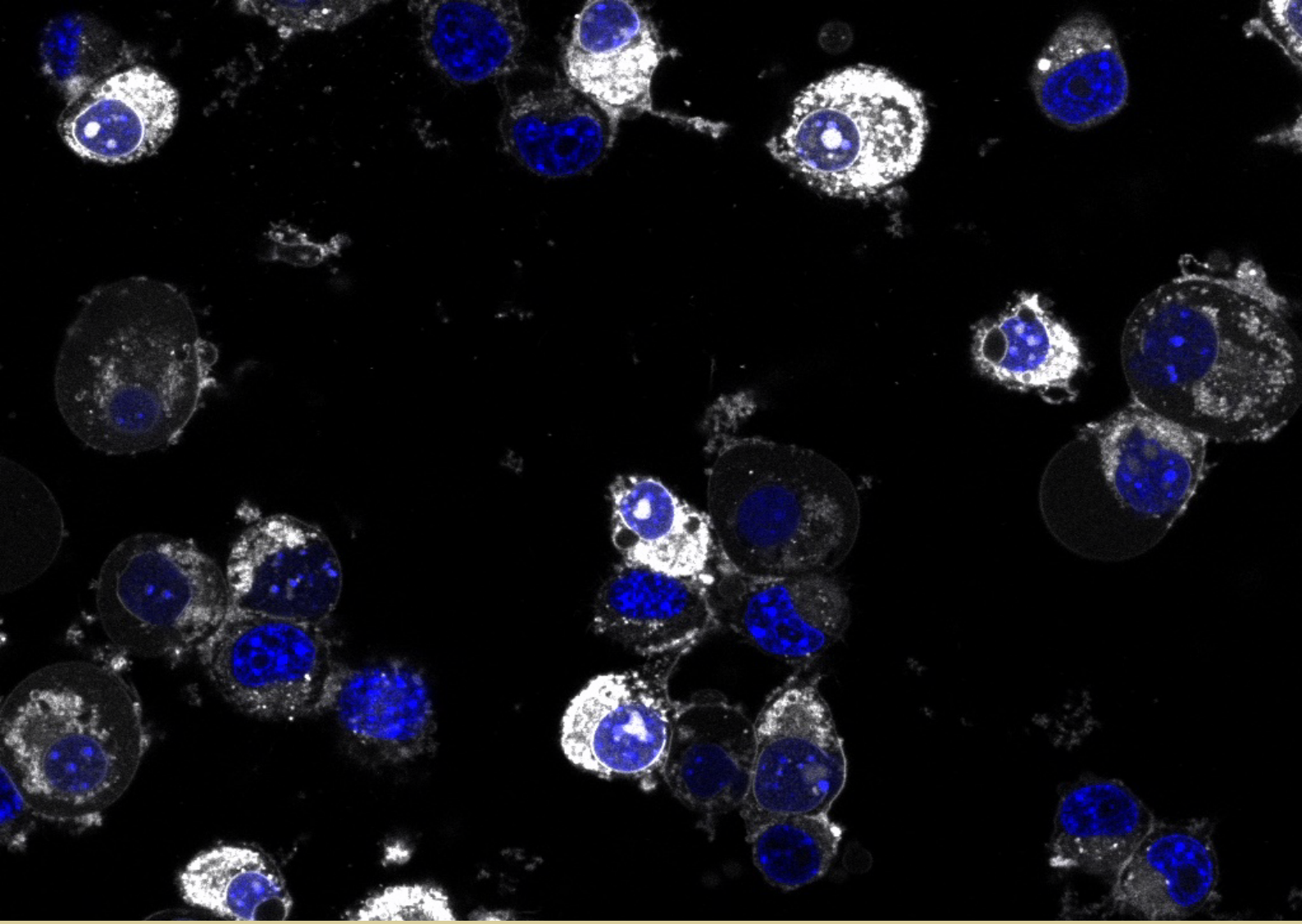

Verónica Resende Figueiredo

# BIOLOGICAL EFFECTS OF NEW BACE1 INHIBITORS FOR ALZHEIMER'S DISEASE TREATMENT

Master's dissertation in Biomedical Engineering, specialty in Neurosciences, supervised by Armanda E. Santos (PhD) and Rosa Resende (PhD), presented to Faculty of Sciences and Technology of University of Coimbra.

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# Biological effects of new BACE1 inhibitors for Alzheimer's disease treatment

Thesis submitted to the
University of Coimbra for the degree of
Master in Biomedical Engineering

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# Resumo

A Doença de Alzheimer (DA) é a maior causa de demência na população idosa. Esta doença é caracterizada por uma perda progressiva de memória e funções cognitivas, que resultam da perda sináptica e neuronal. As duas maiores características histológicas da DA são as placas neuríticas extracelulares constituídas por Péptido Beta Amilóide (A$\beta$) e as tranças neurofibrilares formadas por tau hiperfosforilada. A formação de A$\beta$ por proteólise da Proteína Precursora Amilóide (APP) através da via amiloidogénica é o passo crucial no desenvolvimento da DA. O processamento amiloidogénico da APP resulta de uma clivagem inicial mediada pela Enzima de Clivagem da APP no sítio $\beta$ 1 (BACE1) seguida de uma clivagem mediada pela $\gamma$-secretase. Assim, a BACE1 foi identificada como um possível alvo de intervenção terapêutica na DA visto que a sua inibição irá impedir a formação de A$\beta$ em consequência do processamento da APP.

Resultados prévios revelaram que o novo inibidor da BACE1 Péptido-x (PEPx), desenvolvido pela nossa equipa no Centro de Neurociências e Biologia Celular, reduz os níveis de A$\beta_{40}$, A$\beta_{42}$ e de APP $\beta$ Solúvel (sAPP$\beta$), outro produto da clivagem da APP pela BACE1. De forma a compreender mais profundamente os mecanismos de captação celular do PEPx, bem como os efeitos da inibição da BACE1 em vários eventos neurológicos subjacentes à neurodegeneração na DA, investigámos a captação endocítica do composto *in vitro* e avaliámos *in vivo* num modelo animal da DA, os Murganhos Triplo Transgénicos (3xTg-DA), os efeitos de um tratamento crónico relativamente à perda sináptica, neuroinflamação e morte celular.

Neste trabalho, usando a linha celular Neuro-2a (N2a) avaliámos por microscopia de fluorescência em células vivas a captação celular por via endocítica do PEPx marcado com um fluoróforo usando uma solução hipertónica de sacarose, um inibidor de amplo espectro da endocitose, e a Clorpromazina (CPZ), um inibidor farmacológico da endocitose mediada por clatrina. Por outro lado, os estudos *in vivo* foram realizados em murganhos 3xTg-DA tratados diariamente (injecção i.p.) com solução

salina ou com PEPx durante 4 meses. Murganhos Não Transgénicos (Non Tg) da mesma idade e da mesma estirpe foram usados como controlo das vias patogénicas que os murganhos 3xTg-DA desenvolvem de forma dependente da idade.

Nós observámos que, nas células N2a, o PEPx internalizado se distribuiu em estruturas citoplasmáticas de aparência vesicular. A internalização em estruturas vesiculares foi inibida na presença de uma concentração elevada de sacarose e pela CPZ, sugerindo que o PEPx é internalizado essencialmente por endocitose mediada por clatrina.

*In vivo*, o tratamento crónico com PEPx levou ao aumento dos níveis de SYP em murganhos 3xTg-DA. Visto que a SYP não sofreu alteração em murganhos 3xTg-DA com 8.5 meses tratados com solução salina em comparação com os murganhos Non Tg, os efeitos do tratamento com PEPx sugerem um ganho sináptico. Em relação à neuroinflamação, o inibidor da BACE1 diminuiu os níveis de NLRP3 em murganhos 3xTg-DA, sugerindo uma diminuição da formação do inflamossoma. Os níveis de NLRP3 em murganhos Non Tg não foram detectáveis. Concluindo, estes resultados sugerem que a inibição do processamento da APP, para além de uma diminuição da produção de A$\beta$, tem um efeito benéfico em relação à perda sináptica e à neuroinflamação.

**Palavras-chave:** Doença de Alzheimer; Inibição da BACE1; Endocitose; Perda sináptica; Neuroinflamação; Morte celular.

# Abstract

Alzheimer's Disease (AD) is the major cause of dementia in the elderly population. AD is characterised by a progressive loss of memory and cognitive functions, that arise from synaptic and neuronal loss. The two major histological hallmarks of AD are the extracellular neuritic plaques composed of Amyloid Beta Peptide (A$\beta$) and the intracellular neurofibrillary tangles formed by hyperphosphorylated tau. The A$\beta$ production due to Amyloid Precursor Protein (APP) proteolysis through the amyloidogenic pathway is believed to be the crucial step in the development of AD. The amyloidogenic APP processing is carried out by sequential cleavage mediated by the $\beta$-Site APP-Cleaving Enzyme 1 (BACE1) and $\gamma$-secretase. Thus, BACE1 has been identified as a possible target for therapeutic intervention in AD since its inhibition would halt the formation of A$\beta$ upon APP processing.

The BACE1 inhibitor Peptide-x (PEPx), developed by our team at the Centre for Neuroscience and Cell Biology, has previously showed to decrease the levels of both A$\beta_{40}$ and A$\beta_{42}$, and also Soluble APP $\beta$ (sAPP$\beta$), another product of APP cleavage by BACE1. To more thoroughly understand the mechanisms of cellular uptake of PEPx, as well as the effects of the BACE1 inhibitor upon several neurotoxic events underlying AD neurodegeneration, we investigated *in vitro* the compound's endocytic uptake, and evaluated *in vivo*, using the Triple-Transgenic Mouse Model of AD (3xTg-AD), the synaptic loss, neuroinflammation, and cell death, after a chronic treatment with PEPx

In this work, using the Neuro-2a (N2a) cell line we assessed by live cell fluorescence microscopy the endocytic uptake of fluorophore-labelled PEPx using a hypertonic sucrose solution, a broad-spectrum endocytosis inhibitor, and Chlorpromazine (CPZ), a pharmacological inhibitor of endocytosis mediated by clathrin. On the other hand, *in vivo* studies were performed on the 3xTg-AD daily treated (i.p. injection) with saline or PEPx during four months. Age-matched Non Transgenic Mice (Non Tg) with the same strain background were used as a control of pathological pathways

developing in 3xTg-AD mice in an age-dependent manner.

We observed that, in N2a cells, PEPx internalization gave rise to a cytoplasmic vesicular staining which was reduced in the presence of a high sucrose concentration and with the application of CPZ, thus suggesting that the main pathway of internalization of the peptide is the clathrin-mediated endocytosis.

*In vivo*, the chronic treatment with PEPx lead to an increase in Synaptophysin (SYP) levels in 3xTg-AD mice. Since SYP was not altered in 8.5-month-old 3xTg-AD mice treated with saline regarding Non Tg mice, the effect of PEPx treatment suggests a synaptic gain. Regarding neuroinflammation, the BACE1 inhibitor decreased NOD-Like Receptor Protein 3 (NLRP3) levels in 3xTg-AD mice suggesting diminished inflammasome assembly. The NLRP3 levels in Non Tg mice were undetectable. Taking together, these results suggest that inhibition of the amyloidogenic APP processing, beyond a decrease in A$\beta$ production, has a benefit regarding synaptic loss and neuroinflammation.

**Keywords:** Alzheimer's Disease; BACE1 inhibition; Endocytosis; Synaptic loss, Neuroinflammation; Cell death.

# Abbreviations

**3xTg-AD** Triple-Transgenic Mouse Model of Alzheimer's Disease 21, 22, 26, 27, 41–49, 51, 52

**A$\beta$** Amyloid Beta Peptide 1–5, 7–10, 12–14, 16–18, 21, 22, 26, 33, 34, 42–45, 49, 51

**ACH** Amyloid Cascade Hypothesis 8–10, 26

**AD** Alzheimer's Disease 1–14, 16–18, 21, 22, 24, 26, 33, 42–49, 51

**AICD** APP Intracellular Domain 7

**apoE** Apolipoprotein E 2, 3

**APP** Amyloid Precursor Protein 2–10, 12, 16, 17, 21, 26, 33, 34, 41, 45

**ATP** Adenosine Triphosphate 14

**BACE1** $\beta$-Site APP-Cleaving Enzyme 1 4–7, 11, 16–18, 21, 25, 26, 33, 34, 36, 41, 42, 45, 48, 49, 51

**BBB** Blood–Brain Barrier 2, 5, 8, 17

**BCA** Bicinchoninic Acid 27

**BSA** Bovine Serum Albumine 23, 27, 28, 30, 31

**C83** Carboxyl Terminal Fragment with 83 Amino-Acid 7

**C99** Carboxyl Terminal Fragment with 99 Amino-Acid 7

**CNS** Central Nervous System 2, 12, 17, 44, 45

**CPZ** Chlorpromazine 21, 23, 25, 34, 39, 40, 51

**CSF** Cerebrospinal Fluid 5, 6, 17, 18

**DMEM** Dulbecco's Modified Eagle Medium 23–25

**DNA** Deoxyribonucleic Acid 14, 25

**ECF** Enhanced Chemifluorescence 23, 30, 31

**FBS** Fetal Bovine Serum 23–25

**IL-1** Interleukin-1 12

**IL-1$\beta$** Interleukin-1$\beta$ 12, 13, 21, 22, 44, 45, 51, 52

**LTP** Long-Term Potentiation 8, 26, 43

**MLKL** Mixed-Lineage Kinase Domain-Like 16, 48, 49, 52

**N2a** Neuro-2a 21, 24, 25, 33–35, 37, 39, 40, 51

**NLRP3** NOD-Like Receptor Protein 3 12, 13, 16, 21, 22, 24, 44, 45, 51

**Non Tg** Non Transgenic Mouse 22, 42–49

**PBS** Phosphate-Buffered Saline 25, 26

**PEPx** Peptide-x 21, 22, 25, 26, 33–49, 51, 52

**pRIP3** Phosphorylated Receptor-Interacting Protein Kinase 3 16, 22, 24, 48, 49, 52

**PS** Presenilin 2, 4, 10, 26

**PVDF** Polyvinylidene Difluoride 23, 30

**RHIM** RIP Homotypic Interaction Motif 14, 15

**RIP1** Receptor-Interacting Protein Kinase 1 14–16, 22, 24, 45–47, 49, 51

**RIP3** Receptor-Interacting Protein Kinase 3 14–16, 22, 24, 45–49, 51

**RT** room temperature 30, 31

**sAPP$\alpha$** Soluble APP $\alpha$ 7, 18

**sAPP$\beta$** Soluble APP $\beta$ 7, 17, 18, 41, 42

**SDS** Sodium Dodecyl Sulfate 27, 29

**SYP** Synaptophysin 11, 22, 24, 42–44, 51

**TBS** Tris-Buffered Saline 30, 31

**TEMED** N, N, N', N'-Tetramethyl-ethylenediamine 23, 29

**TLR** Toll-Like Receptor 16

**TNF** Tumor Necrosis Factor 14, 15, 22, 46, 47

**TNFR1** TNF Receptor 1 15, 47

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# Introduction

## 1.1 Alzheimer's Disease

Alzheimer's Disease (AD) is a progressive, unremitting, neurodegenerative disorder [1] and the most common cause of dementia worldwide [2]. Currently, AD or a related dementia affects more than 44 million people [3] and the number is both expected to double every 20 years and to reach 115 million in 2050 [4].

AD is characterized by a progressive loss of memory and cognitive functions [5] and affects wide areas of the cerebral cortex and hippocampus. Abnormalities are usually first detected in the frontal and temporal brain lobes, slowly progressing to other areas of the neocortex and hippocampus at rates that vary between individuals [1].

The typical and most evident characteristic of AD is impairment of anterograde episodic memory. As the disease progresses, other cognitive domains become involved, such as language, behaviour, visuospatial functioning, and executive functioning, which results in a global cognitive decline. A small number of patients present focal cortical symptoms, such as impaired language, or executive function, while memory remains preserved for some time [6].

The two major histological hallmarks of AD are the presence of extracellular neuritic plaques and intracellular neurofibrillary tangles in the brain. Neuritic plaques are spherical lesions that contain extracellular aggregates of Amyloid Beta Peptide (A$\beta$) [7], and are surrounded by an array of abnormal dendrites and axons [8]. Neurofibrillary tangles are insoluble bundles of fibers that locate in the perinuclear cytoplasm and are composed of hyperphosphorylated tau protein [9]. Neuritic plaques and neurofibrillary tangles arise independently [10]. However, neuritic plaques seem to be the primary lesion in AD [11] and it has been suggested that the appearance of tangles in the AD brain could be due to the neuronal response to plaque formation [12]. The average duration of illness is 8–10 years, but the clinical symptomatic

phases are preceded by preclinical and prodromal stages that typically extend over two decades [1].

