# DISSECTING THE ROLE OF PROTEOLYSIS AND CYTOSKELETON REMODELING IN PROTEIN AGGREGATION RELATED DISEASES

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## ABSTRACT

Protein aggregation has been identified as the cause of several neurodegenerative disorders such as Alzheimer's Disease (AD), Parkinson's Disease (PD) and Familial Amyloid Polyneuropathy (FAP). AD is characterized by the extracellular deposition of amyloid-β (Aβ) aggregates mainly in the hippocampal region of the brain. Aβ degradation is one of the promising therapeutic targets in AD. Among the several Aβ-degrading enzymes, the metalloprotease transthyretin (TTR) was shown to be a good candidate with encouraging *in vitro* results. The Aβ cleavage by TTR was shown to occur in multiple positions, resulting in the production of peptides with lower amyloidogenic potential. Moreover, data from our group demonstrated that TTR WT, but not the proteolytically inactive form of the protein, is capable of interfering with Aβ fibrillization by both inhibiting and disrupting fibril formation. In this work, we aimed to further dissect the role of TTR proteolytic activity in AD using both cell based assays and *in vivo* models. We used the TTR proteolytic inactive mutant and compared its neuroprotective effect with TTR WT by: i) analyzing the effect on Aβ clearance and neurotoxicity by using N2A-APPSwe cells and hippocampal neurons, respectively; and ii) assessing its neuroprotective effect *in vivo* using mice and zebrafish as animal models. Using the cell based assays, we verified that TTR proteolytic activity is maintained under physiological conditions and is required for TTR neuroprotective effect in AD by increasing Aβ clearance and decreasing neurotoxicity. The relevance of TTR proteolytic activity *in vivo* could not be clarified by the use of TTR intracerebral administration in a mouse model of AD, what was mainly related with the high variability within animals from the same experimental groups. Using zebrafish as another *in vivo* model, we were able to recapitulate a previously reported methodology using Veins zebrafish (that express GFP in the gut vasculature) treated with Aβ, that will be used in the future to further address TTR proteolytic activity in an *in vivo* system.

Damage to the neuronal cytoskeleton has been observed in several neurodegenerative disorders. Aiming at determining whether neuronal cytoskeleton damage is a common pathogenic mechanism induced by protein aggregates in unrelated neurodegenerative disorders, in this work we used the growth cone morphology of hippocampal neurons (which has a distinctive distribution of both microtubules and actin filaments) as a fine tool to understand the effect of different prone-to-aggregate proteins in the neuronal cytoskeleton of hippocampal neurons. The following species were analyzed: i) Aβ oligomers (the pathogenic protein in AD), untreated or treated with TTR, to determine whether TTR reverts cytoskeleton defects induced by Aβ oligomers; ii) α-synuclein in different aggregations stages (the pathogenic protein in PD); and iii) TTR

amyloidogenic mutants (the pathogenic protein in FAP). In AD, several studies have demonstrated that the neuronal cytoskeleton is also one of the targets of Aβ oligomers-induced neurodegeneration. Therefore, we incubated hippocampal neurons with Aβ oligomers and verified that the toxic species increased the number of dystrophic growth cones; however TTR WT, shown to have a neuroprotective effect in AD, was unable to rescue this phenotype. We also analyzed whether α-synuclein induces similar effects in the neuronal cytoskeleton as the ones observed with Aβ oligomers. We treated hippocampal neurons with α-synuclein at different aggregation states. Although a tendency to an increase in the percentage of dystrophic growth cones was observed with the treatment of the different α-synuclein species, no statistical differences were found. We also tested the effect of TTR variants that are associated with FAP in the growth cone morphology of hippocampal neurons. Although previous data from our group showed that TTR oligomers are able to induce growth cone morphology defects in dorsal root ganglia neurons, similar effects were not observed in hippocampal neurons. In the CNS neurons we observed a tendency to a decrease in the percentage of dystrophic growth cones after treatment with either TTR WT or TTRL55P, although not statistical significant, while TTR V30M has no effect. In conclusion, these results show that further studies should be conducted to unravel the effect of prone-to-aggregate proteins in the neuronal cytoskeleton. This approach would be crucial to determine whether common therapeutic approaches targeting the cytoskeleton would be a valuable strategy for unrelated neurodegenerative disorders caused by protein aggregation.

## RESUMO

A causa de várias doenças neurodegenerativas, como a doença de Alzheimer (AD), de Parkinson (PD) e a Polineuropatia amiloidótica familiar (PAF) tem vindo a ser relacionada com a agregação de proteínas. A AD é caracterizada pela deposição de agregados de β-amilóide (Aβ) extracelulares, maioritariamente na região do hipocampo. Um alvo promissor para o tratamento de AD é a degradação de Aβ. Entre as várias enzimas que o fazem, a metaloprotease transtirretina (TTR) foi identificada por ensaios *in vitro* como um bom candidato. A clivagem de Aβ pela TTR ocorre em múltiplas posições, resultando na produção de péptidos com baixo potencial amiloidogénico. Para além disso, resultados obtidos no nosso grupo demonstram que a TTR wild-type (WT) é capaz de interferir com a fibrilização por inibir e desregular a formação de fibras. A forma proteolicamente inactiva da TTR já não é capaz de interferir neste processo. Neste trabalho, o objectivo foi dissecar o papel da actividade proteolítica da TTR em AD usando tanto ensaios celulares como modelos *in vivo*. Para tal, usamos a forma mutada de TTR (proteoliticamente inactiva) e comparamos o efeito neuroprotector com o da TTR WT ao: i) analizar o efeito na remoção de Aβ e na neurotoxicidade usando células N2A-APPSwe e neurónios do hipocampo, respectivamente; e ii) aferir o efeito neuroprotector *in vivo* usando ratinhos e zebrafish como modelos animal. Com os ensaios celulares, nós verificámos que a actividade proteolítica da TTR é mantida em condições fisiológicas e é requerida para o efeito neuroprotector de TTR em AD pelo aumento da remoção de Aβ e diminuição da neurotoxicidade. A relevância da actividade proteolítica da TTR *in vivo* não pôde ser clarificada pelo uso de injecções intercerebrais de TTR num modelo de AD em ratinho, devido à alta variabilidade entre animais dentro do mesmo grupo experimental. Usando zebrafish como modelo *in vivo*, fomos capazes de recapitular uma metodologia previamente descrita com Veins zebrafish (que expressam GFP na vasculatura do tracto gastrointestinal) tratados com Aβ que serão usados no futuro para analisar a actividade proteolítica da TTR num sistema *in vivo*.

Em várias doenças neurodegenerativas têm sido verificados danos no citoesqueleto neuronal. Com o objectivo de determinar se esse dano é um mecanismo patogénico comum induzido por agregados proteicos em doenças neurodegenerativas não relacionadas usámos, neste trabalho, a morfologia do cone de crescimento de neurónios do hipocampo (que têm uma distribuição distinta em microtúbulos e filamentos de actina) como uma ferramenta para compreender o efeito de diferentes proteínas com capacidade de agregação no citoesqueleto neuronal dos neurónios do hipocampo. As seguintes espécies foram utilizadas: i) oligómeros de Aβ (a proteína patogénica em AD), não tratados ou tratados com TTR, para determinar se a TTR

reverte os defeitos no citoesqueleto induzidos pelos oligómeros de Aβ; ii) α-sinucleína em diferentes estados de agregação (a proteína patogénica em PD); e iii) mutantes amiloidogénicos de TTR (a proteína patogénica de FAP). Diferentes estudos demonstraram que em AD o citoesqueleto neuronal é alvo de neurodegeneração induzida por oligómeros Aβ. Assim, nós incubámos neurónios do hipocampo com oligómeros Aβ e verificámos que as espécies tóxicas aumentaram o número de cones de crescimento distróficos; no entanto, a TTR WT, que tem um efeito neuroprotector em AD, não foi capaz de reverter este fenótipo. Também analisámos se a α-sinucleína induz efeitos semelhantes no citoesqueleto neuronal aos observados com oligómeros de Aβ. Assim, neurónios do hipocampo foram tratados com α-sinucleína em diferentes estados de agregação. Apesar de haver uma tendência para aumentar a percentagem de cones de crescimento distróficos com o tratamento de diferentes espécies de α-sinucleína, diferenças estatisticamente significativas não foram encontradas. Também testámos o efeito das variantes de TTR que estão associadas com FAP na morfologia do cone de crescimento em neurónios do hipocampo. Apesar de dados do nosso grupo demonstrarem que os oligómeros TTR são capazes de induzir defeitos na morfologia do cone de crescimento em neurónios dos ganglios dorsais, efeitos semelhantes não foram detectados nos neurónios do hipocampo. Em neurónios do sistema nervoso central observamos uma tendência para diminuir a percentagem de cones de crescimento distróficos após o tratamento com TTR WT ou com TTR L55P, apesar de não ser estatisticamente significativo, enquanto TTR V30M não tem efeito. Para concluir, estes resultados mostram que mais estudos devem ser conduzidos para determinar o efeito de proteínas com capacidade de agregação no citoesqueleto neuronal. Esta abordagem pode ser crucial para determinar se as abordagens terapêuticas comuns que têm como alvo o citoesqueleto podem ser uma estratégia valiosa para doenças neurodegenerativas não relacionadas causadas por agregação de proteínas.