AD is probably caused by complex interactions among multiple genetic, epigenetic, and environmental factors [5]. Less than 1% of AD cases are caused by an autosomal dominant mutation. Three genes mutations account for these familial types of AD: Presenilin (PS) 1, PS2, and Amyloid Precursor Protein (APP). Familial AD almost invariably has an early onset. The likelihood of a genetic cause is highest in cases with very early onset (normally <45 years) [6]. The PS1 and PS2 mutations affect APP processing, leading to altered production of different A$\beta$ peptides and, thus, their relative ratios [13].

Aging is the most important known non-genetic risk factor for late-onset AD. Potential environmental risk factors include head injury, low educational levels, hyperlipidemia, hypertension, homocysteinemia, diabetes mellitus, and obesity [5].

Apolipoprotein E (apoE) is the primary cholesterol transporter in the Central Nervous System (CNS) and is synthesised in the majority by astrocytes in the Blood–Brain Barrier (BBB) [14]. There are three APOE polymorphic alleles (APOE $\varepsilon$2, APOE $\varepsilon$3, APOE $\varepsilon$4) that encode three protein isoforms (apoE2, apoE3 and apoE4, respectively) with cysteine/arginine polymorphisms [15]. The APOE $\varepsilon$4 allele is the most important genetic risk factor for sporadic late onset AD [16] because of APOE association with an increase in cerebral A$\beta$ accumulation [17]. Thus, APOE $\varepsilon$4 is responsible for an increase in A$\beta$ oligomers aggregation, that could lead to an AD predisposition [18]. Futhermore, apoE, specially apoE4, activates an alternative and unusual MAP kinase signaling cascade that stimulates cFOS phosphorylation containing AP-1 transcription factors, leading to an increase of APP gene transcription 4- to 6-fold in human neurons and A$\beta$ synthesis [19].

The Peripheral Sink hypothesis theorizes that clearance of A$\beta$ from the brain is accelerated by its removal from the plasma through liver and kidneys [20] and that A$\beta$ clearance from APOE $\varepsilon$4 carriers may display some deficits in both compartments [21]. The apoE isoform may influence A$\beta$ degradation in the CNS through regulation of cellular cholesterol enhancing endocytosis of A$\beta$ and lysosomal A$\beta$ degradation in the order apoE4 > apoE3 > apoE2 [22]. On the other hand, A$\beta$ interacts with cell-surface apoE receptors, including low-density lipoprotein receptor related protein 1, low-density lipoprotein receptor and very low-density lipoprotein receptor [23]. Receptor binding to single A$\beta$ or A$\beta$ in complex with apoE either delivers A$\beta$ to the lysosome or leads transcytosis into the plasma through the BBB [24]. The rate of A$\beta$ endocytosis may be disrupted by apoE isoforms from rapid low-

density lipoprotein receptor related protein 1 mediated clearance to very low-density lipoprotein receptor [25].

Only about 20–25% of the general population carries one or more APOE $\varepsilon 4$ alleles, whereas 50–65% of people with AD are APOE $\varepsilon 4$ carriers [6]. The presence of at least one APOE $\varepsilon 4$ allele has been associated with reduced age at onset rather than increases in the lifetime risk of developing AD [26]. Homozygous APOE $\varepsilon 4$ allele carriers develop AD up to 10 years earlier than individuals who do not have this allele [27]. By contrast, some protection is provided by the rarer APOE $\varepsilon 2$ allele [16]. Nonetheless, early-onset AD can also develop in the absence of an APOE $\varepsilon 4$ allele. People with one or two APOE $\varepsilon 3$ alleles might manifest early-onset AD even younger than APOE $\varepsilon 4$ carriers [6].

Making a diagnosis of AD on purely clinical grounds is challenging, not only in the prodromal stage, in which patients only have subtle cognitive symptoms, but also in the dementia phase [1]. Still, with the recent development of molecular imaging techniques, such as Magnetic Resonance Imaging, Positron Emission Tomography and Single Photon Emission Computed Tomography, and the analysis of cerebrospinal fluid biomarkers [28], the diagnosis of AD has been facilitated and can be performed with an elevated degree of certainty. Nevertheless, a definitive histopathological diagnosis of AD can be done after death.

### 1.1.1 Amyloid Precursor Protein

Genetic, biochemical, and behavioral research suggest that generation of neurotoxic A$\beta$ from sequential APP proteolysis is the crucial step in the development of AD [29].

APP is a type I transmembrane protein consisting of an N-terminal 17 residue signaling peptide, a large ectodomain, a 23 residue hydrophobic transmembrane domain, and a 47 residue cytoplasmic domain [7]. This protein is encoded by the gene APP on (human) chromosome 21 [30]. The signal peptide translocates APP to the endoplasmic reticulum where it is bound to the membrane via the 23 residue hydrophobic stretch. APP is then post-translationally modified via N- and O-glycosylation, sulfation, and phosphorylation [8] as it is moved through the secretory pathway by way of the Golgi apparatus and endosomes. Along the way, APP is subjected to different proteolytic events that can release a variety of soluble and membrane bound fragments. Only fully modified and glycosylated APP undergoes proteolytic processing [31].

The canonical processing of APP, which includes the amyloidogenic and the non-amyloidogenic pathways, are mainly performed by $\alpha$-secretase, $\beta$-secretase, and $\gamma$-secretases [9]. APP processing can also occur through non-canonical pathways involving the activity of caspases, meprin-$\beta$, the $\eta$-pathway and $\delta$-pathway [32]. Some of the fragments that are originated by the non-canonical APP processing are implicated in neuronal apoptosis, and may inhibit presynaptic transmitter release contributing to the AD pathogenesis [33], although no information about their physiological relevance is available. Caspase cleavage at Asp664 generates the Carboxyl Terminal Fragment with 31 Amino-Acid fragment (implicated in neuronal apoptosis) [34] and, after subsequent cleavage by $\gamma$-secretase, the Jcasp fragment that may inhibit presynaptic transmitter release.

APP is expressed in many tissues throughout the body, with higher concentrations being found in the kidneys and brain [35]. In the cell, it is found mostly in the late endosomes, and also APP cycling from the cell surface through the endocytic system does occur [36]. In neurons, APP cleavage by $\beta$-Site APP-Cleaving Enzyme 1 (BACE1) occurs mainly in recycling endosomes [37], although in non-neuronal cells it occurs in early endosomes [38]. The main form expressed in neuronal cells is $APP_{695}$, which lacks a 56-amino acid sequence similar to the Kunitz serine protease inhibitors that is present in the longer isoforms of APP, $APP_{751}$ and $APP_{770}$ [39].

While the exact physiological function of APP is not entirely clear, APP and its derivatives have broad functions that include roles in serine protease inhibition, as well as cell adhesion [31], growth promotion, neuroprotection via regulation of intracellular calcium levels, synapse formation and maintenance [9], and synaptic plasticity [40].

### 1.1.2 Amyloid Beta Peptide

A$\beta$ peptides are the main constituents of amyloid plaques [41]. The progressive deposition and aggregation of fibrillary A$\beta$ in the parenchyma and vasculature of the brain characterizes AD and it is implicated in a cascade of neurotoxic events that leads to neuronal loss and dementia [42].

A$\beta$ comes from the sequential processing of APP by the BACE1, a membrane-tethered protease, and the $\gamma$-secretase, a membrane-embedded complex with PS as its catalytic component [30]. Two main species of A$\beta$ are produced, $A\beta_{40}$ and $A\beta_{42}$. $A\beta_{42}$ is more prone to aggregation than $A\beta_{40}$, however both species have been found in neuritic plaques [9]. Once these plaques are formed they are quite stable.

Increases in both A$\beta_{40}$ and A$\beta_{42}$ are seen early on in AD and overall levels of A$\beta$ in the brain have been shown to correlate to the degree of dementia in AD patients [43]. The less aggregative A$\beta_{40}$ is much more abundantly produced in normal cells [10].

Proteolytic processing studies have demonstrated that A$\beta$ is a normal product of APP metabolism and it is generated in neurons throughout an individual's lifetime [1]. A$\beta$ is present in the brains of individuals with no evidence of cognitive impairment and is also produced in the brain in response to neurological injury [44]. The production occurs in mammalian cells during life, and A$\beta$ can be detected in the non-pathological state in plasma and Cerebrospinal Fluid (CSF) [45].

Overproduction of A$\beta$, which appears to be directly neurotoxic [46], can be detected at the earliest stages of AD, even before cognitive dysfunction is detectable [43].

Several lines of evidence suggest that A$\beta$ regulates neuronal and synaptic activities and that accumulation of A$\beta$ in the brain causes an intriguing combination of aberrant network activity and synaptic depression [47]. Impairments of inhibitory interneurons and aberrant stimulation of glutamate receptors, which can result in excitotoxicity, appear to play important upstream roles in the AD pathogenic cascade [48]. Atypical neuronal activity might trigger a vicious cycle by augmenting A$\beta$ production, which is regulated, partially, by neuronal activity [5]. There is also a potential protective role for A$\beta$, which may act as an antimicrobial peptide, by allowing reduced microbial adhesion to host cells [49] in a way similar to evolutionary conserved mechanisms of innate immunity. Thus, A$\beta$ might not simply be a consequence of disease, and its presence might reflect changes within the brain [50].

A$\beta$ is mainly cleared from the brain by proteolytic degradation [51], bulk flow along the perivascular interstitial fluid drainage pathway [52], or by receptor-mediated clearance across the BBB [53].

### 1.1.3 BACE1

BACE1 is a type I transmembrane protein that belongs to the aspartyl protease family. The highest levels of BACE1 expression are seen in the brain and pancreas at early postnatal stages [54], being strongly expressed by neurons, especially at presypnatic terminals [55]. BACE1 is an important enzyme found early in the cascade of biological events that lead to disease progression [35], becoming an interesting therapeutic target for small molecule inhibitors in order to alter the progression of

AD.

BACE1 is formed in the endoplasmic reticulum as an immature, glycosylated propeptide, pro-BACE1 [56]. Pro-BACE1 is processed and cleaved into mature BACE1 in the Golgi apparatus [57]. The propeptide has two conformations: when in the open conformation, pro-BACE1 can exhibit some enzymatic activity; when in the closed conformation, with the pro-domain covering the active site, the activity of pro-BACE1 is diminished, allowing the pro-domain to serve as a weak inhibitor of BACE1 activity [58]. Cleavage of the pro-domain allows BACE1 to have full enzymatic activity by allowing the catalytic active site to be fully accessible to substrate [59]. BACE1 undergoes several post-translational modifications in the cellular secretory pathway, including N-glycosylation, phosphorylation, ubiquitination, S-palmitoylation, and acetylation [60], presenting four potential N-glycosylation sites as well as six cysteine residues that form three disulfide bonds [61], that are important for proper folding and activity of the enzyme.

APP and BACE1 are internalized from the plasma membrane and merge at the early endosomal compartments. BACE1 can then be sorted to lysosomes for degradation or alternatively to recycling endosomes for cell surface reincorporation [62]. Abnormal endosomal enlargement is observed in early AD brain [54].

BACE1 has other substrates besides APP, namely L1, Close Homolog of L1, Contactin-2, and Seizure Protein 6, being involved in axon guidance, neurite outgrowth, and synapse formation [63]. The best understood function is its role in proteolytic processing of Neuregulin-1, that regulates axon myelination in the peripheral nervous system [64].

A mild increase of BACE1 expression and activity was reported in *post-mortem* brains and CSF from AD patients [65]. Accumulation of BACE1 is observed in normal and dystrophic presynaptic terminals surrounding the amyloid plaques in brains of AD mouse models and patients [66].

## 1.2 APP Processing

APP can be proteolytically processed by two different pathways, as shown in Figure 1.1

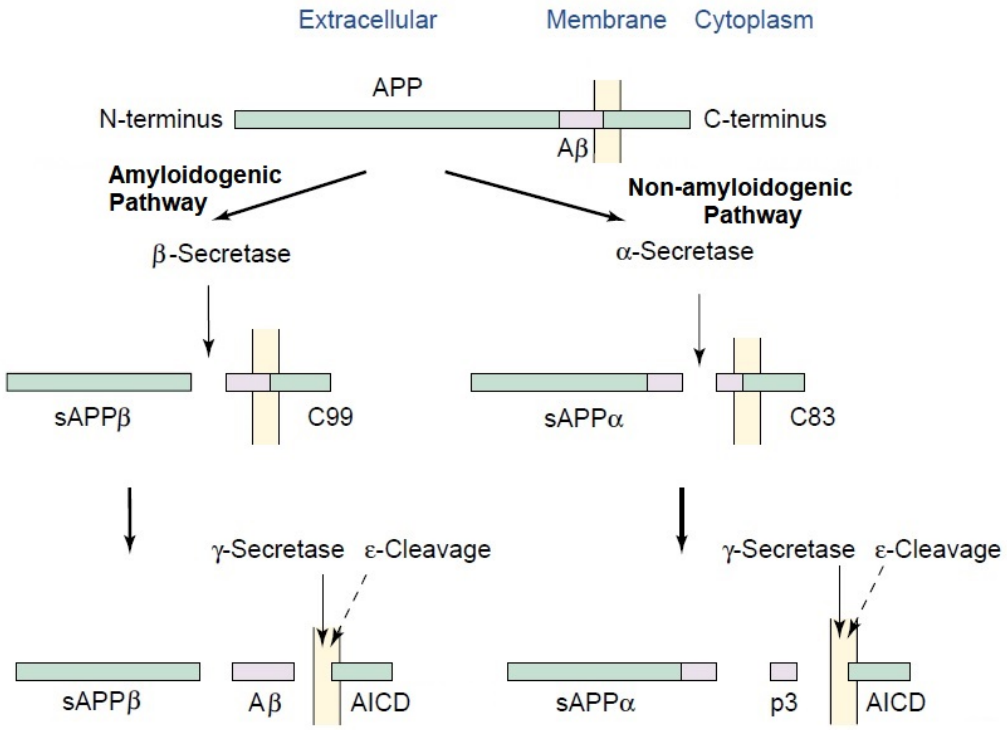


**Figure 1.1:** Amyloidogenic and non-amyloidogenic pathways of APP processing. Adapted from Vardy et al. [44].

In the non-amyloidogenic pathway, APP is cleaved initially by the $\alpha$-secretase at the plasma membrane or in the *trans Golgi* network, generating the N-terminal fragment Soluble APP $\alpha$ (sAPP$\alpha$), which is released into intracellular vesicles or extracellularly [67], and Carboxyl Terminal Fragment with 83 Amino-Acid (C83) [68]. C83 remains membrane-bound and suffers subsequent cleavage by $\gamma$-secretase to produce the p3 peptide and the APP Intracellular Domain (AICD) [44]. The sAPP$\alpha$ has several neuroprotective properties and AICD has nuclear signaling functions [45].

In the amyloidogenic pathway, the one directly related with the formation of A$\beta$ in AD, APP is cleaved by BACE1 in the *trans Golgi*-network [69] and endosomes [37]. In neurons, this processing occurs mainly in the recycling endosomes, generating N-Terminal Soluble APP $\beta$ (sAPP$\beta$), that can be released into intracellular vesicles or extracellularly, and membrane bound Carboxyl Terminal Fragment with 99 Amino-Acid (C99) [67]. C99 is further cleaved by $\gamma$-secretase, to produce A$\beta$ and AICD [70]. $\gamma$-secretase can cleave APP nearer to the boundary of the cytoplasmic membrane, $\varepsilon$-cleavage, and in the middle of the membrane, also named as $\gamma$-cleavage [71]. Thus, the total length of A$\beta$ varies at the C-terminal according with the APP cleavage pattern.

Both the proteolytic processing pathways compete with each other at least in some subcellular loci [72], being mutually exclusive [73]. APP redistribution towards either the cell membrane or subcellular compartments can therefore influence the proteolytic pathway that APP undergoes [73].

## 1.3 Amyloid Cascade Hypothesis

According to the Amyloid Cascade Hypothesis (ACH), the central event in AD pathogenesis is an imbalance between A$\beta$ production and clearance [74], with increased A$\beta$ production in familial AD and decreased A$\beta$ clearance in sporadic AD. The A$\beta$ clearance can be reduced by several reasons, including increased aggregation, defective degradation, disturbed transport across the BBB, or inefficient peripheral removal of the peptide [75].