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Aβ – Amyloid-β peptide

AAV – Adeno-associated Virus

Ac-DEVD-AMC - Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin

AD – Alzheimer's Disease

AIS – Axon Initial Segment

apoA-I – Apolipoprotein A-1

APP – Amyloid Precursor Protein

CNS – Central Nervous System

CRMP - Collapsing Response Mediator Proteins

CSF – Cerebrospinal Fluid

DEAE – Diethylaminoethyl

DIV – Days *in vitro*

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl Sulfoxide

DRG – Dorsal Root Ganglia

EDTA - Ethylenediamine Tetraacetic Acid

ELISA - Enzyme-Linked Immunosorbent Assay

EOAD – Early Onset Alzheimer's Disease

FAD – Familial Alzheimer's Disease

FAP – Familial Amyloid Polyneuropathy

FBS – Fetal Bovine Serum

GSK 3-β – Glycogen Synthase Kinase 3-β

HBSS - Hank's Balanced Salt Solution

HDAC - Histone Deacetylase

HFIP – Hexafluoroisopropanol

hpf – hours post-fertilization

HRP – Horseradish Peroxidase

IDE – Insulin Degrading Enzyme

IF – Intermediate Filaments

KO – Knockout

LB – Lewy Bodies

LOAD – Late Onset Alzheimer's Disease

MAP – Microtubule Associated Proteins

MT - Microtubules

NEP – Neprilysin

NF - Neurofilament

NFT - Neurofibrillary Tangles

NMDAR - N-methyl-D-aspartate Receptor

O/N – Overnight

Opti-MEM – Opti-Minimum Essential Medium

PBS – Phosphate-buffered Saline

PD – Parkinson's Disease

PFA - Paraformaldehyde

PMSF - Phenylmethylsulfonyl Fluoride

PNS – Peripheral Nervous System

PS – Presenilin

PTU - 3-phenyl-thiourea

P/S – Pen/Strep

Rac1/Cdc42 - Ras-related C3 botulinum toxin substrate 1/Cell division control protein 42 homolog

RBP – Retinol-Binding Protein

RT – Room Temperature

SLB – Sample Loading Buffer

$T_4$ – Thyroxine

TBS - Tris buffered saline

TTR – Transthyretin

WT - Wild-type

+TIP - plus-end-tracking proteins

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# CHAPTER 1

## GENERAL INTRODUCTION

# 1. TRANSTHYRETIN

Transthyretin (TTR) is a homotetrameric protein named after its first identified functions: the transport of circulating thyroid hormones (Woeber and Ingbar 1968) and of retinol through retinol-binding protein (RBP) (Goodman 1987). TTR is mainly synthesized in the liver and the choroid plexus of the brain which constitute the source of TTR in the plasma and the cerebrospinal fluid, respectively (Dickson, Howlett et al. 1985). TTR is also known for its role on familial amyloid polyneuropathy (FAP), a neurodegenerative disorder in which mutated TTR accumulates as amyloid fibrils particularly in the peripheral nervous system (PNS) (Saraiva, Magalhaes et al. 2012).

## 1.1. *ttr* gene: structure, expression and evolution

The *ttr* gene is a single copy-gene situated in chromosome 18 in humans (Whitehead, Skinner et al. 1984) and rats (Remmers, Goldmuntz et al. 1993), and in chromosome 4 in mice (Qiu, Shimada et al. 1992), being composed of 4 exons and 3 introns. Although the gene length varies between species (from 6.8kb in humans to 7.3kb in rats), it presents three highly conserved regions: the entire sequence of exons, the 5´proximal region and the flanking region of the exon-intron borders. Exon 1 codes for 20 amino acids long signal peptide and the first three residues of the mature protein; exon 2 encodes for amino acid residues 4-47; exon 3 for amino acid residues 48-92 and, finally, exon 4 codes for amino acid residues 93-127. The introns have 934, 2090 and 3308bp, respectively, and display GT/AG splicing consensus sequences (Sasaki, Yoshioka et al. 1985). The pro-monomer is composed by 127 amino acids plus the signal peptide in the N-terminal region, which is removed in the endoplasmic reticulum, resulting in the native monomer (Soprano, Herbert et al. 1985).

TTR is majorly expressed in the liver and in the epithelial cells of the choroid plexus of the brain and its pattern of gene expression is well characterized in numerous species, namely rat (Dickson, Aldred et al. 1985), human (Dickson and Schreiber 1986), sheep (Schreiber, Aldred et al. 1990), chicken (Southwell, Duan et al. 1991) and pig (Duan, Richardson et al. 1995). In humans, *ttr* expression starts in the tela choroidea and then in the liver (Harms, Tu et al. 1991, Richardson, Bradley et al. 1994) and it is also expressed in human placenta, eye and intestine (Loughna, Bennett et al. 1995, Schreiber and Richardson 1997, Getz, Kennedy et al. 1999). In rats, *ttr* expression occurs since early embryogenesis and is gradually limited to the liver and the choroid plexus during the last phases of embryogenesis and is preserved during adult life. In adult rats, TTR is also expressed in the eye, heart, pancreas, skeletal muscle, spleen and stomach (Soprano, Herbert et al. 1985, Power, Elias et al. 2000). During vertebrate evolution, *ttr* expression

has changed considerably: while in fish it is found in the liver during development (Richardson, Monk et al. 2005), in reptiles it is expressed only in the choroid plexus of adult lizards (Achen, Duan et al. 1993). The preservation of *ttr* expression in the choroid plexus from reptiles to mammals during life suggests a fundamental role for TTR in the brain biology.

## 1.2. TTR structure

TTR is a homotetrameric protein composed of identical subunits of 127 amino acid residues each of approximately 14kDa (Kanda, Goodman et al. 1974). Each monomer is composed by eight β-chains that are organized as two parallel sheets of four strands, acquiring a beta sandwich conformation, and a very short α-helix. Two monomers interact through an extensive hydrogen bond along strands forming a dimer. Two dimers associate and form a tetramer creating a hydrophobic channel that goes through the protein (figure 1). This originates two symmetrical binding sites that are able to accommodate two thyroxine ($T_{4}$) molecules. Furthermore, TTR binds one RBP molecule and its binding sites are located at the surface of the molecule in a region involving interactions between both TTR dimers. The formation of the retinol-RBP-TTR complex stabilizes the TTR tetramer (Saraiva, Magalhaes et al. 2012).

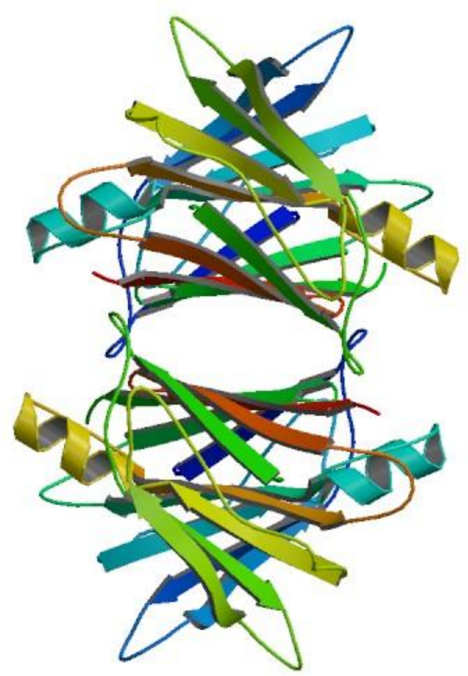

**Figure 1 – Structure of TTR tetramer.** PDB entry 1F41.

### 1.3. TTR metabolism

Plasma-circulating TTR levels range from 170 to 420ug/mL while in the brain its concentration ranges from 5 to 20ug/mL (Vatassery, Quach et al. 1991) and represents 20% of the total CSF proteins (Weisner and Roethig 1983).

In humans, the biological half-life of TTR is about 2-3 days (Socolow, Woeber et al. 1965), while in rats it is 29 hours (Dickson, Howlett et al. 1982). The major degradation sites of TTR in rats are the liver, kidney, muscle and skin, although other sites have been reported namely the testis, kidneys, adipose tissue and gastrointestinal tract. Furthermore, both plasma and CSF TTR are metabolized in the same degradation sites and TTR degradation is not present in the nervous system (Makover, Moriwaki et al. 1988).

Although TTR internalization by some tissues and cell types has been suggested, these mechanisms have not yet been fully discovered. In the kidneys and dorsal root ganglia (DRG), TTR is internalized by via megalin (Sousa, Norden et al. 2000, Fleming, Mar et al. 2009) and, in the liver, its internalization is mediated by an unknown receptor which is an associated protein (RAP)-sensitive receptor (Sousa and Saraiva 2001).

### 1.4. TTR amyloidogenic variants

TTR is known as one of the many proteins which acquire a misfolded conformation and undergo aggregation *in vivo*. Over one hundred TTR mutations have been described so far (Rowczenio and Wechalekar 2015). All mutations, except a single aminoacid deletion at position 122, arise from point mutations in the polypeptide chain (Saraiva 2001). Most amyloidogenic variants of TTR are associated with neuropathies, but other conditions have also been described such as cardiomyopathy (Saraiva, Sherman et al. 1990), carpal tunnel syndrome (Izumoto, Younger et al. 1992), vitreous TTR deposition (Zolyomi, Benson et al. 1998) and leptomeningeal involvement (Petersen, Goren et al. 1997). TTR wild-type (WT) has also propensity for aggregation *in vivo*. Systemic senile amyloidosis, also named senile cardiac amyloidosis, is a highly prevalent late age of onset disease that typically affects elderly men (over 70 years) (Rapezzi, Quarta et al. 2010), characterized by the deposition of TTR WT fibrils specifically in the heart (Westermark, Sletten et al. 1990, Rapezzi, Quarta et al. 2010).

The disease-associated TTR mutations decrease the stability of the tetramer or the monomer or both. Therefore, TTR amyloidogenic potential is governed by the extent of protein stabilization (Johnson, Connelly et al. 2012). In the TTR amyloidogenic cascade, the tetramer dissociates into the natively folded monomer which subsequently undergoes denaturation. The unfolded monomers aggregate very efficiently into a variety of

aggregate morphologies, including oligomers, non-fibrillar aggregates and amyloid fibrils (figure 2) (Johnson, Connelly et al. 2012).