This A$\beta$ abnormal accumulation acts as a trigger for the cascade, enhancing oligomer formation that evolves to insoluble fibrils deposition responsible for microgliosis and astrocytosis [76]. A$\beta$ oligomers could directly inhibit hippocampal Long-Term Potentiation (LTP) and impair synaptic function, causing inflammatory and oxidative stress by aggregating and depositing A$\beta$. Tau pathology with tangle formation is regarded as an important downstream event that may contribute to neuronal dysfunction and cognitive symptoms [77], as can be seen in Figure 1.2.

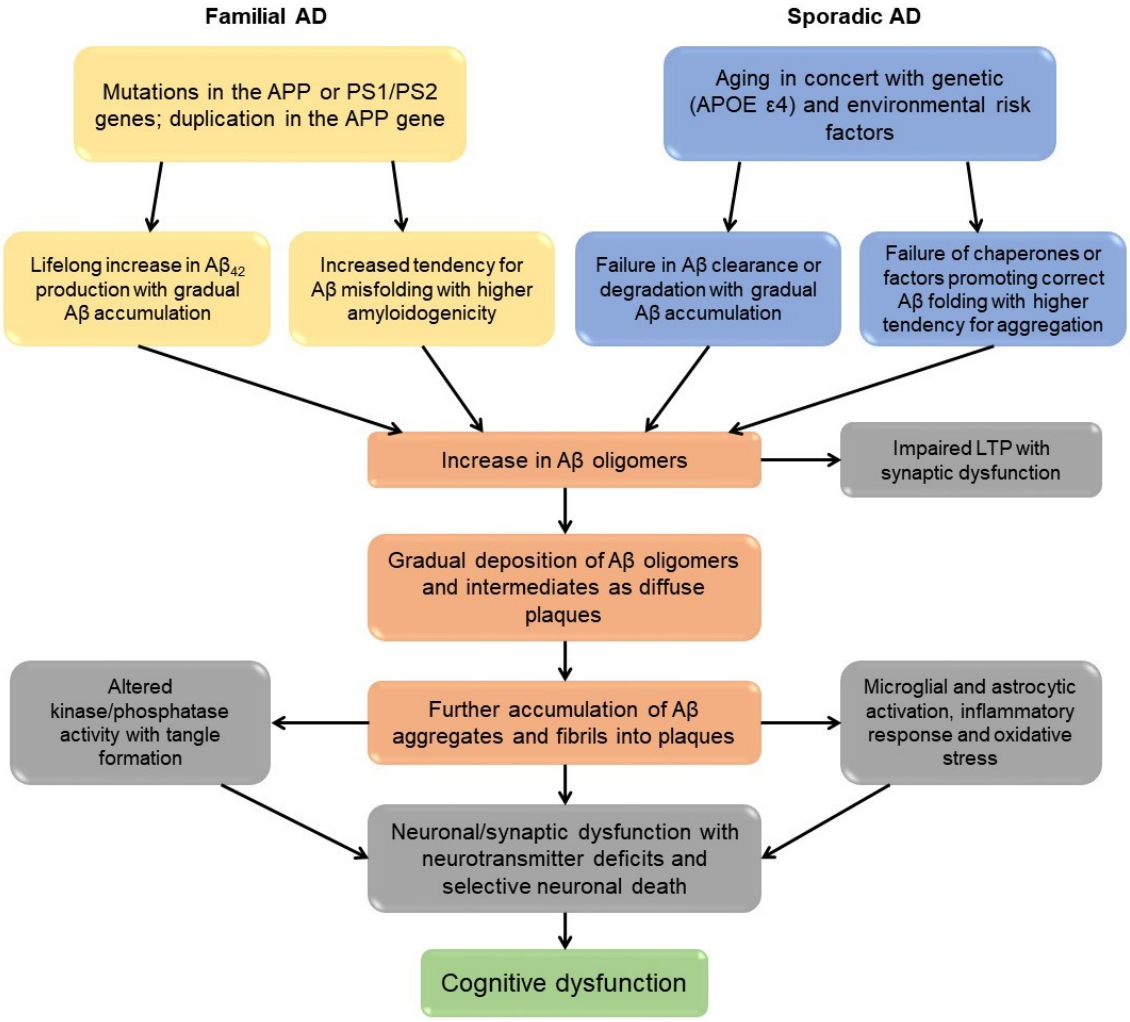


**Figure 1.2:** The sequence of pathogenic events leading to AD proposed by the ACH. Adapted from Hampel et al. [77].

The accumulation of A$\beta$ in various areas of the brain acts as a pathological trigger for a cascade that culminates in cell death, leading to progressive dementia associated with extensive A$\beta$ and tau pathology [76].

The major support for the ACH came from the combination of pathophysiology and human genetics. The origins of the ACH lie in the sequencing of the amino acid sequence of A$\beta$ extracted from cerebral blood vessels [78] and then brain parenchyma [79] of *post-mortem* brains from AD patients. This led to the identification and sequencing of APP gene [7] that encodes the protein from which the A$\beta$ peptide is formed by the sequential action of $\beta$-secretase [80] and $\gamma$-secretase [81].

Although many evidence have been challenging the concepts of the ACH [82], there are still many findings supporting a key role for A$\beta$ dyshomeostasis in AD initiation, thus this hypothesis remains the most commonly accepted [83]. Considering the toxicity of A$\beta$ fibrils for most cells (glial, retina and cerebellar granule cells) [84], the fibrillary A$\beta$ hypothesis did not explain the selective neurodegeneration affecting specifically the hippocampus and parietal lobes. This has led to the concept of soluble toxic oligomers [85], that are intermediary forms between free soluble A$\beta$ and insoluble amyloid fibres, and seem to be toxic both *in vitro* and *in vivo*. However, the molecular nature of these oligomers remains elusive. The extent to which PS1 mutations generate mixtures of A$\beta$ that are more prone to form toxic oligomers remains to be investigated. Nonetheless, this concept could explain cases of AD in which smaller amounts of A$\beta$ are generated [86], although 99.9% of all AD patients have wild type PS [83].

There are findings reporting that the plaque load correlates less well with cognitive decline than do tau pathology [87]. However, A$\beta$ deposition occurs very early and is a widespread event much distant to the clinical dementia, leading to downstream cellular and molecular changes (namely tangles and neuritic dystrophy) that are more forthcoming to and causative of neuronal dysfunction [83]. Furthermore, many non-demented humans show abundant A$\beta$ deposits at death, but some or many of these deposits are diffuse plaques and the levels of oligomeric A$\beta$ are lower than in AD brains [83].

From all the A$\beta$ species, A$\beta_{42}$ oligomers are the most toxic [88], mostly due to their ability to promote excitotoxicity by interacting with different receptors [89], and also to cause endoplasmic reticulum stress and $Ca^{2+}$ levels depletion [90], mitochondrial dysfunction [91], bidirectional axonal transport inhibition [92] and oxidative stress [93] due to interaction with several cellular structures [94].

ACH originally placed amyloid as upstream of tau pathology, although detailed neuropathological studies have shown that tau pathology is present from a very early age in some people and sometimes precedes amyloid pathology [95]. Correlative studies have shown that amyloid pathology likely drives tau pathology from restricted allocortical sites to proliferate throughout the cortex leading to widespread neuronal loss [96]. In addition, human genetics proves that A$\beta$-elevating APP mutations lead to downstream alteration and aggregation of wild type tau, but tau mutations do not lead to A$\beta$ deposition and A$\beta$ related dementia, further supporting the ACH [83].

### 1.3.1 Synaptic Loss

Synapses are the principal unit of intercellular communication in neural circuits, and hippocampal synaptic plasticity underlies memory and learning in humans and in animal models [97]. Synapses are considered the earliest site of AD pathology, and synaptic loss is the best pathological correlate of cognitive impairment in subjects with AD [98]. Assessment of the regional integrity of synapses in *post-mortem* brains relies on quantitative ultrastructural evaluation of synaptic density and immunological detection of synaptic proteins [99]. Synaptic loss in neocortex has been postulated to be associated with the cognitive and memory impairments in normal elderly individuals [100] and are accentuated in subjects with AD [101].

The degree of cognitive decline in patients with AD has been correlated with changes in the presypnatic vesicle protein Synaptophysin (SYP) in the hippocampus and the association cortices [98].

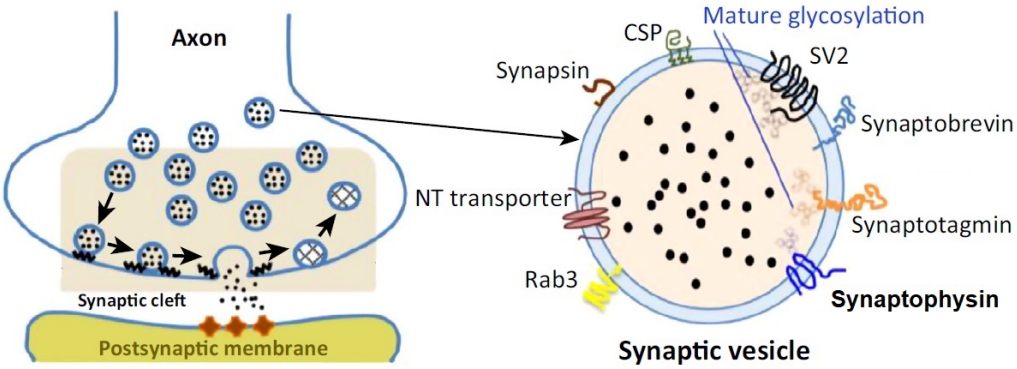


**Figure 1.3:** Representation of the dynamics of synaptic vesicles at the presynaptic terminal (left) with a magnified view of a synaptic vesicle (right) showing the main vesicular proteins, including SYP. Adapted from Abad-Rodríguez et al. [102].

SYP, as seen in Figure 1.3, is a calcium binding glycoprotein located in the membrane of small presypnatic vesicles [103]. The significant decrease in SYP of approximately 25% immunoreactivity in human AD neocortex [104] might maybe be explained as a direct consequence of synaptic loss preceding or following neuronal death [105].

BACE1 has been co-localized with SYP, indicating its presence in synapses [106].

### 1.3.2 Neuroinflammation

Inflammation has been shown to play a critical role in the pathogenesis of AD [107]. Neuroinflammation is the local reaction in the CNS to infection, trauma, toxic molecules or protein aggregates. The brain microglia is able to trigger an appropriate response involving secretion of cytokines and chemokines, resulting in the activation of astrocytes and recruitment of peripheral immune cells. Interleukin-1$\beta$ (IL-1$\beta$) plays an important role in this response [108].

Activated microglial production of the pro-inflammatory cytokine Interleukin-1 (IL-1) is an early event in the generation of A$\beta$ plaques [109], stimulating APP processing and synthesis [110] and A$\beta$ production [111], indicating a role for IL-1 in A$\beta$ fibrillization and aggregation [112]. The chronic deposition of A$\beta$ stimulates the persistent activation of microglial cells in AD [113], leading to increased IL-1$\beta$ levels that have been implicated in the response to A$\beta$ deposition [114].

The molecular steps leading to IL-1$\beta$ maturation take place in an intracellular complex termed the inflammasome [115], as seen in Figure 1.3. The inflammasome is a caspase-1 activating multiprotein platform that results from oligomerization of inactive monomeric proteins from the nucleotide binding domain of the leucine-rich repeat protein family. Different complexes have been described and are usually defined by the core leucine-rich repeat proteins that are implicated, such as NOD-Like Receptor Protein 3 (NLRP3), NLRP1, NLR Family CARD Domain Containing 4 (NLRC4) or the HIN200 family member, interferon-inducible protein AIM2 [108].

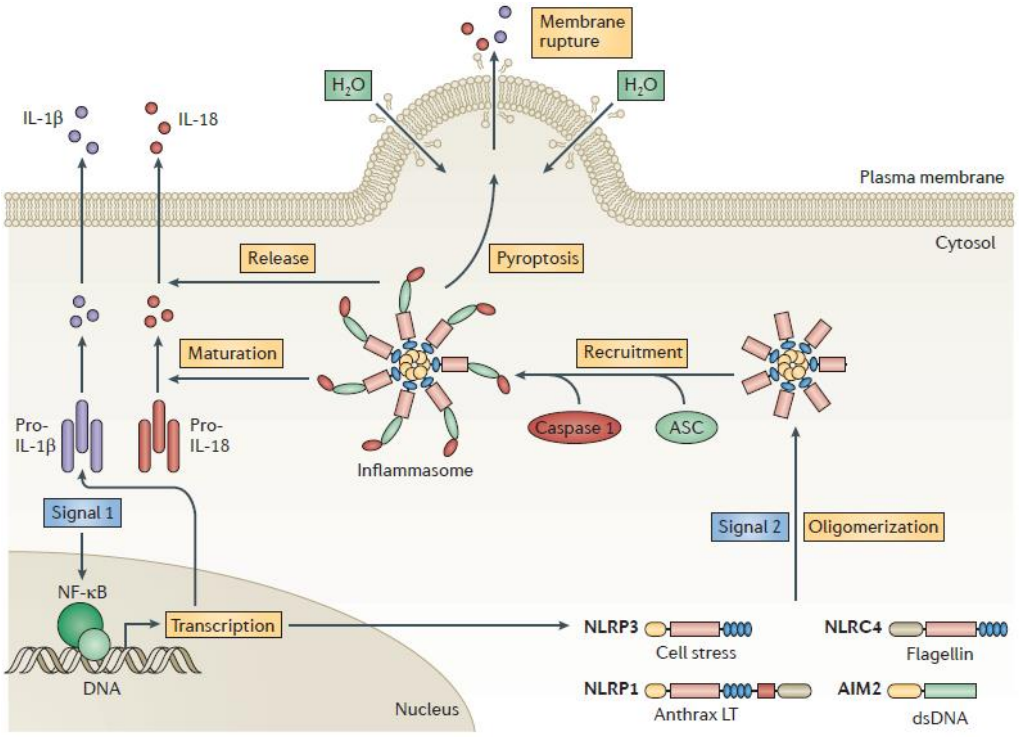


**Figure 1.4:** Inflammasome assembly and cytokines expression [116].

The most intensively studied inflammasome is the NLRP3, which is formed when NLRP3 associates with the adaptor protein Apoptosis-Associated Speck-Like Protein Containing CARD (ASC) and procaspase-1 upon activation by different stimuli, such as pathogen associated molecular patterns (PAMPs) [117] or endogenous danger signals (DAMPs) [116]. Inflammasome assembly results in the cleavage of caspase-1 from its proform to its enzymatically active form. This active caspase-1 then processes the cleavage of several substrates, such as pro-IL-1$\beta$ and pro-IL-18 into the mature cytokines IL-1$\beta$ [118] and IL-18 [119].

The implication of the NLRP3 inflammasome in AD has been shown both *in vitro*, by the ability of A$\beta$ to activate the inflammasome [120], and *in vivo*, in an AD mouse model where NLRP3 knock-out mice were protected from spatial memory impairment and showed decreased A$\beta$ plaque burden [121].

Elevated IL-1$\beta$ level in brains of AD patients are justified due to the A$\beta$ role in inflammasome activation that occurs through its phagocytosis [120]. After phagocytosis, A$\beta$ fibrils localize in intracellular lysosomes causing a compromise in the membrane. This leads to the release of cathepsin B (a lysosomal proteolytic membrane enzyme) into the cytosol, activating the inflammasome. However, the mechanisms by which this process occurs is still not clear [122].

### 1.3.3 Cell Death

Neural and synaptic loss, indicated by gradual cerebral atrophy, are a feature of AD [98]. Neural cell death, mainly in the cortex and hippocampus, contributes to the decline of learning and memory abilities observed in AD patients [123].

Cell death can occur through apoptosis, necrosis, postapoptotic secondary necrosis, necroptosis, autophagic cell death, or mitotic catastrophe [124]. Two extremes of cell death mechanisms are apoptosis and necrosis. Hallmarks of apoptosis include activation of caspases, Deoxyribonucleic Acid (DNA) fragmentation, and membrane blebbing [125]. Necrosis describes cell death with swelling of organelles, such as endoplasmic reticulum, mitochondria, and plasma membrane rupture, resulting in cell lysis. Necrosis was once considered accidental due to overwhelming physical or chemical trauma. Nonetheless, currently, it is known that necrosis can occur in a regulated manner known as necroptosis [126].

Necroptosis is induced by many stimuli, such as intracellular Adenosine Triphosphate (ATP) depletion, disturbance of $Ca^{2+}$ homeostasis, mitochondrial depolarization, poly-(adenosine diphosphate-ribose) polymerase activation, proteolysis by nonapoptotic proteases, increased reactive oxygen species, and cell surface receptor activation. There is evidence that these signals use distinct and yet overlapping mechanisms to induce necroptosis [127].

Death cytokines of the Tumor Necrosis Factor (TNF) family are classic inducers of necroptosis. Receptor-Interacting Protein Kinase 1 (RIP1) and Receptor-Interacting Protein Kinase 3 (RIP3), the two critical kinases that mediate TNF-dependent necrosis, have unique signalling mechanisms and pathological functions [128].

RIP1 consists of an N-terminal kinase domain, an intermediate domain, a RIP Homotypic Interaction Motif (RHIM), and a C-terminal death domain motif [129]. RIP3 has an active kinase domain in its N-terminus that is conserved in other RIP kinases and is essential for necrosis, and a C-terminal RHIM motif [126]. Both RIP1 and RIP3 belong to the RIP family of serine-threonine kinases that are involved in innate and adaptive immunity. The RHIM motif allows for the mediation of their interaction [130] and has been proposed to form an oligomeric amyloid-like necroptotic signaling platform [131]. Recently, evidence of a similarity between RIP3 and A$\beta$ amyloidal properties has been presented, with the presence of A$\beta_{42}$ bound to RIP3 [132].

TNF-induced cell death occurs by three main complexes [134], as seen in Figure 1.5.

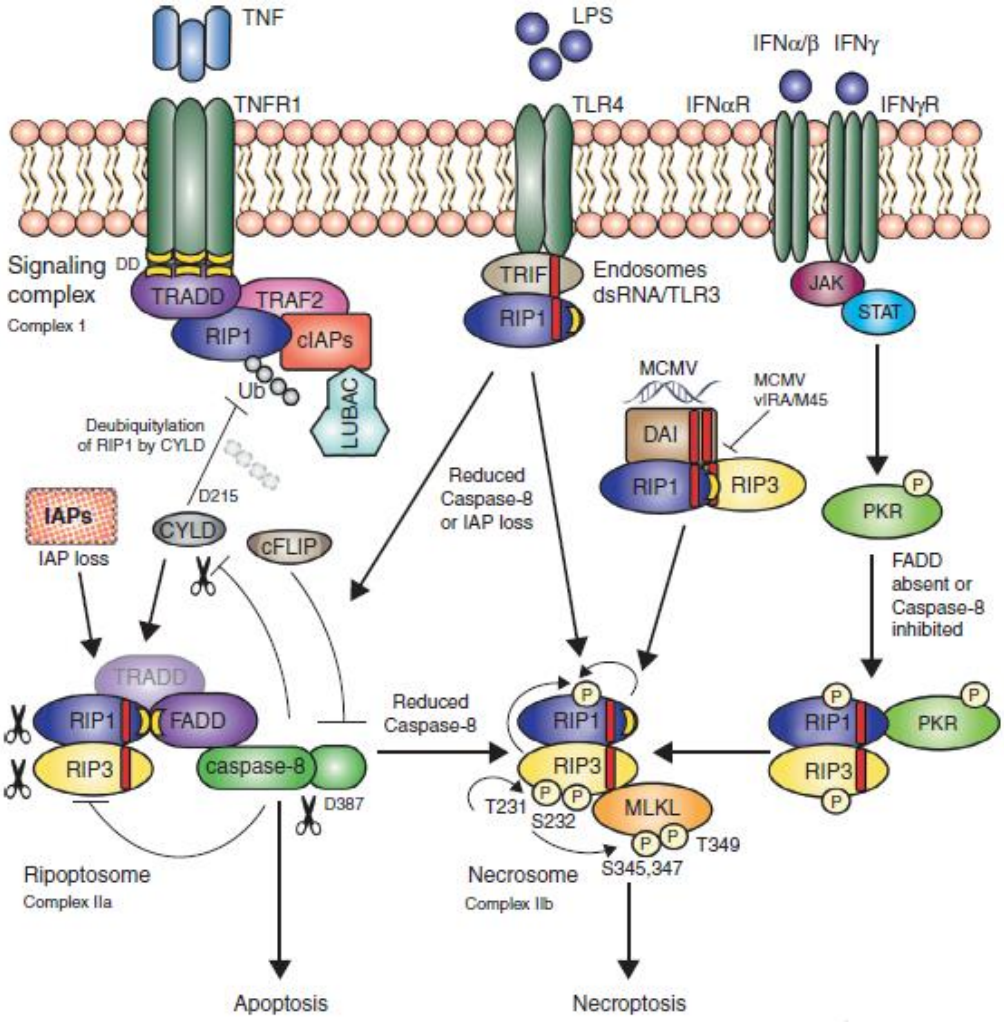


**Figure 1.5:** RIP1 and RIP3 action mechanisms in cell death by apoptosis and necroptosis [133].

TNF Receptor 1 (TNFR1) activation promotes the formation of a pro-survival and inflammation promoter, surface signaling complex, called complex I, comprised of TNFR1-associated DEATH domain (TRADD), RIP1, Cellular Inhibitor of Apoptosis (cIAP) 1 and 2 and Linear Ubiquitin Chain Assembly Complex (LUBAC) [135]. cIAP1/2 bind to the TNFR1 complex via interactions with TNF Receptor Associated Factor 2 (TRAF2) [136]. The ubiquitylation of RIP1 by cIAP1/ 2 and LUBAC [137], and LUBAC ubiquitylation of Nuclear Factor NF-kB essential modifier (NEMO) [138] promotes NF-kB transcription factor activity, and prevents RIP1 forming a cytosolic cell death signaling complex with the adaptor protein FADD and caspase-8, named complex IIa [139]. When caspase-8 activity or an eventual RIP1 ubiquitylation are compromised, the complex IIb, or the necrosome, is formed, causing necroptotic cell death [133]. RIP3 binds to RIP1 through the RHIM domains.

RIP3 is highly phosphorylated upon necroptosis induction, Phosphorylated Receptor-Interacting Protein Kinase 3 (pRIP3), being the determining factor for the necroptotic response [130], with RIP1 being the upstream kinase that initiates necrosis signalling [139]. One phosphorylation site on RIP3, Serine 227 in humans and Threonine 231/Serine 232 in mice RIP3), is particularly important for recruitment and activation of Mixed-Lineage Kinase Domain-Like (MLKL), a crucial downstream substrate of RIP3 in the necroptotic pathway [140]. The activation of MLKL leads to mitochondrial uncoupling, lipid peroxidation, and cell death [141].

A major output of the RIP1 regulated pathways is an inflammatory response, as defined by the production of pro-inflammatory cytokines, which can be mediated in an NF-kB dependent or independent manner. Indeed, the activation of regulated necrotic cell death might also lead to increased inflammation mostly due to the leakage of intracellular content due to cell membrane rupture [142], which suggests a causal role for necroptosis in inflammation associated with AD.

The NLRP3 activation can be mediated by Toll-Like Receptor (TLR) 3 (a pattern recognition receptor that sense a broad range of microbial ligands) through a signalling pathway involving the RIP3/MLKL activity that is controlled by both RIP1 and RIP3, as loss of either kinases leads to inhibition of inflammasome assembly [143].

## 1.4 BACE1 as a Therapeutic Target for AD

The increase in life expectancy is leading to a fast-growing number of patients with dementia, in particular AD. This contributed greatly to an increase in research focused on the discovery of new therapies for prevention of the disease [144]. Unfortunately, there are still no effective treatments to prevent, halt, or reverse AD, but research advances may change this paradigm.

Since the recognition of the central role of secretases in APP processing, they have been labelled as a possible target for therapeutic intervention in AD [145]. Thus, BACE1 has been identified as a significant target since its inhibition would halt the formation of A$\beta$ at the very beginning of the amyloidogenic APP processing. Although it is feasible to target later stages in the cascade, it has been shown that generation of A$\beta$ in excess can be detrimental to cognitive function by limiting synaptic function even before neuronal death [146]. It is then imperative to come up with a therapeutic strategy to slow the progression of AD by inhibiting A$\beta$

formation at an early stage [9].

In order to provide *in vivo* validation that concerns BACE1 as the main $\beta$-secretase enzyme in the brain, BACE1 knockout$^{-/-}$ mice were produced [147]. These mice did not have any detectable abnormalities, were viable and fertile with normal morphology, behaviour, tissue histology, blood cell, and clinical chemistry [60]. Unfortunately, there is also evidence of subtle neuronal phenotypes, such as axon targeting errors [148], reduced myelination [149], memory impairments [150], reduced muscle spindles [151], neurochemical abnormalities [152], alterations in neurogenesis and astrogenesis [153], increased age-related neurodegeneration [154], endophenotypes of schizophrenia [155], reduced spine density [155], seizures [156], and retinal pathology [157]. BACE1 deficiency in mice overexpressing APP rescues memory deficits, which is correlated with a reduction of cerebral A$\beta$ levels, pointing BACE1 as a prime therapeutic target to AD [158].

In order to have clinical potential, BACE1 inhibitors must be of high potency, stable to hydrolysis, present low toxicity, able to cross the BBB, and have selectivity over other aspartyl proteases (namely BACE2, cathepsin D, pepsin, renin, and cathepsin E, among others) [9]. Screening of extensive libraries for non-peptide-based BACE1 inhibitors has resulted in the discovery of a few low-affinity compounds, indicating that this is not an easy target to inhibit effectively *in vivo* because of the extended substrate-binding site requirements [159].

The BACE1 active site is larger and less hydrophobic than other aspartyl proteases, suggesting that enzyme selectivity is indeed possible with a small-molecule inhibitor [160]. One of the major limitations to the CNS penetration of BACE1 inhibitors across the BBB is the active efflux by P-glycoprotein [161].

The BACE1 druggability was first demonstrated with the development of substrate inhibitor OM99-2 and the elucidation of the crystal structure of the BACE1 and OM99-2 complex. This inhibitor was designed based upon a transition-state mimetic concept using non-hydrolyzable dipeptide isostere at the scissile site [162]. Nowadays, even more compounds are being developed. GSK188909 was a potent and selective non-peptidic inhibitor able to block A$\beta$ when co-administered with a P-glycoprotein inhibitor [163]. TC-1, an orally administered inhibitor from Merck, was shown to reduce CSF and plasma A$\beta$ levels in non-human primates [164]. LY2886721, an orally bioavailable small molecule BACE1 inhibitor dose-dependently decreased both plasma and CSF levels of A$\beta_{40}$ and sAPP$\beta$ levels in the CSF [165]. Regrettably, several complications halted these compounds progression in clinical trials.

However, MK-8931, AZD3293 and E2609, are still in clinical trials. MK-8931, a small molecule BACE1 inhibitor, succeeded in both Phase 1 [166] and 2 [167] clinical trials, and progressed to Phase 3. It dose-dependently decreased A$\beta_{40}$, A$\beta_{42}$ and sAPP$\beta$ levels in the CSF of healthy and moderate AD patients. AZD3293 was tested in a Phase 1 study in healthy young and elderly subjects, were it dose-dependently reduced A$\beta_{40}$, A$\beta_{42}$ and sAPP$\beta$, and increased sAPP$\alpha$ levels [168]. It then progressed to combined Phase 2/3 trials. E2609 is an orally bioavailable small molecule BACE1 inhibitor that dose-dependently reduced levels of A$\beta$ in the CSF and plasma, with reduction of sAPP$\beta$ and increase of sAPP$\alpha$ levels in the CSF in Phase 1 trials with healthy individuals [169]. Phase 2 studies are still occurring, and a Phase 3 trial is planned.

To date, none of the created BACE1 inhibitors have made it to commercial use, admitting many have shown clinical potential. Although the development of some BACE1 inhibitors has been hampered, these reports suggest that disease modifying therapy for AD might be a reality in the future. However, given that BACE1 seems to be important in other physiological processes due to the multiple substrates, careful dosing of BACE1 inhibitors is necessary. Consequently, it is crucial to define the level of BACE1 inhibition needed to achieve efficacy, the correct time to start the intervention, whether modulation of A$\beta$ production can modify the course of the disease once symptoms manifest, and the mechanism-based side effects that could be anticipated with BACE1 inhibition [54].

## 1.5 Cellular Uptake of Macromolecules

The cytoplasmic membrane forms a stable barrier between the interior and the exterior of the cell through the phospholipid bilayer, being impermeable for most macromolecules. Nevertheless, some proteins and cell penetrating peptides enter cells in an efficient manner, and it has been proved that endocytosis contributes to the import of these molecules [170]. Endocytosis is a mode of active transport in which a cell transports molecules inside vesicles to its interior to deliver to lysosomes or other organelles. Endocytosis pathways can occur in three different manners: clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis.

The clathrin-mediated endocytosis consists in the production of clathrin-coated vesicles originated from domains of the plasma membrane, dubbed clathrin-coated pits. These pits can concentrate large extracellular molecules bound to different receptors responsible for the receptor-mediated endocytosis of ligands [171]. In caveolae-

mediated endocytosis, caveolae are formed by polymerization of cholesterol-binding protein caveolin, which leads to clustering and invagination of cholesterol lipid rafts [172]. Macropinocytosis consists in a actin-dependent endocytic pathway in which the invagination of the cell membrane into cytoplasmic vacuoles internalizes extracellular liquid, and the formed vesicles fuse with another vesicles, such as endosomes or lysosomes [173].

# 2

# Objectives

BACE1 has been recognized as a therapeutic target for AD [60]. Indeed, BACE1 inhibition should lead to A$\beta$ reduction. However, at present there is not a BACE1 inhibitor in clinical use. Therefore, at Centre of Neuroscience and Molecular Biology, we are developing a new BACE1 inhibitor, which we herein will designate by Peptide-x (PEPx), that showed to reduce A$\beta$ levels due to the inhibition of BACE1 activity.

The objectives of this study are: i) to evaluate *in vitro* PEPx cellular internalization, namely the endocytic pathway since we designed the inhibitor in order to favour its endocytic uptake; ii) and to determine the effect of a chronic treatment with PEPx in synaptic loss, neuroinflammation and neural death in the brain of Triple-Transgenic Mouse Model of Alzheimer's Disease (3xTg-AD). As a mater of fact, pronounced synaptic loss is the best pathological correlate of cognitive impairment in AD patients [98], with synaptic dysfunction being one of the pathological processes involved in the early disease stages [174]. Also, neuroinflammation plays a pivotal role in the pathogenesis of AD [107] due to the actions of the IL-1$\beta$ cytokine, which maturation depends on the activity of the NLRP3 inflammasome [115]. Ultimately, the decline of learning and memory abilities in AD patients are due to the massive neural cell death in the cerebral cortex and hippocampus [123].

To accomplish these objectives we:

**I Accessed the role of endocytosis on the internalization of PEPx in Neuro-2a (N2a) cells.**

The endocytic pathway is of particular relevance, since the accumulation of the BACE1 inhibitor in the endosomes will increase co-localization with the enzyme. Actually, BACE1 cleaves APP in the endosomes. In order to access if PEPx enters the cell through this pathway, we used a fluorophore labelled-PEPx, and we blocked endocytosis using the broad-spectrum inhibitor sucrose [175] and the pharmacological inhibitor Chlorpromazine

(CPZ) [176]. Live cell fluorescence was analysed by confocal microscopy.

**II Evaluated SYP levels in 3xTg-AD mice after chronic administration of PEPx.**

The cognitive decline is intimately associated with synaptic loss [105]. Presypnatic vesicle proteins, namely SYP, suffer a significant decrease in the hippocampus and cerebral cortices in AD patients [98] and in animal models [177]. We hypothesized that PEPx chronic administration in 3xTg-AD mice would increase SYP levels in comparison to 3xTg-AD control (saline injected) mice. The SYP protein levels were determined by Western Blot in total brain homogenates of Non Transgenic Mouse (Non Tg), and 3xTg-AD mice treated with saline or PEPx.

**III Determined NLRP3 inflammasome levels in 3xTg-AD mice after chronic administration of PEPx.**

The NLRP3 inflammasome has been shown to lead to an increase in IL-1$\beta$ in AD animal models [121]. Since the NLRP3 inflammasome can be activated by A$\beta$ [120], we hypothesized that PEPx chronic administration would decrease NLRP3 levels in comparison to 3xTg-AD controls. To answer this question we evaluated the protein levels of NLRP3 by Western Blot in total brain homogenates obtained from Non Tg and both treated and control 3xTg-AD mice.

**IV Elucidated RIP1, RIP3 and pRIP3 levels in 3xTg-AD mice after chronic administration of PEPx.**

Cell death by necroptosis is induced by death cytokines of the TNF family, and is mediated by RIP1 and RIP3 [128]. Phosphorylated RIP3 (pRIP3) is crucial for the necroptotic response [130]. Although apoptosis is already proven to occur in AD brains [178], the contribution of necroptosis to the massive cell death occuring in this disease is not completely understood. Thus, we evaluated whether PEPx treatment could alter the levels of RIP kinases that mediate necroptosis. For this purpose, we determined by Western Blot analysis the levels of RIP1, RIP3 and pRIP3 in Non Tg mice, and saline or PEPx treated 3xTg-AD mice in total brain homogenates.