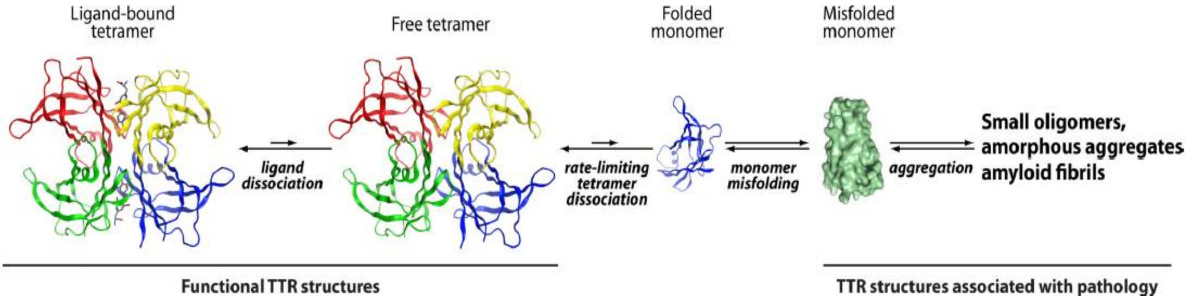


**Figure 2 - TTR amyloidogenic cascade.** The TTR tetramer dissociates into monomers which undergo denaturation, becoming an aggregation-prone amyloidogenic intermediate. Adapted from (Johnson, Connelly et al. 2012)

The most common amyloidogenic TTR mutation is a substitution of a methionine for a valine at position 30 (TTR V30M) that is associated with FAP (Saraiva, Birken et al. 1984) (see section 3.2.3). Other aggressive amyloidogenic variants such as TTR Leu55Pro (TTR L55P) were also described. TTR L55P is a highly amyloidogenic variant that induces a progressive form of neuropathy. Biochemical studies showed that this mutant presents decreased tetramer stability (McCutchen, Colon et al. 1993, Lashuel, Lai et al. 1998) due to alterations in the dimer-dimer contact regions (Sebastiao, Saraiva et al. 1998). Both these mutations were shown to destabilize the tetramer, generating partially unfolded monomers that have a high tendency for aggregation (Quintas, Vaz et al. 2001). Although some cases of homozygous individuals were described (Skare, Yazici et al. 1990), most carriers are heterozygous for the amyloidogenic TTR variants. For instance, double mutant individuals carrying the heterozygous V30M/T119M variants were identified with a less severe form of the disease, suggesting that T119M acts as a protective mutation (Alves, Altland et al. 1997).

## 1.5. TTR physiological functions

### *1.5.1. Transport of $T_4$ and retinol*

The most acknowledge functions for TTR are the transport of thyroid hormones like $T_4$ and vitamin A (retinol), in the latter case through binding to the RBP. In the human plasma, almost all $T_4$ is bound to plasma proteins, namely $T_4$-binding globulin (70%), TTR (15%) and albumin (10%) and some lipoproteins. In mice, TTR is the major $T_4$ carrier, transporting 50% of total $T_4$ (Hagen and Solberg 1974). TTR was proposed as being involved in thyroid hormone homeostasis and hormone delivery. However, the first studies regarding this interaction demonstrated that although the plasma levels of free $T_4$ of TTR knockout (KO) mice were decreased, the liver and kidney of these mice didn't show

differences in $T_4$ levels (Palha, Episkopou et al. 1994, Palha, Hays et al. 1997). Furthermore, the absence of TTR did not alter the distribution of $T_4$ in the brain. These results suggest that despite the fact that TTR is a transporter of $T_4$, its absence doesn't affect the normal thyroid hormone function.

In the serum, retinol circulates bound to RBP and TTR, creating a complex that allows the delivery of retinol to tissues. Studies using TTR KO mice showed the existence of decreased levels of plasma retinol and RBP when comparing with WT mice (Episkopou, Maeda et al. 1993). However no differences were found in the tissue levels of total retinol between WT and TTR KO mice, with TTR KO mice lacking symptoms of vitamin A deficiency (Episkopou, Maeda et al. 1993, van Bennekum, Wei et al. 2001). The reduced levels of plasma RBP-retinol complex in TTR KO animals were related to an increased renal filtration, suggesting that TTR prevents RBP-retinol loss through renal filtration (van Bennekum, Wei et al. 2001).

### *1.5.2. TTR as a nerve regeneration enhancer*

Trying to understand the preferential deposition of TTR in the PNS of FAP patients, several reports have assessed a role for TTR in the biology of the nervous system. In this respect, TTR was shown to enhance nerve regeneration (Fleming, Saraiva et al. 2007). TTR KO mice showed a decreased regeneration capacity after sciatic nerve crush when compared to WT littermates (Fleming, Saraiva et al. 2007). Transgenic TTR KO mice expressing TTR in neurons showed a rescue in the phenotype, strengthening that TTR is responsible for the enhancement of nerve regeneration (Fleming, Mar et al. 2009). *In vitro*, the same effect was observed as neurite outgrowth was decreased in primary cultures of DRG neurons from TTR KO mice, what might explain the impaired regenerative capacity of TTR KO mice (Fleming, Saraiva et al. 2007). It was also shown that TTR KO axons present a compromised retrograde transport what might account for the delayed regenerative capacity of TTR KO mice and decreased neurite outgrowth in the absence of TTR (Fleming, Mar et al. 2009).

### *1.5.3. TTR as a novel protease*

In the plasma, a fraction of TTR is carried in high density lipoproteins through binding to apolipoprotein A-I (apoA-I), its major protein component. This interaction was investigated and TTR was found to be a novel plasma protease which is able to cleave the C-terminus of apoA-I (Liz, Faro et al. 2004). The relevance of apoA-I cleavage by TTR was determined; upon TTR cleavage, high-density lipoproteins display a reduced capacity to promote cholesterol efflux and cleaved apoA-I displays increased amyloidogenicity,

suggesting that TTR might impact in the development of atherosclerosis (Liz, Gomes et al. 2007).

TTR proteolysis was also shown to impact on nervous system biology. *In vitro* work showed that TTR is able to cleave neuropeptide Y and that TTR proteolytic activity is necessary for its ability to enhance neurite outgrowth, suggesting the existence of additional TTR substrates in the nervous system (Liz, Fleming et al. 2009). The catalytic machinery behind the proteolytic activity of TTR was also revealed. The analysis of three-dimensional structures of TTR complexed with $Zn^{2+}$ and site-directed mutagenesis of selected amino acids confirmed that TTR is a metallopeptidase with $His^{88}$, $His^{90}$ and $Glu^{92}$ being the residues constituting the active site (figure 3) (Liz, Leite et al. 2012). This finding not only strengthens the establishment of TTR as a novel protease but also provides the possibility to modulate the proteolytic activity in order to analyze its relevance in both physiological and pathological conditions.

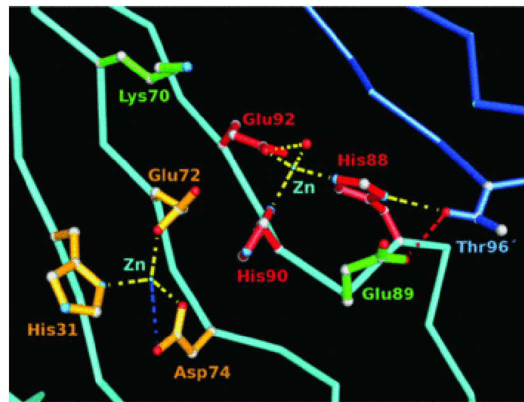


**Figure 3 - Structure of TTR active site**. In cyan and dark blue are represented two different TTR monomers. The metallic ion zinc is linked by His88 and His90 and Glu92 is contacting with a water molecule. A second Zn2+ binding site consists of Glu72, His31 and Asp74. Glu89 and Thr96 of a second monomer contact by a hydrogen bond which is also in contact with His88. Attractive forces are showed in dashed lines. Adapted from: (Liz, Leite et al. 2012)

## 1.6. TTR is neuroprotective in Alzheimer’s Disease

TTR and Amyloid-β (Aβ) peptide interaction has been address by many groups since the CSF and plasma TTR levels of Alzheimer’s disease (AD) patients are decreased, proposing a neuroprotective action of TTR in AD (Riisoen 1988, Hansson, Andreasson et al. 2009, Ribeiro, Santana et al. 2012). The ability of TTR synthesized by the choroid plexus and secreted into the CSF to interact and impact on Aβ levels, aggregation state and/or toxicity has been questioned since Aβ is mainly present in the hippocampus and cortex. Therefore, the presence of TTR in brain areas other than its site of synthesis and secretion has been subject of study. Although TTR expression was demonstrated in the hippocampus of both AD patients and AD mice models (Schwarzman and Goldgaber

1996, Stein, Anders et al. 2004, Li, Masliah et al. 2011) and was also verified *in vitro* using SH- SY5Y cells (Kerridge, Belyaev et al. 2014, Wang, Cattaneo et al. 2014), a study using a mouse model with compromised heat-shock response showed that, in situations of injury such as ischemia, TTR is present in the brain, but it is derived from CSF-TTR (Santos, Fernandes et al. 2010). Thus, there is still debate on whether or not TTR is synthesized by neurons. Nevertheless, the referred studies support the importance of TTR in AD, reinforcing the need for a better understanding of this interaction.

TTR is able to sequester Aβ, thereby preventing amyloid formation and toxicity *in vitro* (Schwarzman, Gregori et al. 1994). More recently, it was shown that TTR protective capacity is related to its binding to toxic/pretoxic Aβ aggregates in both intracellular and extracellular environment in a chaperone-like manner (Buxbaum, Ye et al. 2008). Further studies revealed that the inhibition and disruption of Aβ fibrils by TTR was the possible mechanism behind the protective role of this protein, since TTR binds to soluble, oligomeric and fibrillar Aβ with similar affinities and is capable of interfering with Aβ fibrillization (Costa, Goncalves et al. 2008).

The nature of TTR/Aβ interaction was further investigated and TTR was found to be able to cleave Aβ *in vitro* in multiple positions which are also cleavage sites for other Aβ degrading enzymes. The proteolytic activity of TTR over Aβ generates peptides with lower amyloidogenic potential than the full length counterpart, suggesting that TTR contributes to its clearance (Costa, Ferreira-da-Silva et al. 2008). Nevertheless, TTR cleavage of Aβ was only demonstrated *in vitro* by SDS-PAGE using the two purified proteins, thus further studies should address the ability of TTR to cleave Aβ in a physiological environment.

## 2. ALZHEIMER'S DISEASE

AD is the most common form of dementia representing 60-70% of all cases and is increased among people over 65 years old. AD is a progressive neurodegenerative disorder of the central nervous system (CNS) characterized by the gradual decline in memory, thinking, language and learning capacity, ultimately leading to death (Duthey 2013). Clinically, AD can be divided into three different stages: preclinical AD, mild cognitive impairment due to AD and dementia due to AD. Based on its age of onset, AD is classified into early onset AD (EOAD) and late onset AD (LOAD). The most common form of AD (~95% of all cases) is LOAD in which patients present an age at onset later than 65 years. EOAD represents ~5% of all cases and the age at onset varies from 30 years to 65 years (Reitz and Mayeux 2014).

## 2.1. Genetics of Alzheimer's Disease

AD can be divided into familial cases that follow Mendelian inheritance (Familial AD) and sporadic cases in which there is no familial link. There are three principal genes that present rare and highly penetrant mutations which lead to FAD: amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2). These mutations culminate in alterations in Amyloid β Precursor Protein (APP) breakdown and Aβ peptide generation (Reitz and Mayeux 2014). Although the sporadic form is usually related with the combination of genetic and environmental factors, several studies have pointed out several genetic risk factors such as the epsilon4 allele in apolipoprotein E (Piaceri, Nacmias et al. 2013, Reitz and Mayeux 2014).

## 2.2. Pathology of Alzheimer's Disease

AD is a devastating incurable neurodegenerative disorder characterized by the occurrence of extraneuronal amyloid plaques (figure 4A), consisting of aggregates of Aβ peptide and intraneuronal neurofibrillary tangles (NFT) (figure 4B) composed of aggregates of abnormally hyperphosphorylated tau protein particularly in the hippocampus and cortex, the regions responsible for cognition and memory (Castellani, Rolston et al. 2010, Li and Buxbaum 2011). The senile plaques can be distinguished into multiple subtypes, being the neuritic plaques the most pathogenically relevant and the ones used to diagnose AD at autopsy (Castellani, Rolston et al. 2010). NFT are mostly characterized in terms of localization since they can be related to lesions such as neuropil threads (thread like accumulations within neuropil of gray matter and white matter) and dystrophic neuritis (terminal neuritic swellings) that arise within neuritic plaques (Castellani, Rolston et al. 2010). Other hallmarks of AD are neuronal and dendritic loss, synaptic loss, granulovacuolar degeneration, Hirano bodies and cerebrovascular amyloid (Castellani, Rolston et al. 2010).

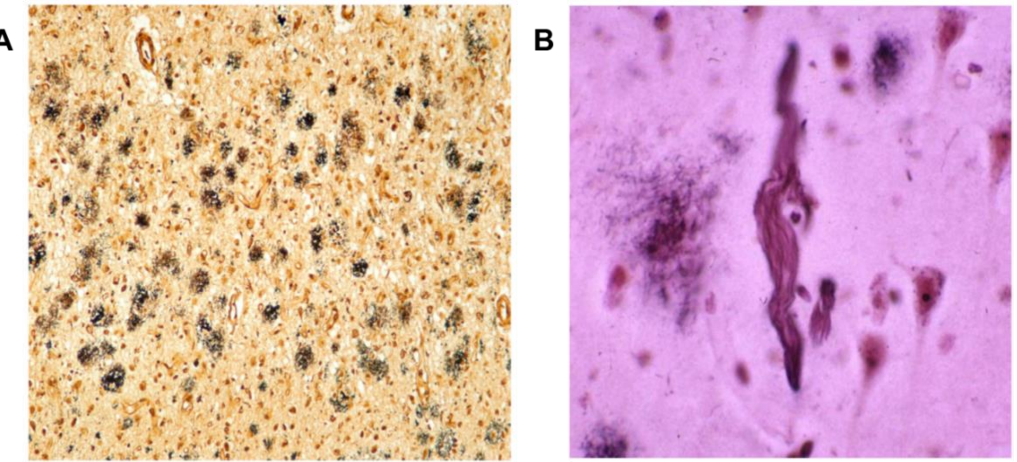


**Figure 4 - Two pathological features of AD**: senile plaques **(A)** and neurofibrillary tangles **(B)** (both seen with Bielshowsky silver staining). Adapted from: (Castellani, Rolston et al. 2010)

## 2.3. The Amyloid β Precursor Protein processing and Aβ generation

### *2.3.1. The amyloidogenic pathway*

Senile plaques are formed by Aβ aggregates that result from the cleavage of a larger precursor protein named APP. APP is a transmembrane protein which is abundantly expressed in the CNS, but is also present in peripheral tissues such as epithelium and blood cells (Paula 2009). The cleavage and processing of APP involves two distinct pathways: the non-amyloidogenic and the amyloidogenic routes. In the first, APP is cleaved by the α-secretase, producing a large amino (N)-terminal ectodomain (sAPPα) that is secreted into the extracellular environment. This cleavage occurs within the Aβ region, thus preventing its formation. The produced fragment (named C83) is engaged in the membrane and is then cleaved by γ-secretase, producing a short fragment termed p3. In the amyloidogenic pathway, the first proteolytic step occurs through the action of β-secretase, releasing sAPPβ into the extracellular medium and keeping a fragment named C99 within the membrane. This fragment is then cleaved by the γ-secretase, resulting in the formation of an intact Aβ peptide (figure 5) which is released into the extracellular space (LaFerla, Green et al. 2007).

Mutations in APP, PS1 and PS2 (two γ-secretases), as mentioned above, affect the metabolism and stability of Aβ (LaFerla, Green et al. 2007). The most common APP mutation is the Swedish mutation (APPswe), in which a double amino acid change (K670N, M671L) results in increased APP cleavage by β-secretase (Haass, Lemere et al. 1995). Also, mutations in the presenilin, such as the PS1A246E mutation, lead to increased levels of $A\beta_{42}$ (Aβ peptide composed of 42 aminoacids) (Jankowsky, Fadale et al. 2004).

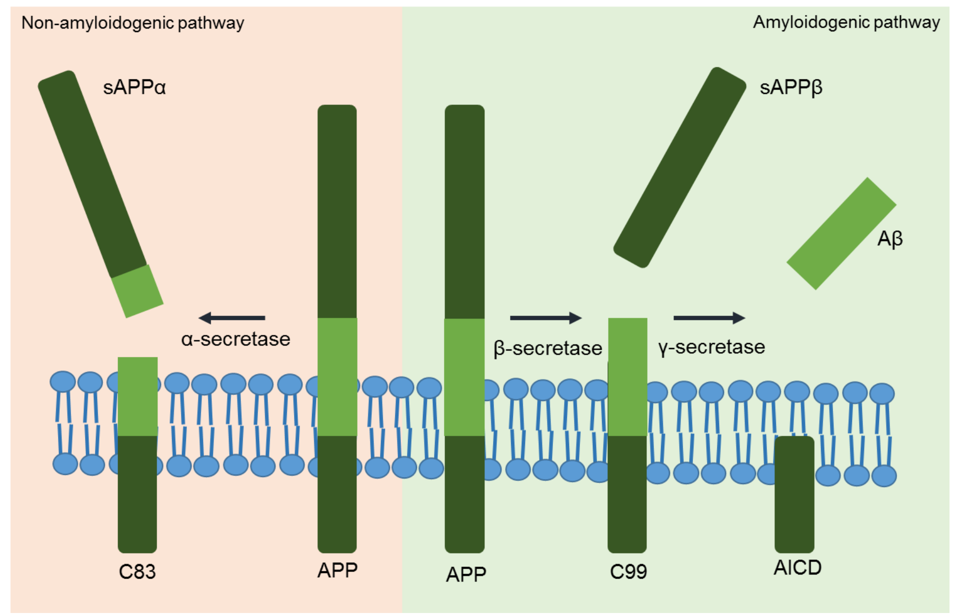


**Figure 5 – Schematic diagram of APP processing and Aβ formation.** While in the non-amyloidogenic pathway, APP is cleaved by α-secretase forming sAPPα (nontoxic), in the amyloidogenic pathway, the subsequent proteolysis of APP by β- and γ-secretases gives rise to Aβ peptide that accumulates in the extracellular environment. Adapted from (LaFerla, Green et al. 2007).

### *2.3.2. Aβ peptide and its assembly states*

As stated before, extracellular Aβ fibrils are the major constituent of senile plaques. Amyloid is characteristically congophilic, thioflavin S positive and highly insoluble in most solvents (Castellani, Rolston et al. 2010). The protein was shown to be composed of 42-43 amino acids derived from the larger APP (Glenner and Wong 1984). This 4kDa peptide has a β-pleated sheet configuration and its length can vary at the c-terminus, depending on where the γ-secretase cleaves APP (Perl 2010). In the brain, the most abundant Aβ forms are $A\beta_{40}$ and $A\beta_{42}$, being the levels of $A\beta_{40}$ higher than $A\beta_{42}$ ones (LaFerla, Green et al. 2007). The latter is more hydrophobic and more prone to fibril formation, being the major isoform in senile plaques (Jarrett, Berger et al. 1993). Aβ can exist in different assembly states: it is released as monomers which gradually aggregate into dimers, trimmers, oligomers, protofibrils and fibrils to finally deposit and form amyloid plaques (Paula 2009) (figure 6).

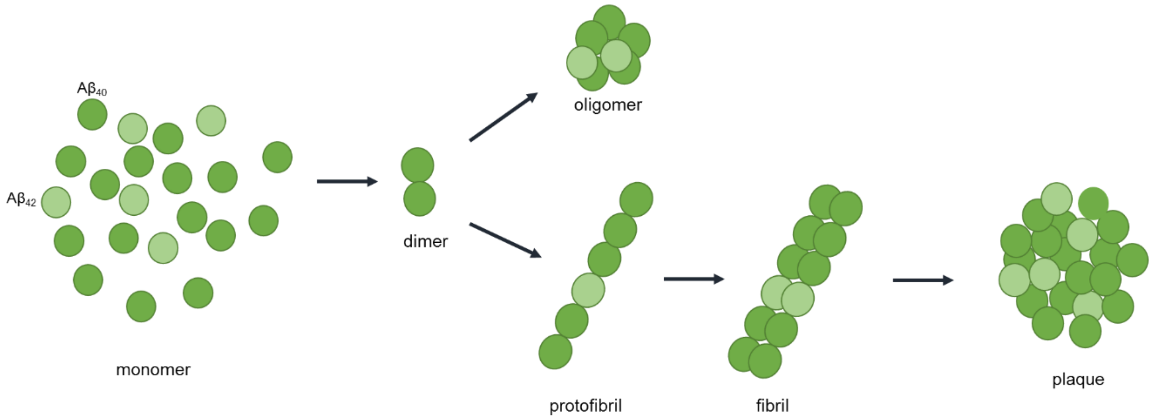


**Figure 6 – Schematic diagram of Aβ assembly states.** Aβ exists in several assembly states – monomers, dimers, oligomers, protofibrils, fibrils and plaques.