# 3

# Materials and Methods

## 3.1 Materials

30% Acrylamide/Bis Solution 29:1 was acquired from Bio-Rad Laboratories (Hercules, California, USA). Enhanced Chemifluorescence (ECF) Substrate for Western Blotting and Polyvinylidene Difluoride (PVDF) Transfer Membrane Amersham Hybond-P were obtained from GE Healthcare (Little Chalfont, UK). $\mu$-Slide 8 Well (ibiTreat) was obtained from Ibidi (Martinsried, Germany). NZYColour Protein Marker was obtained from NZYtech (Lumiar, Lisboa, Portugal). Bovine Serum Albumine (BSA), Fetal Bovine Serum (FBS) and Pierce BCA Protein Assay Kit were obtained from Thermo-Fisher Scientific (Waltham, Massachusetts, EUA). Chlorpromazine Hydrochloride (CPZ), Dulbecco's Modified Eagle Medium (DMEM) (D5648), DMEM Without Phenol Red (D5030), Nonessential Amino Acids, Penicillin - Streptomycin, Protease Inhibitor Cocktail (P2714-1BTL), Sodium Pyruvate, Sucrose, N, N, N', N'-Tetramethyl-ethylenediamine (TEMED) and the remaining reagents were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Methanol was obtained from VWR Prolabo Chemicals (Radnor, Pennsylvania, USA).

All the primary and secondary antibodies used in Western Blot are indicated in Tables 3.1 and 3.2, respectively.

**Table 3.1:** Primary antibodies information.

| Primary Antibodies | Host | Dilution | Molecular Weight | Supplier |
|---|---|---|---|---|
| $\beta$ Actin | Mouse | 1:10000 | 42 kDa | Sigma |
| NLRP3 | Rat | 1:250 | 117 kDa | R&D Systems |
| pRIP3 | Rabbit | 1:5000 | 53kDa | abcam |
| (Phospho T231+S232) | | | | |
| RIP1 | Mouse | 1:200 | 74 kDa | BD Biosciences |
| RIP3 | Rabbit | 1:500 | 57 kDa | abcam |
| SYP | Mouse | 1:1000 | 38 kDa | Sigma |

**Table 3.2:** Secondary antibodies information.

| Secondary Antibodies | Host | Dilution | Supplier |
|---|---|---|---|
| Anti-Mouse | Sheep | 1:10000 | GE Healthcare |
| Anti-Rabbit | Goat | 1:20000 | GE Healthcare |
| Anti-Rat | Goat | 1:5000 | Santa Cruz Biotechnology |

## 3.2 Neuro-2a Cell Line

The N2a cells are a fast-growing neuroblastoma cell line [179]. The cells were derived from a spontaneous tumour in an albino strain A mouse. N2a cells have been used to study neurite outgrowth [180], neurotoxicity [181], AD (pathological mechanisms and pharmacological studies) [182], and asymmetric division of mammalian cell lines [183].

N2a cells are highly relevant in the field of AD studies, since they represent the most practical cellular system to study the molecular mechanisms underlying this disease [182].

The N2a cells used in this work were obtained from Dr. Ciro Isidoro, Università del Piemonte Orientale "A. Avogadro", Novara, Italy [184].

N2a cells were cultured in DMEM, supplemented with 10% (w/v) heat inactivated FBS, 1% (w/v) nonessential amino acids, 1 mM sodium pyruvate, and 1% (w/v) Penicillin-Streptomycin.

### 3.2.1 N2a Cell Line Maintenance and Sub-Culturing

The N2a cells cultivated in 75 $cm^2$ flasks were maintained at 37°C in a humidified incubator with 5% $CO_2$. At confluence, the cell medium was removed and cells washed with 5 ml Phosphate-Buffered Saline (PBS) (pH 7.4) and then incubated in 2 ml of trypsin. After a short incubation, allowing the cells to detach from the bottom of the cell culture flask, 8 ml of cell culture medium with FBS were added. Afterwards, 1 ml of the cell suspension was transferred to a new cell culture flask containing culture medium supplemented with FBS in order to make up 15 ml.

### 3.2.2 Cellular Uptake of the New BACE1 Inhibitor

For the analysis of the cellular uptake of the BACE1 inhibitor, the N2a cells were cultured in the $\mu$-Slide 8 Well ibiTreat at a density of 1x$10^5$ cells/$cm^2$ 24 hours after plating.

Cells were washed twice with PBS, and incubated for 10 minutes with 2 $\mu$g/ml Hoechst prepared in PBS. Hoechst 33342 is a cell-permeable nucleic acid dye that emits blue florescence when bound to DNA [185].

To visualize the cellular uptake of the peptide, cells were incubated with 20 $\mu$M or 75 $\mu$M PEPx fluorescent labelled at the N-Terminal with Cy5.5 (Cy5.5-PEPx) for 30 minutes at 37°C in Phenol Red-free DMEM supplemented with 1% (w/v) nonessential amino acids, 1 mM sodium pyruvate, 1% (w/v) Penicillin-Streptomycin, 4.5 g/L Glucose, and 4 mM L-glutamine. In order to investigate whether the cellular uptake of PEPx is carried out by endocytosis, cells were pre-incubated for 30 min at 37°C with 0.4 M sucrose or 30 $\mu$M CPZ, followed by the incubation of the PEPx in the presence of the inhibitors.

Sucrose is a disaccharide, resulting from the combination of monosaccharides glucose and fructose. Hyperosmotic sucrose solutions are used as a non-specific inhibitor of receptor-mediated endocytosis [175], being known to inhibit shallow coated pits formation and preventing interaction of clathrin and adaptors [186]. CPZ is a cationic amphiphilic drug that was initially developed as an anti-psychotic medication that blocks dopamine receptors [187]. CPZ inhibits clathrin-coated pit formation by a reversible translocation of clathrin and its adapter proteins from the cytoplasmic membrane to intracellular vesicles and receptor recycling [188].

After treatments, cells were washed three times with PBS and kept in Phenol Red free medium. The fluorescence was visualized by confocal microscopy (Zeiss LSM 710 inverted microscope) and processed with the Zen lite software from Zeiss (Oberkochen, Germany).

For image analysis, we used FIJI [189]. We splitted the colour channels and, in the image corresponding to PEPx fluorescence, adjusted the detection threshold. The integrated intensity was obtained by multiplying the area by the mean gray value and then normalized to the total cell number in each image.

## 3.3 Triple-Transgenic Mouse Model of Alzheimer's Disease

The 3xTg-AD mice harbour the $PS1_{M146V}$, $APP_{Swe}$ and $tau_{P301L}$ transgenes, developing both plaque and tangle pathology in the hippocampus, amygdala and cerebral cortex, the brain regions associated with AD. This model was obtained by directly introducing human $APP_{Swe}$ and human $tau_{P301L}$ transgenes into the germline of homozygous mutant $PS1_{M146V}$ knockin mice by comicroinjection [177].

These mice develop age-related extracellular A$\beta$ deposits and LTP deficits prior to tangle formation, consistent with the ACH [177].

At 2 months of age, the mice do not present A$\beta$ nor tau pathology, have normal spatial reference and contextual fear memory. At 4 months of age, the 3xTg-AD mice present increased levels of intraneural A$\beta$, manifesting subtle impairments in long-term memory. At 6 months of age, the 3xTg-AD mice are neuropathologically characterized by diffuse amyloid plaques in the neocortex, and intraneural A$\beta$ buildup in pyramidal neurons of the hippocampus, cortex, and amygdala, with the onset of short-term memory impairment [2].

### 3.3.1 3xTg-AD Mice Chronic Treatment With the New BACE1 Inhibitor

At 4 months old, 3xTg-AD mice were subjected to a chronic treatment with the new BACE1 inhibitor, PEPx. For that purpose, mice were daily injected intraperitoneally (i.p.) with 1.25 mg/kg PEPx or with saline (0.9% NaCl) for 4 months.

After treatment, the mice were sacrificed under anaesthesia followed by cervical dislocation. The brains were dissected out on ice and the samples immediately stored at –80°C.

All animal procedures were done in accordance with the European Union Guidelines for Animal Research, Directive 2010/63/EU of September 22, 2010.

### 3.3.2 3xTg-AD Mice Brain Homogenates Preparation

After chronic treatment and sacrifice, the 3xTg-AD mice brains were homogenized in ice cold RIPA Lysis Buffer (250 mM NaCl, 50 mM Tris-Base, 1% Nonidet P-40, 0.5% DOC, 0.1% Sodium Dodecyl Sulfate (SDS), pH 8.0) supplemented with 1% Protease Inhibitor Cocktail. After 15 minutes on ice, the samples were centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatant was collected and stored at -80°C.

## 3.4 Western Blotting

### 3.4.1 Protein Quantification

The protein quantification method used was the Bicinchoninic Acid (BCA) for the colorimetric detection and quantification of total protein, and the selected kit the Pierce™ BCA Protein Assay from Thermo-Fischer Scientific (Waltham, Massachusetts, EUA) [190].

This method combines the biuret reaction (reduction of $Cu^{2+}$ to $Cu^{+}$ by protein in an alkaline medium) with the colorimetric detection of $Cu^{+}$ using a reagent containing BCA [191]. The resulting purple-coloured reaction product of this kit is formed by the chelation of two BCA molecules with one $Cu^{+}$. The complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range of 20-2000 $\mu$g/ml.

The colour formation with BCA are due to the macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) [192]. A series of dilutions of known concentration of BSA are used to obtain a standard curve and calculate the concentration of each sample.

The standard and samples were prepared in 96-well plates in triplicate and according to the Tables 3.3 and 3.4.

**Table 3.3:** Standard protein quantification protocol.

| BSA 2mg/ml ($\mu$l) | RIPA ($\mu$l) | $H_2O$ ($\mu$l) | Reagent ($\mu$l) | Protein ($\mu$g) |
|---|---|---|---|---|
| 0 | 1 | 99 | 100 | 0 |
| 1 | 1 | 98 | 100 | 2 |
| 2 | 1 | 97 | 100 | 4 |
| 3 | 1 | 96 | 100 | 6 |
| 4 | 1 | 95 | 100 | 8 |
| 5 | 1 | 94 | 100 | 10 |
| 6 | 1 | 93 | 100 | 12 |
| 7 | 1 | 92 | 100 | 14 |

**Table 3.4:** Sample protein quantification protocol.

| BSA 2 mg/ml ($\mu$l) | Sample ($\mu$l) | $H_2O$ ($\mu$l) | Reagent ($\mu$l) | Protein ($\mu$g) |
|---|---|---|---|---|
| 0 | 1 | 99 | 100 | ? |

For the standard, it was added by order, $H_2O$, RIPA Lysis Buffer supplemented with Protease Inhibitor Cocktail, BSA 2 mg/ml, and A:B reagent solution in the proportion of 50:1.

For the samples quantification, it was added $H_2O$, sample (after vortex), and A:B reagent solution in the proportion of 50:1.

In both cases the final volume was 200 $\mu$l. The multi-well was incubated at 37°C in the dark for 30 minutes, and after that the absorbance was read at 562 nm in the Spectrophotometer Spectramax Plus 384 Microplate Reader with the SoftMax Pro V5.0 from Molecular Devices (California, USA).

The mean of the obtained absorbance values was calculated and used to obtain the parameters of a standard curve, with the respective known concentrations: $y=ax+b$, where $y=absorbance$ and $x=protein$. The protein concentration ($\mu$g/ml) in the brain homogenates was obtained using the mean of the triplicated absorbance values.

### 3.4.2 Sample Preparation

Samples were denaturated at 95°C for 5 minutes in 6x concentrated sample buffer (0.5 M Tris, 30% (v/v) glycerol, 10% (w/v) SDS, 0.6 M DTT, 0.012% bromophenol blue). Equal amounts of protein of each sample (50 $\mu$g) were separated by electrophoresis on 10% acrylamide gels.

### 3.4.3 Gel Preparation and Electrophoresis

Once the samples were prepared, we mounted the electrophoresis system for 1.5 mm gels and checked with distilled water if there were no leaks. The resolving gel was prepared in a falcon tube (in the order of table 3.5) and applied after a small vortex. The remaining solution was kept as a control of the polymerization time.

**Table 3.5:** 10% acrylamide resolving gels.

| Resolving Gel | One gel | Two gels | Three gels | Four Gels | Six gels |
|---|---|---|---|---|---|
| 2x Resolving Buffer | 4 ml | 8 ml | 12 ml | 16 ml | 24 ml |
| 30% Acrylamide/Bis | 2.66 ml | 5.34 ml | 8 ml | 10.66 ml | 16 ml |
| Distilled $H_2O$ | 1.34 ml | 2.66 ml | 4 ml | 5.2 ml | 8 ml |
| 10% APS | 60 $\mu$l | 120 $\mu$l | 180 $\mu$l | 240 $\mu$l | 360 $\mu$l |
| TEMED | 4 $\mu$l | 8 $\mu$l | 12 $\mu$l | 16 $\mu$l | 24 $\mu$l |

The resolving buffer (2x) composition is 0.75 M Tris-HCl, 0.2% SDS, pH 8.8.

We levelled the gel and removed the air bubbles by application of isopropyl alcohol. After polymerization, isopropyl alcohol was discarded and the gel washed with distilled water.

We prepared the stacking gel accordingly to Table 3.6, applied it over the resolving gel and placed the combs with the desired number of wells.

**Table 3.6:** 4% acrylamide stacking gels.

| Stacking gel | One gel | Two gels | Three gels | Four gels |
|---|---|---|---|---|
| 2x Stacking Buffer | 1.25 ml | 2.50 ml | 3.75 ml | 5 ml |
| 30% Acrylamide/Bis | 0.38 ml | 0.76 ml | 1.140 ml | 1.52 ml |
| Distilled $H_2O$ | 0.90 ml | 1.8 ml | 2.7 ml | 3.6 ml |
| 10% APS | 15 $\mu$l | 30 $\mu$l | 45 $\mu$l | 60 $\mu$l |
| TEMED | 2.76 $\mu$l | 5.5 $\mu$l | 8.26 $\mu$l | 11 $\mu$l |

The stacking buffer (2x) composition is 0.25 M Tris-HCl, 0.2% SDS, pH 6.8.

After the stacking gel polymerization, we gently removed the combs and washed the wells with distilled water. The Mini Protean III system was mounted with special attention to the colour correspondence and running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3) was added in accordance with the number of gels.

The run starts at a 100 V, and is then increased to 120-130 V until the ladder bands of interest are all visibly separated.

### 3.4.4 Electro-Transference

After the electrophoresis, the proteins were electroblotted onto a PVDF membrane.

Membranes were activated by soaking in methanol for 10 seconds, in distilled water for 5 minutes and in transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol at pH 8.3) for 10 minutes. In the cassette, on the black side over the sponge, it was placed, by order and without air bubbles:

1. One sheet of filter paper soaked in transfer buffer;
2. The gel;
3. The membrane, properly marked to know the sample order and which side is in contact with the gel;
4. Another sheet of filter paper, also soaked in transfer buffer.

The other sponge is laid, the cassette closed and placed in the tin. Since the proteins have a negative charge, the gel must be placed in the black negative pole in order to the proteins migrate toward the red positive pole and be adsorved into the membrane placed on the side of the positive pole by application of an electric current.

The system was connected to a power source with a constant amperage of 750 mA for 2 hours.

### 3.4.5 Immunodetection

After protein transfer to the PVDF membrane was completed, each membrane was incubated in 5 ml of 5% BSA (w/v) blocking agent in Tris-Buffered Saline (TBS) (150 mM NaCl, 25mM Tris-HCl pH 7.6) with 0.1% (v/v) Tween 20 for 1 hour at room temperature (RT). The membranes were incubated overnight at 4°C in 5 ml of primary antibody prepared in 5% BSA.

After the incubation period, the membranes were washed six times for 5 minutes in TBS/Tween, incubated for 1 hour at RT with 5 ml of the secondary antibody prepared in TBS/Tween, and washed again in TBS/Tween six times, 5 minutes each.

The membranes were then incubated with ECF substrate in a smooth surface, the proteins facing down in contact with 300 $\mu$l of the ECF reagent for a maximum of 5 minutes at RT.

Immunoreactive bands were detected on a Versadoc Imaging System from Bio-Rad Laboratories (California, USA).