## 2.4. Aβ oligomers: the main toxic form

Soluble Aβ oligomers are defined as Aβ assemblies which stay in solution after high-speed centrifugation, however these forms can bind to other macromolecules or to cell membranes, becoming insoluble. There are several types of Aβ oligomers that can derive from natural and synthetic Aβ (Table 1) (Haass and Selkoe 2007). Dimers and trimmers have been indicated as the building blocks of larger oligomers and insoluble amyloid fibrils since they were found in soluble fractions of human brain and amyloid plaque extracts (Walsh, Tseng et al. 2000). These low molecular weight oligomers are highly toxic *in vitro*, being dimers threefold more toxic than monomers and tetramers 13-fold more toxic (Ono, Condron et al. 2009). Also, different molecular-weight synthetic Aβ oligomers have been developed and their toxic nature has been confirmed. For instance, small Aβ globular oligomers (5 nm in diameter) known as Aβ-derived diffusible ligands strongly bound to the dendritic arbors of cultured neurons, leading to neuron death and blocking long-term potentiation (a typical model for synaptic plasticity and memory loss) (Lambert, Barlow et al. 1998).

Aβ oligomers have been pointed out as key players in AD pathology. Several studies suggest that soluble Aβ, including soluble oligomers, are the species linked to the presence and degree of cognitive deficits rather than the amyloid plaques (McLean, Cherny et al. 1999, Wang, Dickson et al. 1999, Naslund, Haroutunian et al. 2000). The larger contact between the neuronal membranes and a multitude of small oligomers rather than a low contact with larger fibrillar plaques (due to a small Aβ surface area) might explain why soluble assembly forms are much likely able to induce neuronal and/or synaptic dysfunction than plaques (Haass and Selkoe 2007).

**Table 1 – Oligomeric assemblies of Aβ**. Adapted from (Haass and Selkoe 2007)

| OLIGOMERIC ASSEMBLY | CHARACTERISTICS |
|---|---|
| Protofibril | Intermediates of synthetic Aβ fibrillization; up to 150 nm in length and ~5 nm in width; β-sheet structure: bind Congo red and Thioflavin T |
| Annular assemblies | Doughnut-like structures of synthetic Aβ; outer diameter of ~8–12 nm; inner diameter of ~2.0–2.5 nm |
| Aβ-derived diffusible ligands | Synthetic Aβ oligomers smaller than annuli; might affect neural signal transduction pathways |
| Aβ*56 | Apparent dodecamer of endogenous brain Aβ; detected in the brains of an APP transgenic mouse line and might correlate with memory loss |
| Secreted soluble Aβ dimers and trimers | Produced by cultured cells; resistant to SDS; alter synaptic structure and function |

## 2.5. Models of Alzheimer's Disease

The development of transgenic mouse models of AD has opened new insights in the development of the pathology and in the discovery of new therapeutic targets. Several AD mice models have been described so far and most of them carry mutations in APP, PS1 or PS2, displaying features characteristic of AD. Since TTR has been suggested as a neuroprotector in this pathology, AD mouse models transgenic for TTR have been created. An AD mouse model that carried the APP Swedish mutation (APP23 mice) showed co-localization of TTR and Aβ in the amyloid deposits (Buxbaum, Ye et al. 2008). The APP23/hTTR$^{+}$ mice, that resulted from the crossing of a mouse strain that overexpresses human TTR WT with the APP23 mice, showed normalized cognitive function and spatial learning besides presenting a reduction in the amounts of deposited Aβ (Buxbaum, Ye et al. 2008). In this study, authors also showed that the existence of two copies of *ttr* gene has a great impact in the development of the disease than having only one copy (Buxbaum, Ye et al. 2008). However, other studies have proven the opposite. TgCRND8/TTR$^{+/-}$ mice presented reduced Aβ plaque burden in the hippocampus when compared with TgCRND8/TTR$^{+/+}$ mice (Doggui, Brouillette et al. 2010). In studies using APPswe/PS1A246E transgenic mice (that presented accelerated Aβ deposition in the hippocampus and in the cortex) crossed with TTR null mice, females mice showed increased Aβ levels when comparing with their male counterparts and mice with only one

copy of *ttr* gene also exhibited higher Aβ levels, suggesting then a gender-dependent modulation of Aβ levels (Oliveira, Ribeiro et al. 2011).

Although mice are the leading animal model in the neurodegeneration field, zebrafish has emerged as new valuable animal model for disease modelling, mechanistic studies and therapeutic testing due to its unequaled advantages: the ease to quickly and cheaply generate a large number of animals, their transparency during development and the large number of available techniques to modulate their genetics and phenotypes (Martin-Jimenez, Campanella et al. 2015). Notably, zebrafish possesses the orthologues of the genes known to be involved in AD, i.e., *ps1, ps2* and *app* (Newman, Verdile et al. 2011). Since these genes have not yet been fully studied, the development of an AD zebrafish model that presents the AD pathological characteristics and that relies on the mutation of such genes has not been achieved yet. Nevertheless, an AD zebrafish model was created, showing cognitive defects and increased tau phosphorylation after Aβ injection in the brain ventricle of 24 hours post-fertilization (hpf) zebrafish embryos (Nery, Eltz et al. 2014). A different approach in which zebrafish embryos are incubated in an Aβ-containing medium was also developed. Using a zebrafish line that expresses GFP in the vessels, Aβ was demonstrated to induce vessel reduction, impairment of angiogenesis and an increase reactive oxygen species, phenotypes that are frequently found in AD (Lu, Liu et al. 2014).

### 2.6. Therapeutic approaches for Alzheimer's Disease

The current therapeutic approaches for AD are based on the modulation of the effects created by specific hallmarks such as hyperphosphorylation of tau and the low levels of acetylcholine. Drugs that target the components of the amyloidogenic pathway are also being developed (Kumar, Nisha et al. 2015). The treatments approved so far aren't able to delay the progression of neurodegeneration, relaying only in the maintenance of the patient functional ability and with less symptoms (Rabins and Blacker 2007). Cholinesterase inhibitors such as donepezil have been approved by the Food and Drug Administration for the treatment of the cognitive impairment found in AD. Other drugs such as non-steroidal anti-inflammatory drugs have also been proposed to have beneficial effect at this level. However these treatments showed mild side effects and low efficacy in clinical trials (Rabins and Blacker 2007). Therefore, the development of new therapies that target other neurodegenerative processes found in this disease might be of great interest.

It is currently believed that, in particular in the sporadic cases of AD which represent the majority of the cases, elimination of Aβ is compromised rather than its increased production (as it seems to be the case of familial cases). Therefore, amyloid-

degrading enzymes have been suggested as valuable tool to modulate AD pathology. Neprilysin (NEP), a peptidase present in the neuronal surface, has been implicated in the degradation of Aβ peptide both *in vitro* and *in vivo* (Nalivaeva, Beckett et al. 2012). The lentiviral delivery of NEP to the brain of AD mice lead to a diminished amyloid pathology in these mice (Marr, Rockenstein et al. 2003). Other reports also stated that the early neuronal overexpression of NEP is beneficial since it diminishes Aβ levels and delays plaque formation and AD pathology (Leissring, Farris et al. 2003). A recent study, in which recombinant soluble NEP was administrated to an AD mice model, through a guide cannula placed in the hippocampal region, showed that soluble NEP is able to reduce Aβ plaque burden and to improve the memory defects on these mice (Park, Lee et al. 2013). Nevertheless, the role of other enzymes has also been addressed in this context. The involvement of insulin-degrading enzyme (IDE), a zinc endopeptidase present in neuronal cells, in Aβ clearance has been the focus of extensive research (Nalivaeva, Beckett et al. 2012).

## 3. Neuronal cytoskeleton remodeling in protein aggregation diseases

The neuronal cytoskeleton has been identified as an essential component in the neurodegenerative process caused by the accumulation of specific prone-to-aggregate proteins. The most studied neurodegenerative disease with a strong cytoskeleton dysfunction is AD, since tau, that is a microtubule-associated protein (MAP), accumulates and forms NFTs. Nevertheless, Aβ has been also related to the neuronal cytoskeletal defects found in AD. Other disorders have also been investigated, such as Parkinson's Disease (PD) and FAP, however there still is a lack of information regarding the effect of the accumulation of the disease-related proteins in the neuronal cytoskeleton.

### 3.1. The neuronal cytoskeleton

The cytoskeleton of eukaryotic cells is the structure that helps cells maintaining their morphology and internal organization. There are three main types of cytoskeletal polymers that can be distinguished by their diameter, type of subunit and subunit arrangement: microtubules (MTs), actin filaments and intermediate filaments (IFs).

### *3.1.1. Structure and organization of microtubules, actin filaments and intermediate filaments*

MTs are long protein polymers composed by subunits formed by α and β tubulins. MTs consist of 13 linear protofilaments assembled around a hollow core (25nm in diameter), that forms a polar structure with two different ends: a fast-growing plus end and a slow-growing minus end. This polarity is crucial to determine the movement along MTs (Cooper 2013).

Both α-tubulin and β-tubulin bind to GTP that regulates polymerization. Shortly after polymerization GTP is hydrolyzed and the affinity of tubulin for adjacent molecules weakens, favoring depolymerizating and resulting in the dynamic state. MTs also undergo treadmiling, a dynamic process in which the plus end of the filament grows in length while the other one shrinks, due to the addition of tubulin molecules bound to GTP lost from the minus end to the plus end of the same MT (Cooper 2013). MT dynamics is tightly regulated by post-translational modifications of tubulin such as detyrosination/tyrosination, acetylation and polyglutamylation, that not only state the dynamic state of MTs but also were suggested to regulate binding affinity to motor proteins (Janke and Bulinski 2011). The molecular motors were shown to bind with higher affinity to acetylated and polyglutamylated MTs which constitute the most stable pools (Janke and Bulinski 2011). Furthermore, microtubule-associated proteins (MAPs) have also an important role in MT stabilization (for example, MAP1, MAP2, tau and collapsing response mediator proteins (CRMPs)) and destabilization (such as spastin and katanin) (Janke and Bulinski 2011). Besides their role on MT dynamics, a group of MAPs namely MT plus end-tracking proteins (+TIPs) control the interaction between MT and cellular organelles (Akhmanova and Steinmetz 2008).

Due to their dynamic nature and their interaction with all these intracellular players, MTs are a key cellular component in the determination of the polarity of neurons and form the trails for intracellular transport of a high variety of structures. Intracellular transport is crucial for neuronal morphogenesis, function and survival (Hirokawa, Niwa et al. 2010). The long distance transport of cargos along the axon is carried out by three essential molecular motor superfamilies: kinesin, dynein and myosin (Hirokawa and Noda 2008). Both kinesin and dynein use ATP to move along MTs in different directions: kinesins walk toward the plus end (anterograde transport) and dyneins walk toward the minus end (retrograde transport) (Hirokawa, Niwa et al. 2010). In the axon, two types of transport exist: fast transport of membranous organelles and slow transport of cytosolic proteins and cytoskeletal proteins.

The actin cystoskeleton is composed of actin monomers (globular (G) actin) that have tight binding sites that enable head-to-tail interactions with two other actin monomers, so actin monomers polymerize to form thin, flexible fibers of approximately 7 nm in diameter actin filaments (filamentous (F) actin). These filaments are organized into higher-order structures, forming bundles or three-dimensional networks. Since all the actin monomers are oriented in the same direction, actin filaments have a distinct polarity and the plus and minus ends are distinguishable. The reversible addition of monomers happens in both ends, but the plus end elongates five to ten times faster than the minus end. Actin monomers bind ATP which leads to a faster polymerization and is hydrolyzed to ADP after assembly (Cooper 2013). This process is tightly regulated by elongation factors such as profilin and formins and by depolymerization factors such as cofilin. Together these features make actin the engine behind the generation of the force necessary to regulate the neuronal shape and cellular internal and external movements (Edwards, Zwolak et al. 2014).

IFs differ from MTs and actin by their size and primary structure of their constitutive proteins, their non-polar architecture and their relative insolubility. Each IF protein is composed of a conserved α-helix central region, called rod domains, which is flanked by non-α-helical head (amino-terminal) and tail (carboxy-terminal) domains (Lepinoux-Chambaud and Eyer 2013). The assembly of IF proteins consists in two anti-parallel dimmers, which form protofilaments. A filament is finally composed of eight protofilaments, resulting in a diameter of 10 nm. IFs are divided into six sequence homology groups (types I to VI) depending on the cellular type in which they are present. Type IV IFs (neuronal-specific) are neurofilaments (NFs) and their related proteins (NF-L, NF-M, NF-H, nestin, synemin, syncoilin and α-internexin) (Szeverenyi, Cassidy et al. 2008). The regulation of these proteins is achieved through post-translational modifications, such as phosphorylation, glycosylation and transglutamination (Lepinoux-Chambaud and Eyer 2013). The main role of NFs in neurons is the stabilization of the other components of the cytoskeleton.

#### *3.1.2. Cytoskeleton organization in neurons*

During neuronal development and regeneration, each neuron has an axon which will continue to growth till its final destination. Growth cones, which are the tips of each axon, play a fundamental role in the elongation of the axon. The cytoskeleton organization has a crucial impact on nervous system injury. The adult CNS and the PNS differ greatly in the regenerative capacity, responding differently in terms of cytoskeleton reorganization after injury. While PNS neurons form a growth cone which allows regeneration, adult CNS

neurons, which are not able to grow after injury, develop dystrophic end bulbs or retraction bulbs, a hallmark of degenerating axons (Bradke, Fawcett et al. 2012).

The growth cone can be separated into three domains based on the cytoskeletal components distribution. The central domain (C-domain), where the axon shaft terminates, is composed by bundles of stable MTs that enter into the growth cone and polymerize with their plus end toward the leading edge, and by vesicles, organelles and other proteins that are being transported into this domain. The actin filaments, that exist as both filopodia (packed actin bundles) and lamellipodia (sheet-like actin meshwork), constitute the peripheral domain (P-domain) and shape the growth cone and direct its propagation. Both types of actin filaments have their depolymerizing ends directed to the basal region of the growth cone, forming the transition zone (T-zone). In this thin band between the central and the peripheral domains, MTs and actin filaments interact what is crucial for axon extension (figure 7) (Neukirchen and Bradke 2011).

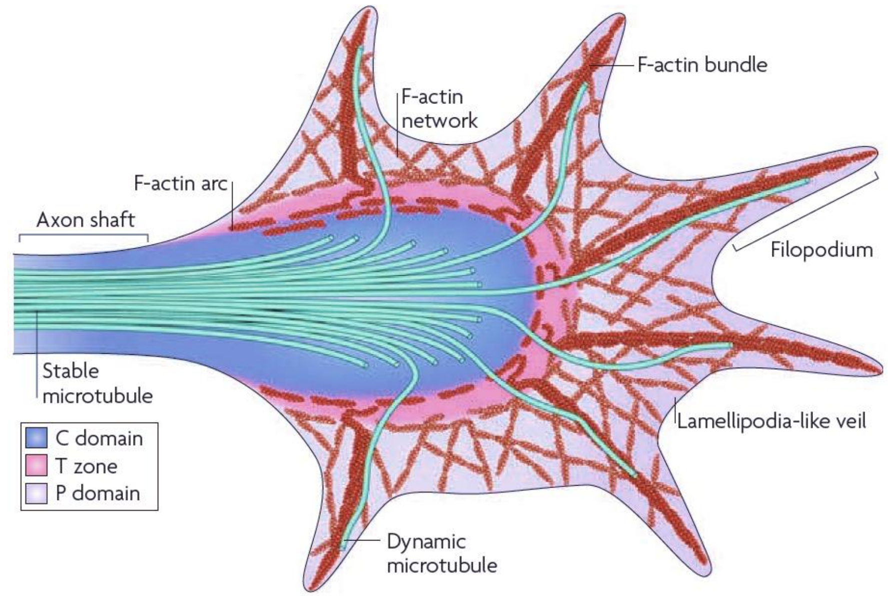


**Figure 7 - The structure of the neuronal growth cone.** The growth cone is composed of three different zones: the central zone (C), the peripheral zone (P) and the transitional zone (T), which have different cytoskeletal elements composition. Adapted from (Lowery and Van Vactor 2009)

Patches of actin filaments are present on the initial portion of the axon – axon initial segment (AIS) – and along the more distal axon shaft and in the dendrites. The presence of these structures in the AIS has been proposed to capture axonal transport cargoes, limiting their entrance into the axon, while their presence in the distal part is related with the formation of axonal collateral branches (Arnold and Gallo 2014). Recently, a novel form of actin filament organization was found: actin rings where actin is disposed in isolated rings associated with adducin and separated by spectrin tetramers were demonstrated along the shaft of the axon. In the axon shaft, MTs are aligned in parallel bundles that then splay apart within the growth cone (Xu, Zhong et al. 2013). In the

dendrites, MTs have a mixed polarity with both plus and minus ends towards these structures, being mainly involved with synaptic activity. Actin, in other hand, governs morphological and functional synaptic plasticity (Shirao and Gonzalez-Billault 2013). NFs are mainly present in axons, being essential for radial growth, the maintenance of axon caliber and the transmission of electrical impulses along axons (Yuan, Rao et al. 2012). These proteins, as long as actin and tubulin, are synthethized within the cell body and travel long distances to reach their sites of action (figure 8).

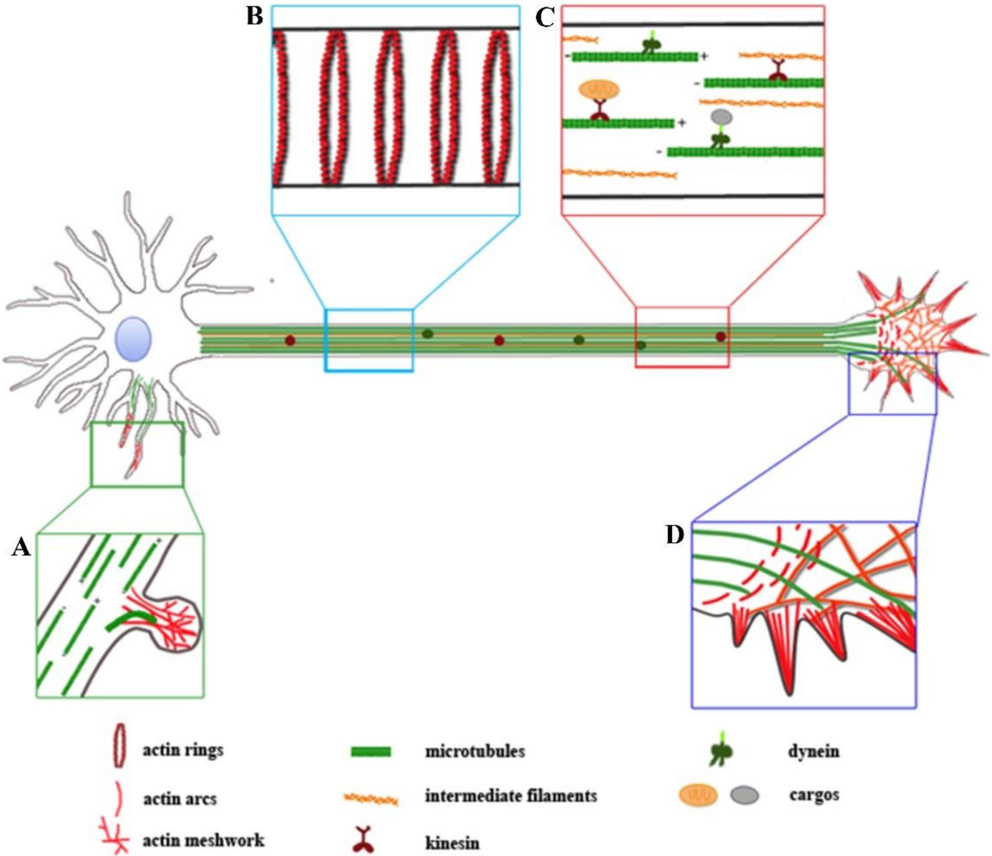


**Figure 8 – Schematic depiction of the neuronal cytoskeleton of developing neuron. A -** The dendritic spines present mixed orientated microtubules and patches of actin; **B -** Actin rings are one of the main actin-based structures in the axon; **C –** Axonal transport occurs through the action of molecular motors such as kinesin and dynein that move along microtubules; **D –** The growth cone is composed by highly motile microtubules, actin meshwork and actin arcs.

## 3.2. Cytoskeleton alterations in neurodegenerative diseases

Neurodegenerative disorders are a wide group of chronic neurological disorders that, depending on its origin, can be characterized clinically by slow progressive loss of motor/sensory functions and/or decreased perceptual function which might be associated with cognitive and behavioural deficits (Pal, Alves et al. 2014).