### 3.4.6 Membrane Stripping and Reprobing

In order to reprobe the membranes with another antibody, we removed the ECF and the previous antibodies.

For the ECF removal, we washed the membranes overnight with TBS/Tween at 4°C or for 30 minutes with 40% Methanol at RT.

To remove antibody, the membranes were washed, with continuous agitation, with water for 5 minutes, with NaOH (0.2 M) during 5 minutes, and with water again for 5 more minutes.

After the required strippings, we incubated the membranes in 5 ml of 5% BSA (w/v) in TBS with 0.1% (v/v) Tween 20 for 1 hour at RT, being then ready for new antibody incubation.

## 3.5 Statistics

Statistical significance was determined by ordinary one-way ANOVA followed by the Sidak's post-hoc test for multiple groups or Students' t-test, as described in figure legends. Data were analysed by using Excel 2016 (Microsoft, Seattle, WA, USA) and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) software. Data were expressed as the mean ± SD of the number of experiments indicated in figure legends. $p < 0.05$ was considered significant.

# 4

# Results and Discussion

## 4.1 *In vitro* Studies

The new BACE1 inhibitor PEPx, developed in our laboratory, demonstrated to decrease the production of A$\beta$ peptides, A$\beta_{40}$ and A$\beta_{42}$, in an *in vitro* model of AD, the N2a cells expressing human APPswe [184]. The levels of both A$\beta$ species were previously evaluated by sandwich ELISA in the conditioned medium of N2a-APPswe cells treated with vehicle or PEPx.

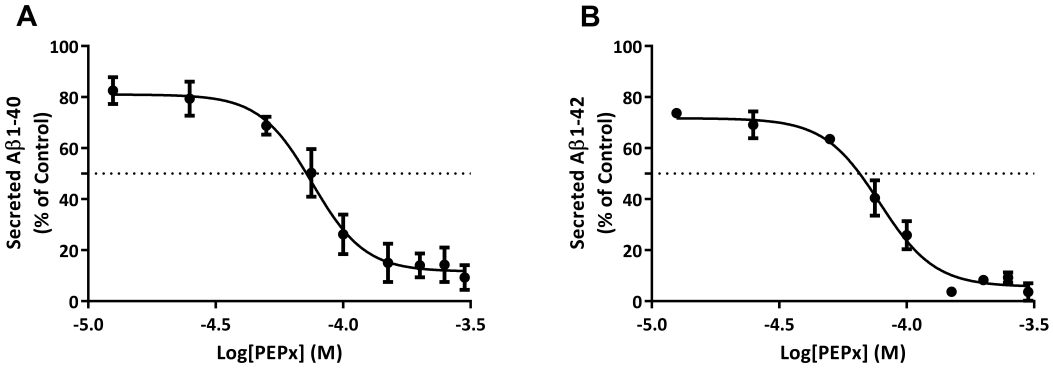


**Figure 4.1: Dose-response curves regarding the effect of the new BACE1 inhibitor PEPx on A$\beta_{40}$ and A$\beta_{42}$ levels in N2a cells expressing APPswe.** Effect of PEPx on secreted A$\beta_{40}$ (A) or A$\beta_{42}$ (B) levels. Twenty-four hours after plating, N2a cells constitutively expressing human APPswe were incubated in FBS free medium with 12.5 to 300 $\mu$M of the peptides for 24 hours, at 37°C, in a humidified incubator with 5 % $CO_2$. At the end of the incubation period, the conditioned medium was collected and stored at -80°C until analysis of A$\beta_{40}$ and A$\beta_{42}$ levels by a sandwich ELISA test (Invitrogen), according to manufacturer's protocol. Control cells were subjected to the same experimental procedures but in the absence of peptide. The results represent the mean ± SEM of n = 2-7 independent experiments performed in duplicate, and are expressed as percentage of control (Resende et al., unpublished results).

As can be seen in Figure 4.1, PEPx decreased in a dose-dependent manner the production A$\beta_{40}$ (Figure 4.1A) and A$\beta_{42}$ (Figure 4.1B) in N2a-APPswe cells (Resende et al., unpublished results). The IC50, or half maximal inhibitory concentration, refers to the peptide concentration that inhibits, in this instance, endogenous A$\beta_{40}$ and A$\beta_{42}$ production in N2a-APPswe cells by 50%. From the results above in Figure 4.1, the IC50 determined in a cellular assay for A$\beta_{40}$ is $7.562 \times 10^{-5}$ M and for A$\beta_{42}$ is $7.823 \times 10^{-5}$ M.

These results are in accordance with the reductions in A$\beta_{40}$ and A$\beta_{42}$ levels observed in rodents and large animal species treated with BACE1 inhibitors that are in clinical trials, namely E2069, MK-8931, and LY2886721 [60]. The results validated our premise, therefore we next investigated the mechanisms of PEPx cellular uptake.

### 4.1.1 PEPx Cellular Uptake in N2a Cells

One of the limitations of previously developed BACE1 inhibitors has been the low cellular permeability [9]. Therefore, PEPx, a cationic peptide, was designed in order to favour the endocytic uptake of the compound. On the other hand, BACE1 cleaves APP in the endosomes [193], thus the location of the new BACE1 inhibitor PEPx in the endosomal compartment promotes the co-localization with BACE1, increasing the probability of its inhibition. Since we hypothesized that PEPx will be internalized by endocytosis, we proposed to inhibit this pathway in order to investigate whether PEPx cellular uptake is affected.

For that purpose, we incubated N2a cells with PEPx coupled to the fluorophore Cy5.5, and followed its internalization in the absence or in the presence of a high concentration of sucrose or CPZ, which have been described to inhibit endocytosis [170].

We incubated the N2a cells in $\mu$-Slide 8 Well ibiTreat for 30 minutes in the presence or in the absence of 0.4 M sucrose (Figure 4.2). Hypertonic sucrose represents the most common chemical inhibitor of clathrin-mediated endocytosis [194], but also affects other endocytic pathways [175].

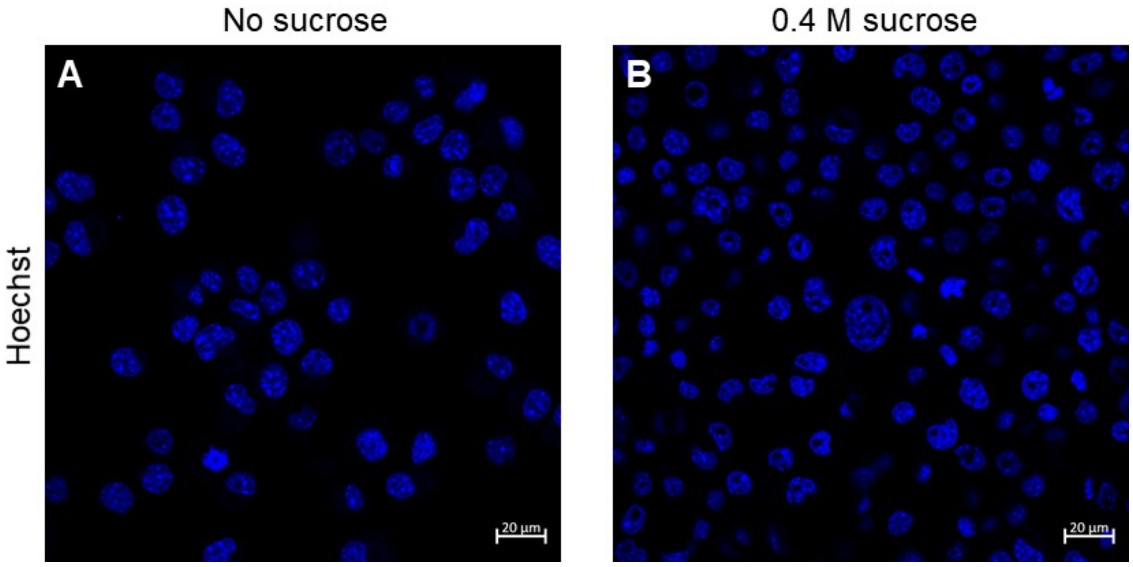


**Figure 4.2: N2a cells visualization in $\mu$-Slide 8 Well ibiTreat.** Nuclei were visualized by Hoechst staining (blue). Cells were pre-incubated with sucrose when this endocytic inhibitor was used. N2a cells were incubated with Hoescht in A, and with Hoechst and 0.4 M sucrose in B. Images were processed using Zen program. Confocal images were obtained with a 40x objective in A and B on a Zeiss LSM 710 inverted microscope. Scale bar: 20 $\mu$m.

As seen in Figure 4.2, 30 minutes incubation with 0.4 M sucrose [194] does not have major apparent negative effects regarding the N2a cell line.

To investigate the cellular uptake pattern of PEPx in the N2a cell line, we tested two different concentrations of PEPx, 20 $\mu$M and 75 $\mu$M, in order to determine whether the uptake mechanism and its subsequent inhibition may depend on peptide concentration.

N2a cells were incubated with Cy5.5-PEPx at 20 $\mu$M in the presence or in the absence of hypertonic sucrose (Figure 4.3).

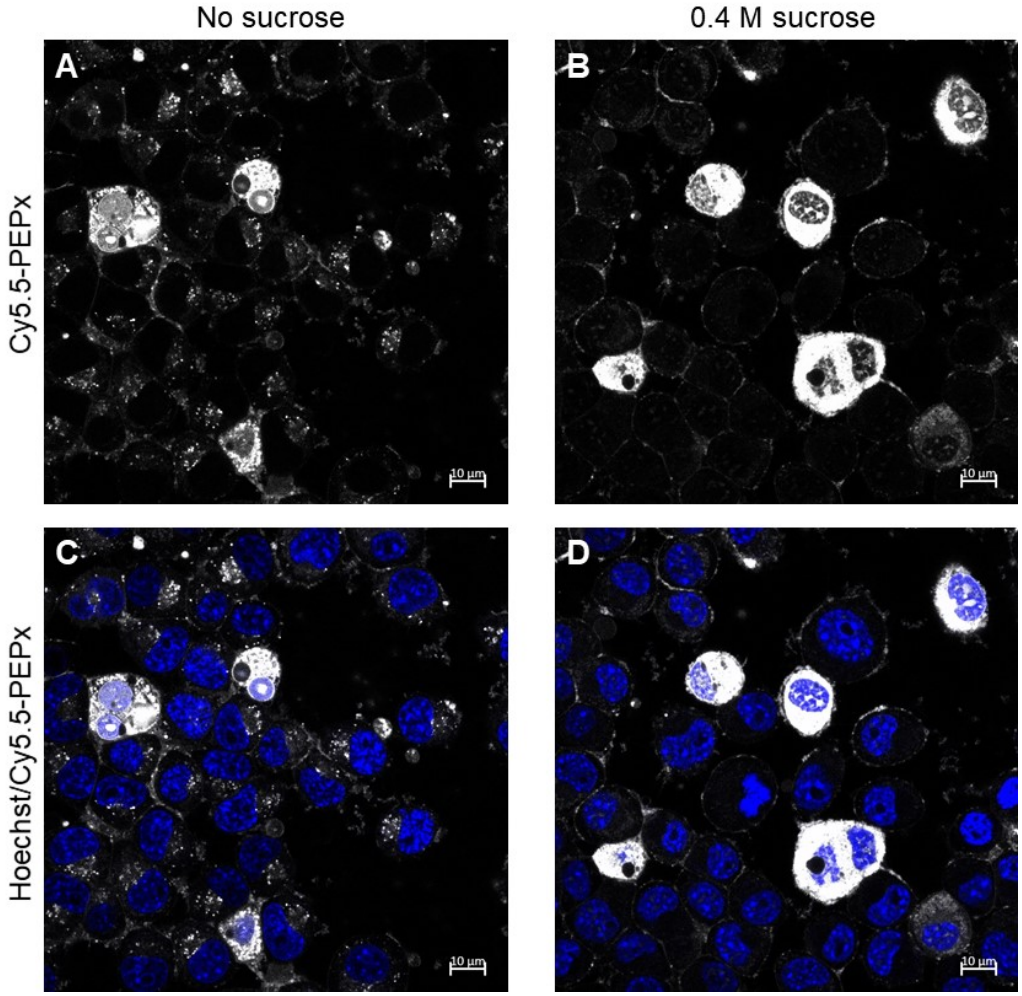


**Figure 4.3: Hypertonic sucrose inhibits the cellular uptake of 20 $\mu$M of Cy5.5-PEPx into vesicles.** Nuclei were visualized by Hoechst staining (blue) whereas Cy5.5-PEPx fluoresce is assigned white in the images. Cells incubated with 20 $\mu$M Cy5.5-PEPx for 30 minutes in the absence (A and C) or in the presence of 0.4 M sucrose (B and D). Cells were pre-incubated with sucrose when this endocytic inhibitor was used. Images were acquired using Zen program. Confocal images were obtained with a 40x objective on a Zeiss LSM 710 inverted microscope. Scale bar: 20 $\mu$m.

As expected in the absence of sucrose, the large majority of cells showed intracellular fluorescence. The BACE1 inhibitor was predominantly localized within vesicles near the cell surface membrane as well as scattered in the cytoplasm, giving the cells a vesicular pattern, as can be seen in Figures 4.3A and 4.3C. Also, a few cells were extremely fluorescent with PEPx both in the cytoplasm and in the nucleus. This goes in accordance with the pattern visualized by Duchardt et al. [170], where they studied the internalization of the cationic cell-penetrating peptide derived from the antennapedia homeodomain.

Hypertonic sucrose is one ot the most used chemical inhibitors of clathrin-mediated endocytosis [194], involving the removal of membrane-associated clathrin lattices resulting in the disappearance of clathrin-coated vesicles, plasma membrane coated pits and clathrin-coated buds on the *trans* Golgi Network [186]. However, this treatment may also affect non-clathrin mediated internalization endocytosis pathways [194].

As can be observed in Figures 4.3B and 4.3D, the pattern of PEPx internalization in high sucrose medium has shifted. Although some cells had intense fluorescence all over the cells, suggesting a massive load of Cy5.5-PEPx, the intracellular vesicular pattern seen in Figures 4.3A and 4.3C obtained in the absence of sucrose ceased to exist. As well, a few cells had diffuse fluorescence. Importantly, in the majority, the cells presented a faint fluorescence surrounding the external cell membrane suggesting that the peptide did not enter the cell. This observation allows us to assume that, besides the cellular uptake being inhibited, the N2a cells cytoplasmic membrane is intact in this experimental conditions.

Afterwards, we accessed the uptake pattern of a higher concentration of Cy5.5-PEPx in N2a cells. The concentration we used was 75 $\mu$M, the IC50 for this peptide. We incubated N2a cells in the presence and absence of 0.4 M sucrose, as shown in Figure 4.4.

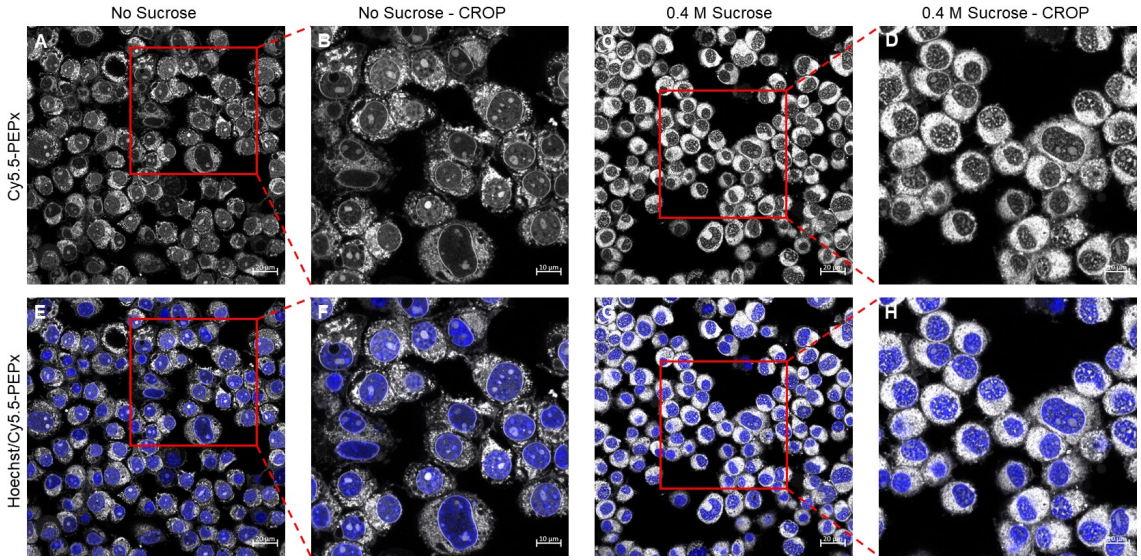


**Figure 4.4: Hypertonic sucrose alters the cellular uptake pathway of Cy5.5-PEPx at 75 $\mu$M.** Nuclei were visualized by Hoechst staining (blue), whereas Cy5.5-PEPx fluorescence is assigned white in the images. Cells were incubated with 75 $\mu$M Cy5.5-PEPx for 30 minutes in the absence (A, B, E and, F) or in the presence of 0.4 M sucrose (C, D, G, and H). Cells were pre-incubated with sucrose when this endocytic inhibitor was used. Images were acquired using Zen program. Confocal images obtained with a 40x objective in A, C, E and G, and with a microscope crop in B, D, F, and H, on a Zeiss LSM 710 inverted microscope. Scale bar: 20 $\mu$m for A, C, E and G, and 10 $\mu$m in B, D, F, and H.