In several neurodegenerative disorders, such as AD and Parkinson's Disease (PD), affecting the CNS and FAP, which mainly affects the PNS, the presence of

aggregated forms of a given protein are a hallmark. Protein aggregation can be caused by a mutation in a disease-associated protein, by a genetic alteration that augments the amounts of a normal protein or by environmental stress or aging (Ross 2005). Protein-aggregated structures can also be due to the inexistence of a defined tertiary structure or can also result from the partial unfolding of a proteins which usually has a well-defined tertiary and/or quaternary structure (Johnson, Connelly et al. 2012). In some diseases, large intracellular or extracellular deposits of aggregated protein are often formed (Ross 2005). Many reports have been published showing the high toxicity of these structures that lead to neurodegeneration. Some of the potential causes of neurodegeneration investigated include: excitotoxicity, astroglia and/or microglia dysfunction, oxidative stress and mitochondrial dysfunction, endoplasmic reticulum stress, defects in axonal transport and RNA-processing and deregulation of metabolic and degradation pathways. Additionally, several studies showed that major axonal and cytoskeletal alterations are seen in diverse models of neurodegenerative diseases (Vickers, King et al. 2009).

#### *3.2.1. Alzheimer's Disease*

AD is characterized by abnormal decline in cognition, which is associated with cytoskeletal changes in neurons and subsequent neurodegeneration (Suchowerska and Fath 2014). In AD patients, accumulation of cytoskeletal proteins is a common phenomenon. Hirano bodies, eosinophilic inclusions that are frequently observed in postmortem AD brains, were discovered to be composed by bundles of F-actin (Galloway, Perry et al. 1987). Furthermore, senile plaques within the brains of sufferers of AD are typically surrounded by dystrophic neurites (Tanzi, St George-Hyslop et al. 1989). Further studies using human AD tissue presented NFs accumulation in ring-like whorls or bulbous conformations (Vickers, King et al. 2009).

Aβ aggregation is acutely toxic to neurons. Concerning cytoskeletal alterations, *in vitro* stimulation of the Ras-related C3 botulinum toxin substrate 1/ Cell division control protein 42 homolog (Rac1/Cdc42) pathway (proteins of the Rho GTPase family, the major regulators of actin dynamics) with fibrillar $A\beta_{1-42}$ increased actin polymerization, as shown by increased lamellipodia and filopodia formation in rat hippocampal neurons (Mendoza-Naranjo, Gonzalez-Billault et al. 2007). Aβ aggregates were suggested to be sufficient to induce the formation of neuritic abnormalities in rat hippocampal neurons (Pike, Cummings et al. 1992) and, more recently, it was demonstrated that in SH-SY5Y cells and in a mouse model of AD, Aβ aggregates lead to reduction in the length of neurites by promoting CRMP-2 phosphorylation via Rac1 (Petratos, Li et al. 2008). Moreover, studies conducted with hippocampal brain tissue of AD patients showed increased Rac1/Cdc42 expression when compared to age-matched controls (Zhu, Raina et al. 2000) what might

account for an excessive F-actin accumulation resulting in the formation of the observed Hirano bodies (Henriques, Oliveira et al. 2014). Cofilin-actin rods are intracellular inclusions primarily formed in the axons and dendrites of neurons due to the hyperactivitation by dephosphorylation of cofilin and that block transport within neurites (Bamburg and Bloom 2009).The existence of cofilin-actin rods in AD was verified both *in vitro* using neurons that were exposed to Aβ oligomers (Maloney, Minamide et al. 2005) and *in vivo* using human AD brains (Minamide, Striegl et al. 2000).

Nevertheless, the axonal transport defects are also due to alterations in the MT cytoskeleton and the deregulation of specific signaling pathways. Regarding the first, it was demonstrated that Aβ impacts on MT cytoskeleton. Deacetylated tubulin is related to a dynamic form of tubulin that blocks binding of motor proteins, thus axonal transport (Reed, Cai et al. 2006). Aβ decreases α-tubulin acetylation (Henriques, Vieira et al. 2010) and toxic concentrations of oligomeric $Aβ_{1-42}$ were shown to promote MT stability independently of tau in primary neurons, being this effect mediated by RhoA pathway (Pianu, Lefort et al. 2014). Furthermore, the levels of histone deacetylase 6 (HDAC6), which is able to deacetylate α-tubulin, are increased in AD patients brains (Ding, Dolan et al. 2008). Additionally, Aβ oligomers were also shown to induce tau (that is usually in the axonal compartment) missorting to dendrites and a drastic reduction in the number of MTs (Zempel, Thies et al. 2010). Regarding the deregulation of signaling pathways, hippocampal neurons treated with Aβ oligomers exhibited vesicle and mitochondria transport defects via a mechanism that involves N-methyl-D-aspartate receptors (NMDARs) and glycogen synthase kinase 3-β (GSK3-β) (Decker, Lo et al. 2010). The transport of vesicles containing brain-derived neurotrophic factor after treatment with Aβ oligomers was also assessed and an impairment of the fast axonal transport related independently of tau was observed (Ramser, Gan et al. 2013).

#### ***3.2.2. Parkinson's Disease***

Parkinson's Disease (PD) is a progressive neurodegenerative disorder of the CNS. PD commom symptoms include resting tremor, rigidity, gait impairment, bradykinesia and postural instability. The histopathological hallmarks in PD are the degeneration of dopaminergic neurons in the substantia nigra along with Lewy bodies (LB) - intracellular protein accumulations (Suchowerska and Fath 2014) being these structures mainly composed by α-synuclein, tubulin, MAPs and NFs (Galloway, Mulvihill et al. 1992).

α-synuclein is a member of the synuclein family of proteins that has 140 amino acids and has no defined structure. When bound to negatively charged lipids, it acquires an α-helical structure, while, in longer incubation times, it gains a β-sheet-rich structure (Stefanis 2012). This protein is abundantly expressed in the nervous system and localizes

preferentially in the presynaptic terminals of neurons (George 2002). There are two well-studied disease related-mutations of α-synuclein: A30P and A53T (Polymeropoulos, Lavedan et al. 1997, Kruger, Kuhn et al. 1998). As other β-sheet-rich proteins, α-synuclein has a high propensity for aggregation. Indeed, WT as well as disease-related mutants form amyloid-like fibrils in long incubations, structures known for being the basis of the mature LB and lewy neurites. The α-synuclein aggregation cascade is similar to other known aggregation cascades: the natively unfolded protein initially forms soluble oligomers that assemble in protofibrils that eventually form fibrils (Stefanis 2012).

Several reports have established the importance of the neuronal cytoskeleton in PD. Although the precise mechanisms by which α-synuclein act were not yet discovered, it is known that it has an important role on neuronal plasticity and synaptic regulation (Cheng, Vivacqua et al. 2011). Most studies have addressed the effect of α-synuclein on MTs and axonal transport. α-synuclein and tubulin were shown to interact with each other *in vitro* (Zhou, Huang et al. 2010), however conflicting effects on tubulin polymerization have been found (Alim, Ma et al. 2004, Zhou, Huang et al. 2010). Furthermore, MT instability has been proposed as an early event in the degeneration of dopaminergic neurons in a PD mice model (Cartelli, Casagrande et al. 2013).

Axonal transport defects were also identified as one of the mechanisms behind α-synuclein pathogenesis (Hunn, Cragg et al. 2015) and since dopaminergic neurons have long axons, cargo trafficking is vital for their life-maintaining processes. Cortical neurons overexpressing α-synuclein mutants (A30P and A53T) exhibited reduced axonal transport and transfection of these cells with A30P resulted in the accumulation of the protein proximal to the cell body, a process that might contribute to LB formation and neuritic defects (Saha, Hill et al. 2004).

Efforts have been made to unravel the role of α-synuclein in the actin cytoskeleton in PD. Zhou et al demonstrated that α-synuclein interacts with the actin cytoskeleton components such as actin, cofilin and F-actin capping proteins (Zhou, Gu et al. 2004). Furthermore, the A30T mutant was shown to alter the actin cytoskeletal structure and dynamics in primary hippocampal neurons (Sousa, Bellani et al. 2009). Since, in this report, the aggregation status of this mutant was not assessed, further studies should verify if the aggregation status of α-synuclein impact on the actin cytoskeleton remodeling.

#### ***3.2.3. Familial Amyloid Polyneuropathy***

TTR is a highly amyloidogenic protein that is related to FAP. In this autosomal dominant disease, TTR deposits preferentially in the PNS and forms fibril aggregates leading to nerve lesions. FAP typically causes a nerve length-dependent sensory-motor polyneuropathy and autonomic dysfunction (Plante-Bordeneuve and Said 2011, Saraiva,

Magalhaes et al. 2012). The systemic extracellular deposition of mutated TTR aggregates and amyloid fibrils is present in the connective tissue, with the exception of brain, liver and parenchyma, and has a major impact in the PNS. TTR has access to the nerve through the blood and CSF. In the peripheral branch, the fibrils are diffusely distributed, involving nerve trunks, plexuses and sensory and autonomic ganglia. Consequently, axonal degeneration becomes a feature of this disease, beginning in unmyelinated and myelinated fibers of low diameter and ending up in neuronal loss of ganglionic sites (Plante-Bordeneuve and Said 2011).

The involvement of the neuronal cytoskeleton in this disorder is still under investigation. Unpublished *in vitro* data from our group revealed that mouse WT DRG neurons incubated with TTR oligomers show a marked reduction of the growth cone area, with disruption of the typical morphology of the growth cone presenting dystrophic MTs and lacking the lamellipodial actin structures (figure 9). Furthermore, using *Drosophila Melanogaster* in which TTR V30M is expressed in the photoreceptors cells resulting in roughening of the eye, our group has also verified a decreased axonal projection of photoreceptor neurons, that presented more compact growth cones lacking the spread distribution of filopodia and lamellipodia actin structures. Our group also performed a genetic screening in which TTR V30M flies were crossed with flies for the knockdown or overexpression of candidate genes that are related to the neuronal cytoskeleton. It was verified that overexpression of the major regulator of actin dynamics Rac1 reverted the TTR-induced rough eye phenotype, reinforcing the important role of the actin cytoskeleton and the associated signaling pathways in neurodegenerative process in FAP.