In the absence of high sucrose, the cellular uptake of 75 $\mu$M PEPx had a vesicular uptake pattern at almost all the cytoplasmic area (Figures 4.4A, 4.4B, 4.4E, and 4.4F) instead of localizing in the vesicles mainly near the cell surface membrane as seen for the lower Cy5.5-PEPx concentration (20 $\mu$M). Also, the peptide is present in the nuclear compartment of some cells with a vesicular staining.

In the presence of a high sucrose concentration, the pattern of Cy5.5-PEPx internalization was shifted to a non-vesicular appearance. Indeed, cells presented an intense but mainly diffuse fluorescence in the cytoplasmic area (Figures 4.4C, 4.4D, 4.4G, and 4.4H). Also, these results indicate that high sucrose concentration mainly inhibits the endocytic peptide uptake at 20 $\mu$M but favour cellular uptake, possibly by another pathway than endocytosis, when peptide is present at a higher concentration.

These results suggest that at 20 $\mu$M and 75 $\mu$M Cy5.5-PEPx is internalized into vesicles, probably by clathrin-mediated endocytosis at least for the lower concentration we used in this study. Duchardt et al. [170] observed that above a critical threshold, peptide internalization may occur through highly efficient non-endocytic

pathways originating a diffuse cellular distribution [195], through direct penetration pathways such as inverted micelle formation, pore formation, carpet-like model, and the membrane thinning model [196]. However, the peptide we are developing at both tested concentrations was internalized into vesicles, indicating an endocytic uptake.

### 4.1.2 PEPx Cellular Uptake is Inhibited by CPZ

CPZ is a cationic amphipathic drug that, in a micromolar range, inhibits clathrin-mediated endocytosis [176], although its effect varies in different cell lines [197]. The concentration commonly used to inhibit cellular peptide uptake is 30 $\mu$M for 30 minutes [198].

We assessed the cellular uptake pattern of PEPx in the N2a cell line with and without the presence of 30 $\mu$M CPZ.

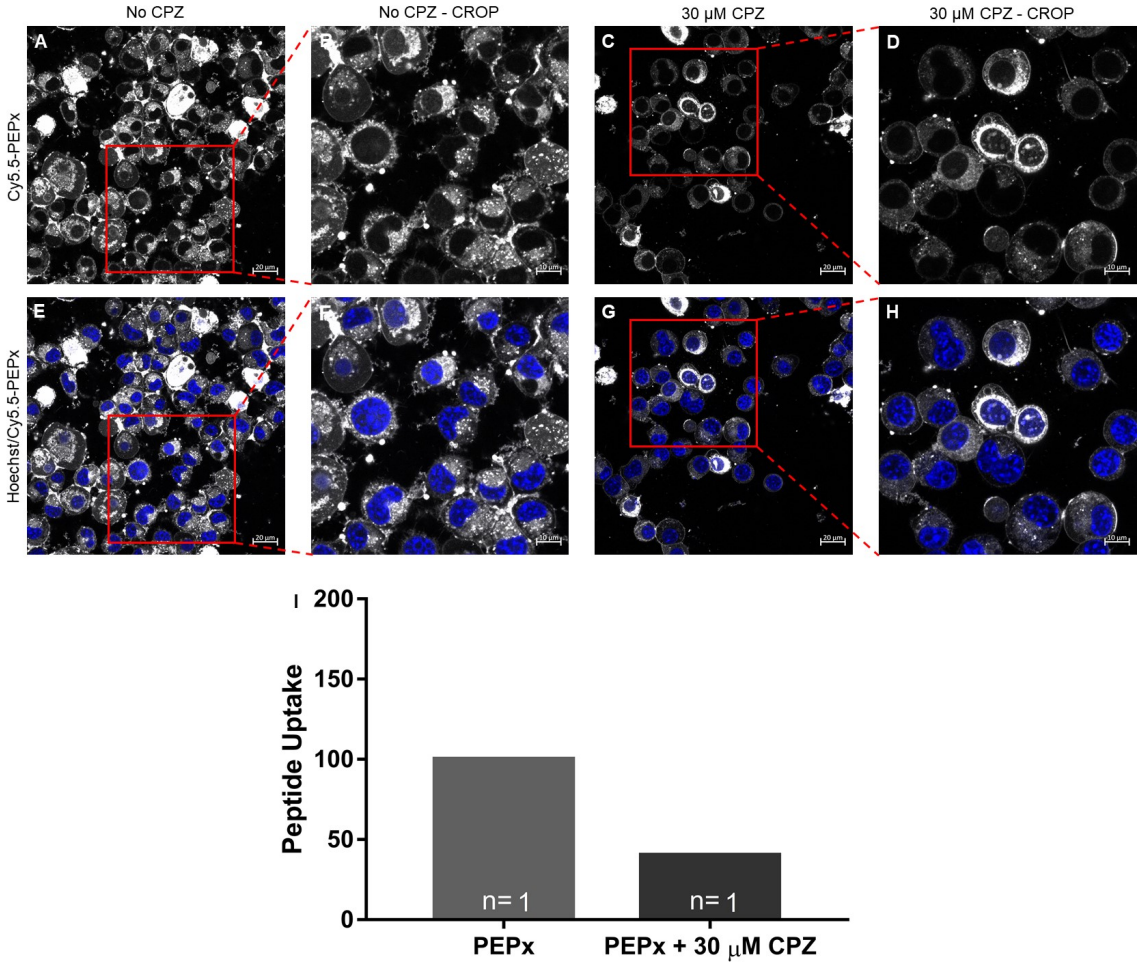


**Figure 4.5: CPZ inhibits the cellular uptake of 20 $\mu$M Cy5.5-PEPx.** Nuclei were visualized by Hoechst staining (blue) whereas Cy5.5-PEPx fluorescence is assigned white in the images. Cells incubated with 20 $\mu$M PEPx for 30 minutes in the absence (A, B, E, and F), or in the presence of 30 $\mu$M CPZ (C, D, G, and H). Cells were pre-incubated with CPZ when this endocytosis inhibitor was used. Images were acquired using Zen program. Confocal images obtained with a 40x objective in A, C, E, and G, and with a microscope crop in B, D, F, and H, on a Zeiss LSM 710 inverted microscope. Scale bar: 20 $\mu$m for A, C, E, and G, and 10 $\mu$m in B, D, F, and H. Peptide uptake quantification in the presence or in the absence of CPZ is shown in I. In each condition the relative fluorescence was calculated in 3-5 images and normalized to the total cell number of each image. The relative fluorescence of N2a cells incubated with Cy5.5-PEPx in the absence of CPZ was considered 100%.

Incubation of the N2a cells with CPZ strongly decreased 20 $\mu$M Cy5.5-PEPx endocytic internalization, suggesting that CPZ inhibited the endocytic uptake of the peptide. Still, a reduced number of cells exhibited diffused faint fluorescence, suggesting a non-endocytic uptake.

Regarding the quantitative analysis seen in Figure 4.5I, the reduction of Cy5.5-PEPx cellular uptake is evident, with the values reducing to less than half.

Both the quantitative and qualitative analysis are in accordance with another study by Duchardt et al. [170], where there is strong evidence that this endocytic pathway is a key internalization mechanism of cationic peptides. These results suggest that PEPx enters the cells through clathrin-mediated endocytosis, as expected.

## 4.2 *In vivo* Studies

Previous results from our laboratory showed that the new BACE1 inhibitor PEPx significantly decreased the amyloidogenic APP processing *in vivo* after chronic treatment of 3xTg-AD mice. Indeed, the new BACE1 inhibitor decreased the levels of the sAPP$\beta$ fragment, which is the BACE1-cleaved ectodomain of APP [67]. The levels of sAPP$\beta$ were evaluated by Western Blotting in total brain homogenates of 3xTg-AD mice daily treated with 1.25 mg/Kg of PEPx (n=11) or the vehicle (n=13) for 4 months, as shown in Figure 4.6 (Resende et al., unpublished results).

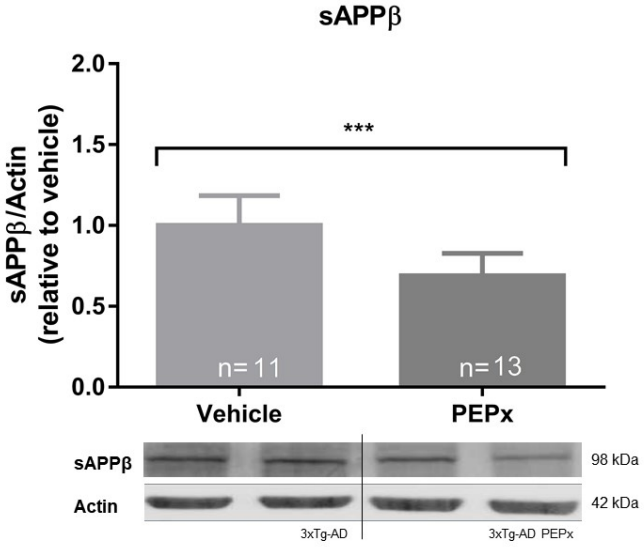


**Figure 4.6: sAPP$\beta$ brain levels in 3xTg-AD mice treated with vehicle and PEPx.** Total brain homogenates from 3xTg-AD mice daily treated with saline or PEPx (1.25 mg/Kg) during 4 months were evaluated by Western Blotting for the total protein levels of sAPP$\beta$. The quantitative results represent the mean ± SD of n = 11-13 mice per group, and are normalized to control (vehicle injected 3xTg-AD mice). Statistical analysis was performed by t-test. The results showed ***$p < 0.001$ for PEPx versus vehicle (Resende et al., unpublished results).

In agreement with PEPx ability to decrease A$\beta_{40}$ and A$\beta_{42}$ production, the 3xTg-AD mice chronically treated with PEPx had diminished sAPP$\beta$ fragment levels relatively to mice treated with vehicle. This reduction is directly correlated with a diminution in BACE1 activity as observed in a cell free essay used to evaluate *in vitro* BACE1 activity in the presence of PEPx (Resende et al., unpublished results), and is in accordance with results from other BACE1 inhibitors, namely E2069, MK-8931, and LY2886721 [60].

Regarding the promising previous results obtained with PEPx, the effects of the chronic treatment in synaptic loss, neuroinflammation and necroptosis were assessed in Non Tg mice and 3xTg-AD mice treated with saline or PEPx.

The Non Tg mice are age matched with the 3xTg-AD, and have the same strain background. Non Tg mice were used as a control of pathological pathways of 3xTg-AD mice.

### 4.2.1 PEPx Chronic Administration Increases SYP levels in 3xTg-AD Mice

The synaptic loss that precedes or follows neuronal death in the AD brain explains the diminished values of the pre-synaptic membrane protein SYP in AD [105].

In order to evaluate the levels of SYP, we analysed by Western Blotting total brain homogenates from male or female Non Tg mice (n=6) and 3xTg-AD mice daily treated with 1.25 mg/Kg of PEPx (n=6) or the vehicle (saline) (n=8) for 4 months, as seen in Figure 4.7.

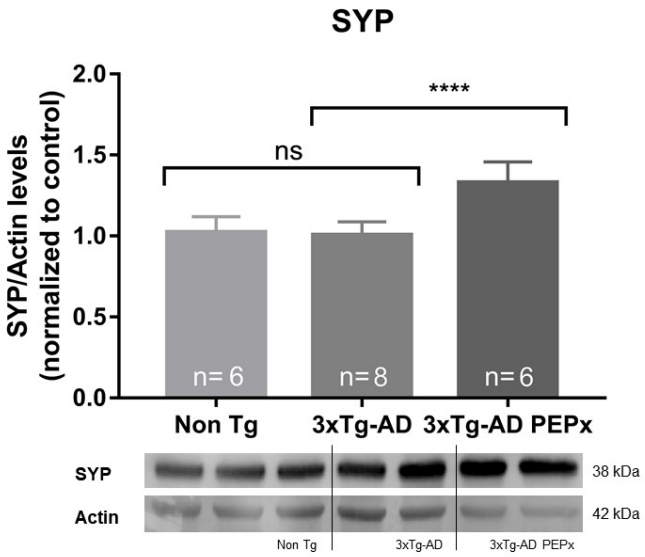


**Figure 4.7: SYP total levels in Non Tg mice, 3xTg-AD and 3xTg-AD PEPx-treated mice.** Total brain homogenates from Non Tg and 3xTg-AD mice daily treated with saline or PEPx (1.25 mg/Kg) during 4 months were evaluated by Western Blotting for the total protein levels of SYP. The quantitative results represent the mean ± SD of n = 6-8 mice per group, and are normalized to control (Non Tg mice). Statistical analysis was performed with one-way ANOVA followed by Sidak's *post-hoc* test. The results showed non-significant differences for Non Tg versus 3xTg-AD and ****$p < 0.0001$ for 3xTg-AD versus 3xTg-AD treated with PEPx.

As shown in Figure 4.7, there was no significant difference between Non Tg and 3xTg-AD (vehicle) mice. This might be due to 3xTg-AD mice age by the end of treatment (8.5 months), since it has been described that synaptic loss does not manifest until 13 months of age [199]. As well, an eventual synaptic loss in mice 8.5-months old might occur only at specific brain areas which could be detectable by immunohistochemistry [200, 201].

Even though the levels of SYP remained similar to Non Tg in saline treated 3xTg-AD mice, there was a significant increase of SYP protein levels in the mice chronically treated with PEPx. This increase suggests a gain in pre-synaptic vesicles, and can be considered beneficial taking in account that loss of SYP is considered the best brain correlate of cognitive decline in human AD [98]. There are studies that report a SYP loss of about 25% in human brains with mild AD, being even further decreased in moderate to severe cases [104]. In the 3xTg-AD mice, there are reports of deficits in synaptic plasticity, including LTP, that occurs before extracellular A$\beta$ deposition but is associated with an increase in intracellular immunoreactivity against A$\beta$ [177].

It is suggested that early accumulation of intracellular A$\beta_{42}$, even before plaque formation, might trigger neuronal injury and synaptic damage [195]. Thus a decrease in A$\beta$ species might lead to an increase in SYP levels, which may translate in better synaptic function.

### 4.2.2 Chronic PEPx treatment causes a decrease in NLRP3 inflammasome levels in 3xTg-AD mice

Neuroinflammation has an important role in AD progression [107]. On the other hand, there is a direct relationship between the NLRP3 inflammasome and IL-1$\beta$ cleavage, leading to CNS inflammation [115].

In order to evaluate the effect of chronic administration of PEPx in neuroinflammation, we accessed by Western Blot analysis, the levels of NLRP3 in total brain homogenates from male or female Non Tg mice (n=6), and 3xTg-AD mice treated with saline (n=6) or PEPx (n=4) for 4 months, as shown in Figure 4.8.

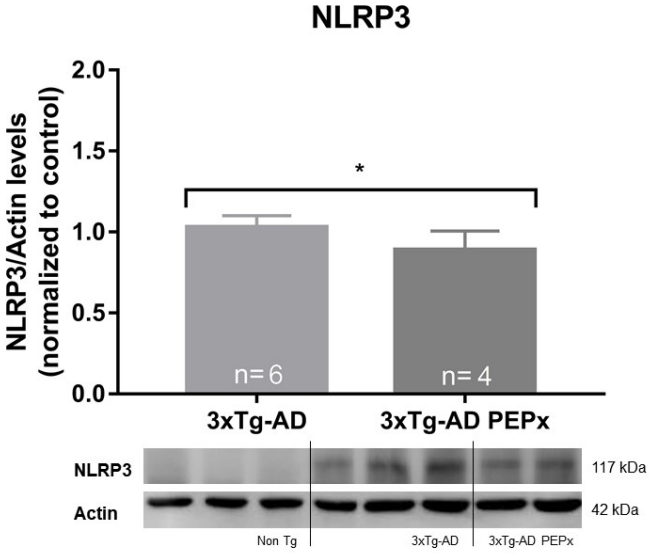


**Figure 4.8: NLRP3 total levels in Non Tg, 3xTg-AD and 3xTg-AD PEPx-treated mice.** Total brain homogenates from Non Tg mice and 3xTg-AD mice daily treated with vehicle (saline) or PEPx (1.25 mg/Kg) during 4 months were evaluated by Western Blotting for the total protein levels of NLRP3. The quantitative results represent the mean ± SD of n = 4-6 mice per group, and are normalized to non-treated 3xTg-AD (vehicle) mice. Statistical analysis was performed by t-test. The results showed *$p < 0.05$ for 3xTg-AD PEPx versus 3xTg-AD.

As we expected, NLRP3 levels in Non Tg mice were null, probably because there was no inflammation in the CNS of this mice. For this reason, the quantitative analysis of these mice was not included in Figure 4.8.

As a result, we normalized the levels to the mean of the 3xTg-AD values, since this mice show inflammation pathology from an early age [202]. The depicted results showed a significant decrease in NLRP3 levels in the PEPx treated mice as compared to 3xTg-AD treated with saline. This suggests a reduction in inflammasome assembly levels. The end result might be IL-1$\beta$ or caspase-1 diminished values, and thus a reduction in neuroinflammation.

Indeed, in APP/PS1/NLRP3$^{-/-}$ mice, caspase-1 cleavage was absent [121], this was accompanied with a strong reduction of A$\beta_{40}$ and A$\beta_{42}$. It was shown the inflammasome activation occurred in an age and A$\beta$ dependent manner. This dependence was already expected since NLRP3 inflammasome activation negatively impacts the microglial clearance function in AD [121].

### 4.2.3 Necrosome assembly is compromised with PEPx chronic treatment in 3xTg-AD mice

Neural cell death in the cortex and hippocampus correlates with the decline of both learning and memory abilities in AD patients [123]. However, the cell death programs that may be involved in AD are not completely understood. Therefore, we decided to investigate whether necroptosis contributes to cell death in the AD brain and if this event could be downstream BACE1-mediated APP processing. In cell death by necroptosis, the major intervenients are the RIP1 and RIP3 kinases [128]. The necroptosis signalling is usually mediated upstream by RIP1 [139], and the phosphorylation of RIP3 is a crucial factor for necroptotic death [140].

To investigate the levels of RIP1 and RIP3 kinases, we accessed by Western Blot analysis RIP1 levels in total brain homogenates from male or female Non Tg mice (n=6), and 3xTg-AD mice treated with saline (n=9) or PEPx (n=10) for 4 months.

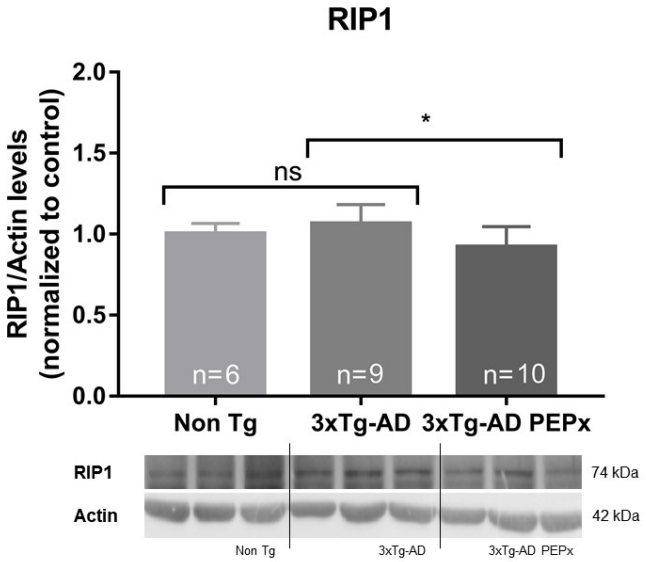


**Figure 4.9: RIP1 total levels in Non Tg, 3xTg-AD and 3xTg-AD PEPx-treated mice.** Total brain homogenates from Non Tg mice and 3xTg-AD mice daily treated with saline or PEPx (1.25 mg/Kg) during 4 months were evaluated by Western Blotting for the total protein levels of RIP1. The quantitative results represent the mean ± SD of n = 6-10 mice per group, and are normalized to Non Tg mice. Statistical analysis was performed by one-way ANOVA followed by Sydak's *post-hoc* test. The results showed non-significant differences for Non Tg versus 3xTg-AD and *$p < 0.05$ for 3xTg-AD PEPx versus 3xTg-AD.

As depicted in Figure 4.9, there was no significant differences in RIP1 levels in Non Tg compared to 3xTg-AD. This may be due to the age of mice, not manifesting considerable neuronal loss at 8.5-months [177]. We can not exclude that at a later age, in a more severe disease state, we would find a significant increase in RIP1 levels in 3xTg-AD mice. Indeed, an emerging study revealed an overall increase in RIP1 levels in human AD brains [141].

Importantly, the RIP1 levels of 3xTg-AD mice significantly decreased with the PEPx chronic treatment. RIP1 has been implicated as a key switch of cell regulation depending on the cellular context, controlling whether TNF induces NF-kB activation, apoptosis, or necroptosis [203]. Therefore, the significant decrease observed in RIP1 levels in 3xTg-AD PEPx by itself may not directly reflect an alteration in the necrosome levels, because of the vast set of functions on other cell death pathways. Therefore, we investigated RIP3 levels, a kinase selective of the necroptotic pathway.

RIP3 is an essential requirement for the execution of TNF induced necroptosis downstream of RIP1 [139]. In order to access the effect of chronic administration of PEPx in the levels of RIP3, we accessed the levels by Western Blot analysis in total brain

homogenates from male of female Non Tg mice (n=6), and 3xTg-AD treated with saline (n=10) or 1.25 mg/Kg PEPx (n=8) for 4 months.

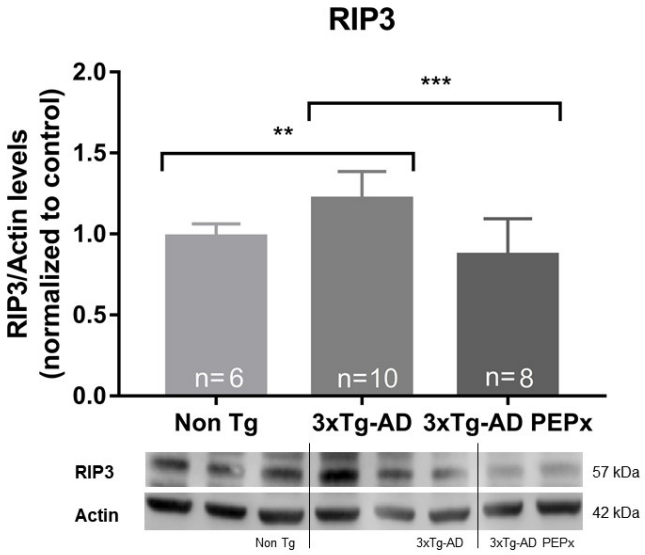


**Figure 4.10: RIP3 total levels in Non Tg, 3xTg-AD and 3xTg-AD PEPx-treated mice.** Total brain homogenates from Non Tg mice and 3xTg-AD mice daily treated with saline or PEPx (1.25 mg/Kg) during 4 months were evaluated by Western Blotting for the total protein levels of RIP3. The quantitative results represent the mean ± SD of n = 6-10 mice per group, and are normalized to Non Tg mice. Statistical analysis was performed by one-way ANOVA followed by Sydak's *post-hoc* test. The results showed **p $<$ 0.01 for Non Tg versus 3xTg-AD and ***p $<$ 0.001 for 3xTg-AD versus 3xTg-AD PEPx.

As shown in Figure 4.10, there was a significant increase in RIP3 levels in 3xTg-AD compared to Non Tg mice. The increase in RIP3 levels suggest necroptosis may contribute to cell death in AD. Cell death was observed in different animal models. For example, there are reports on synaptic degeneration associated with neuronal loss in 5XFAD mice [204], and $APP^{SL}$PS1KI mice exhibit significant neuron death in area CA1/2 of the hippocampus [205]. Importantly, this increased RIP3 expression has been proven to bypass the requirement for RIP1 in TNFR1 induced necroptotic signalling, since cells that over-expressed additional RIP3 died upon addiction of TNF [206].

With the PEPx chronic treatment there was a considerable decrease of RIP3 levels, reflecting an effective and positive action from the treatment. This could result in lower necrosome assembly levels.

Since RIP3 phosphorylation is required for RIP3 to form a stable complex with MLKL in order to occur death by necroptosis [130], we appraised the effect of chronic administration of PEPx in pRIP3 levels. In order to do this evaluation, we accessed the levels of pRIP3 by Western Blot analysis in total brain homogenates from male or female Non Tg mice (n=6) and 3xTg-AD mice daily treated with 1.25 mg/Kg of PEPx (n=9) or saline (n=11) for 4 months as seen in 4.11.

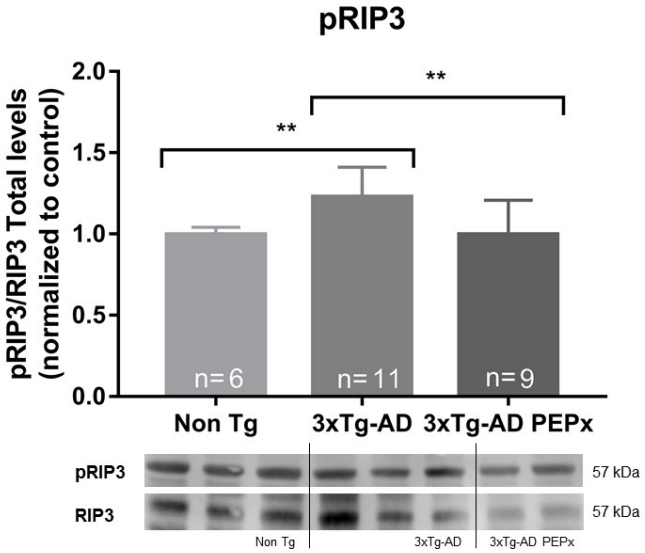


**Figure 4.11: pRIP3 total levels in Non Tg, 3xTg-AD and 3xTg-AD PEPx-treated mice.** Total brain homogenates from Non Tg mice and 3xTg-AD mice daily treated with saline or PEPx (1.25 mg/Kg) during 4 months were evaluated by Western Blotting for the total protein levels of pRIP3. The quantitative results represent the mean ± SD of n = 6-11 mice per group, and are normalized to Non Tg mice. Statistical analysis was performed by one-way ANOVA followed by Sydak's *post-hoc* test. The results showed **p $<$ 0.01 for Non Tg versus 3xTg-AD and **p $<$ 0.01 for 3xTg-AD versus 3xTg-AD PEPx.

In accordance with the previous results in Figure 4.10, there was a significant increase in pRIP3 levels between Non Tg and 3xTg-AD (vehicle) (Figure 4.11), supporting the view that programmed cell death by necroptosis might occur in this animal model of AD.

As we shown in Figure 4.10, RIP3 levels significantly diminished in mice treated with PEPx. Following the same pattern, the levels of pRIP3 were also decreased in 3xTg-AD mice. Taken together, this results indicated that BACE1 inhibition leads to a reduction in RIP3 levels, as well to a significant reduction in RIP3 phosphorylation, lowering its role and efficacy as the determining factor of necroptosis.

Necroptosis is an inflammatory form of cell death with important functions in certain viral infections, trauma-induced tissue injury, brain ischemia [207] and, very recently, it was shown to occur as well in AD [141].

In our study, although in 8.5-months old 3xTg-AD mice there was no changes in RIP1 levels compared to Non Tg, both the RIP3 and pRIP3 levels increased significantly. Taking in consideration the role of RIP3 in inducing necroptotic cell death, which may occur independently of RIP1 when RIP3 is over-expressed, the results suggest that necroptosis may contribute to cell death in this animal model of AD.

Interestingly, our study reveals for the first time that BACE1 inhibition leads to a reduction in RIP1, RIP3 and pRIP3 levels, pinpointing that a reduction in A$\beta$ may contribute to decrease necroptotic signalling. There are reports of a co-localization between RIP1 and RIP3 in human brains with AD [208]. Its then conceivable to hypothesize that, with the reduction of both their levels, the co-localization also diminishes and thus the necrosome levels decrease, leading to a reduction of cell death by necroptosis.

RIP3 phosphorylation is mediated directly or indirectly by RIP1, promoting its kinase activity [208]. The human Serine 227 and mouse Threonine 231/Serine 232 auto-phosphorylation sites of RIP3 are required for the interaction of RIP3 with its substrate MLKL [130], essential for necroptotic cell death. With the reduction of pRIP3 following a chronic treatment with PEPx, its interaction with MLKL should be reduced, decreasing the subsequent necroptotic cell death.

# 5

# Conclusions

BACE1 has a crucial role in the amyloidogenic pathway. The final product of this pathway is the A$\beta$ peptide, whose accumulation in extracellular plaques is one of the hallmarks of AD. With no effective treatment for AD, novel therapies need to be studied, with BACE1 being a significant target. BACE1 inhibition would halt A$\beta$ formation, and consequent accumulation. Thus, to a more deep understanding of the benefits of the new BACE1 inhibitor we are developing, PEPx, we investigated its cellular uptake mechanism as well as its effects in diverse pathological mechanisms involved in AD, namely synaptic loss, neuroinflammation and neuronal death.

In N2a cells, the internalization of BACE1 inhibitor PEPx originates a vesicular pattern, as expected since the BACE1 inhibitor was designed in order to favour the uptake by the endocytic pathway. Namely, our work suggests PEPx is internalized mainly by clathrin-mediated endocytosis, as CPZ inhibits peptide internalization.

Regarding the effects of a chronic treatment with PEPx in 3xTg-AD mice, the synaptic marker SYP increased significantly its levels with the PEPx treatment, reflecting an increase in pre-synaptic vesicles and, possibly, in synaptic transmission. On the other hand, the NLRP3 levels decreased with PEPx administration, which may decrease IL-1$\beta$ cleavage and thus diminish neuroinflammation. Interestingly, the levels of the RIP1 and RIP3 kinases, and the phosphorylated form of RIP3, decreased with chronic PEPx administration. This suggests a decrease in necrosome assembly and necroptotic signalling, and therefore of ensuing necroptotic cell death. Our study reveals for the first time that necroptotic signalling is downstream of BACE1 activation in AD.

Overall, the BACE1 inhibitor PEPx shows promising results. Not only it decreases both A$\beta_{40}$ and A$\beta_{42}$ levels, the major histological hallmark of AD, but has beneficial effects in other pathological mechanisms related with the disease.

## 5.1 Future Work

In order to complement the peptide cellular uptake studies and demonstrate that in our experimental conditions the cell membrane is not damaged, the integrity of the membrane should be tested using a trypan blue exclusion test or by incubation with propidium iodide.

The use of other pharmacological endocytosis inhibitors are required to rule out other pathways of PEPx internalization, for example, 5-(N-ethyl-N-isopropyl)amiloride for inhibition of macropinocytosis and methyl-beta-cyclodextrin for disruption of import through caveolae. The use of tracers for different endocytic pathways is also a good approach. For example, transferrin for clathrin-mediated endocytosis, cholera toxin beta subunit for caveolae-mediated endocytosis and dextran for macropinocytosis. This way, a timed co-localization between this tracers and the Cy5.5-labelled PEPx could indicate other secondary internalization pathways.

Regarding the method applied to cellular uptake quantification, flow cytometry would provide more uniform results considering the decrease of human error. On the other hand, to evaluate specifically the endocytic uptake a possible approach would be to quantify both the endocytic vesicle area and the fluorescence within. It would also be interesting to evaluate the co-localization of PEPx in early endosomes by expressing an endosomal marker coupled to Green Fluorescent Protein.

Regarding *in vivo* studies, the levels of IL-1$\beta$, caspase-1 and Glial Fibrillary Acidic Protein, should be assessed by Western Blot analysis or an ELISA assay in order to have more information about neuroinflammation in the brain of 3xTg-AD mice, and the Microtubule-Associated Protein 2 levels to access synaptic loss. To have a more profound understanding of the influence of PEPx in necroptosis, the MLKL levels and oligomerization state should be accessed by Western Blot, since MLKL oligomerization is the most proximal step to membrane disruption and cell death. Also, the co-localization of the pRIP3/MLKL complex by immunoprecipitation could be evaluated. The levels of caspase-3 could be accessed as an apoptosis marker, since it is an executioner caspase.

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