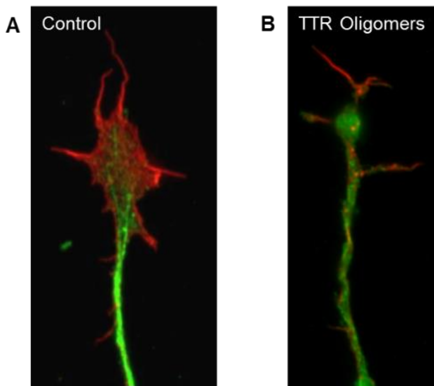


**Figure 9 - TTR oligomers induce alterations in the growth cone morphology of DRG neurons. (A)** control DRG neurons present a typical growth cone morphology with lamellipodia and filopodia (red) and splayed microtubules (green). **(B)** WT DRG neurons incubated with TTR oligomers show an altered growth cone morphology being dystrophic and lackig lamellipodial actin structures.

In the diseases described above, the neuronal dysfunction was suggested to occur as a result of the action of the noxious oligomers instead of the fibrils themselves. It has been proposed that oligomers composed by Aβ, α-synuclein and other prone-to-aggregate proteins share a common structure regardless of the differences present in their amino

acid side chains (Kayed, Head et al. 2003). This discovery might imply that a common pathogenic mechanism is behind the neurodegenerative process associated with protein misfolding and aggregation and this mechanism might be associated with the hydrophobic interaction of the oligomeric species with different cellular targets, such as the neuronal cytoskeleton (Agorogiannis, Agorogiannis et al. 2004).

## OBJECTIVES

Protein aggregation is a hallmark of several neurodegenerative disorders such as AD, PD and FAP. Aβ accumulation is considered the major pathological change in AD progression constituting a therapeutic target involving the use Aβ degrading enzymes. In this respect, TTR is a metalloprotease that was shown to cleave both soluble and aggregated forms of Aβ *in vitro*. Nevertheless, the relevance of TTR proteolysis in AD was not previously addressed and constituted the first main goal of this thesis (Chapter 2). For that we established the following objectives:

i) Dissect the effect of TTR proteolytic activity on Aβ clearance and Aβ-mediated neurotoxicity using both cell lines and primary neuronal cultures;
ii) Investigate the role of TTR proteolytic activity *in vivo* using mice and zebrafish as AD models.

The second aim of this thesis was to analyze the effect of different prone-to-aggregate proteins, related with AD, PD and FAP, in the neuronal cytoskeleton (Chapter 3), since common pathological mechanisms might be involved in the neurotoxicity induced by these proteins. In order to accomplish that, the following objectives were defined:

i) Analyze the effect of Aβ oligomers in the growth cone morphology of hippocampal neurons and verify if TTR WT is able to rescue the resulting phenotype;
ii) Dissect the effect of the different aggregation states of α-synuclein in the growth cone morphology of hippocampal neurons;
iii) Study the effect of TTR WT and TTR mutants (V30M and L55P) in the growth cone morphology of hippocampal neurons;

# CHAPTER 2

## TTR NEUROPROTECTIVE EFFECT IN AD DEPENDS ON TTR PROTEOLYTIC ACTIVITY

# THEORETICAL BACKGROUND

Alzheimer's Disease (AD) is a neurodegenerative disorder clinically characterized by the progressive memory deficits and cognitive decline of the elderly. One of the hallmarks of AD is the extraneuronal accumulation of amyloid-β (Aβ) oligomers that results in the formation of amyloid plaques inducing neuronal dysfunction and cell death (Castellani, Rolston et al. 2010). Aβ is produced by the subsequent cleavage of the amyloid precursor protein (APP) by β- and γ-secretases (LaFerla, Green et al. 2007). Its levels are regulated by a balance between *de novo* synthesis of Aβ, aggregation and degradation. The dysfunction of these mechanisms occurs with aging and disease as AD. Transthyretin (TTR), a protein with metalloprotease activity, was suggested as having a neuroprotective role in AD as the protein was shown to be able to sequester Aβ, preventing amyloid formation and toxicity *in vitro* (Schwarzman, Gregori et al. 1994). Further studies verified that TTR protective capacity was related to its binding to toxic Aβ intermediates in both intracellular and extracellular environment in a chaperone-like manner (Buxbaum, Ye et al. 2008). Additionally, TTR was shown to be able to cleave Aβ *in vitro* in multiple positions, producing peptides with lower amyloidogenic potential (Costa, Ferreira-da-Silva et al. 2008). Unpublished data from our group in collaboration with the MIND group (IBMC) suggested that TTR proteolytic activity is required for the inhibition of Aβ fibril formation (figure 10) and for the disruption of Aβ fibrils (figure 11). This was shown by co-incubating either with TTR WT or a proteolytic inactive form (TTR H90A) with soluble or fibrillar Aβ. These results suggested that TTR proteolysis might underlie the neuroprotective effect of the protein, although this hypothesis was lacking support from *in vivo* data. In this work, we aimed at further dissecting the role of TTR proteolytic activity in AD both in cell based systems, using cell lines and primary neuronal cultures, and *in vivo* using mice and zebrafish, as AD models.

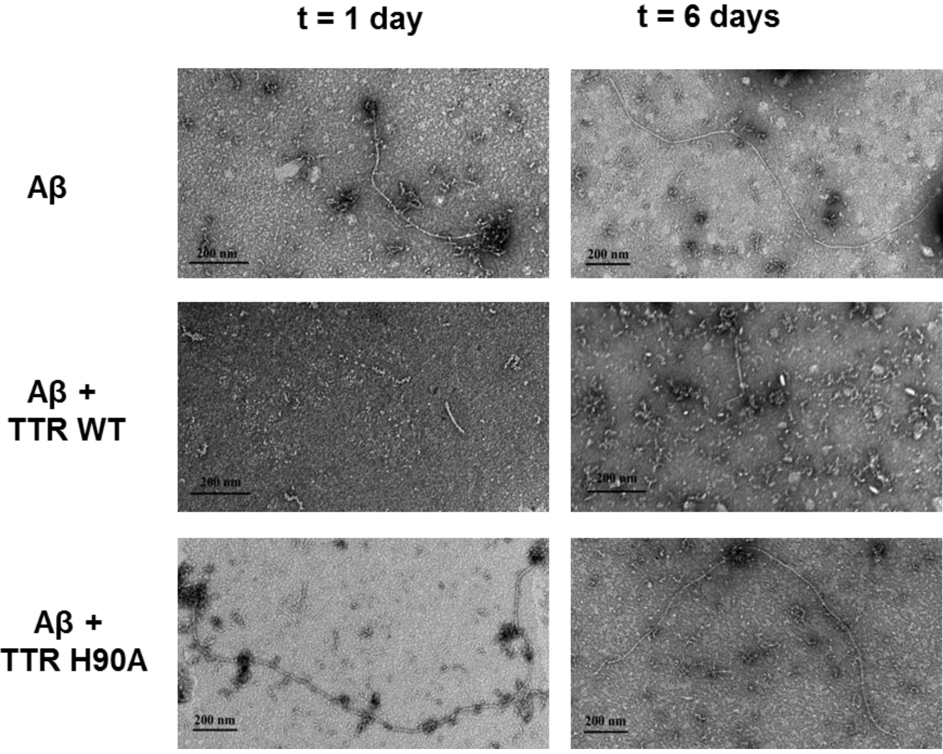


**Figure 10 – TTR proteolytic activity impacts on Aβ fibrillization.** Aβ peptide (soluble at t = 0) was incubated with or without TTR (either TTR WT or TTR H90A) and analyzed by TEM at days 1 and 6. The incubation of TTR WT with soluble Aβ showed an inhibition of Aβ fibrilization *in vitro*, an effect that was not seen with the incubation with TTR H90A.

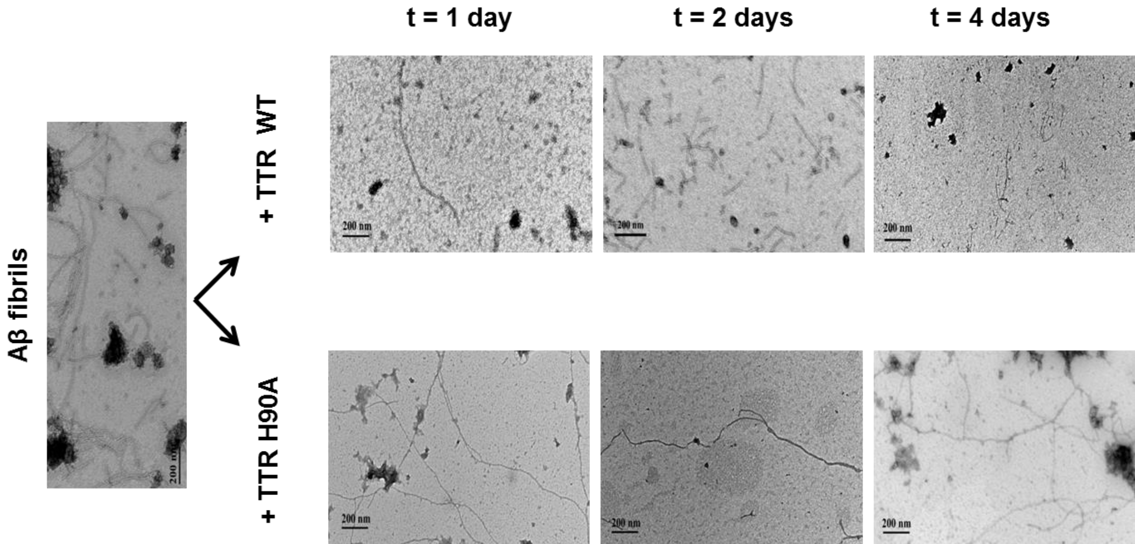


**Figure 11 – TTR proteolytic activity is required for the disruption of Aβ fibrils.** Aβ fibrils were grown for 10 days at 37 ºC (t=0); then either TTR WT or TTR H90A was added and further incubated at 37ºC and analyzed at different time points (1, 2 and 4 days) by TEM. The incubation of TTR WT with Aβ fibrils led to the generation of Aβ fragments of decreased size, while the incubation with TTR H90A did not have this effect.

## MATERIALS AND METHODS

### TTR production, purification and labeling

Recombinant TTR WT and TTR H90A (TTR proteolytically inactive) were produced in BL-21 pLys *Escherichia coli* cells transformed with pET plasmids carrying TTR cDNA as previously described (Furuya, Nakazato et al. 1989). The protein was isolated and purified as previously described (Liz, Faro et al. 2004). Briefly, after osmotic shock of bacteria, protein extracts were run on a diethylaminoethyl (DEAE)-cellulose (Whatman) ion exchange chromatography, dialyzed, lyophilised and isolated in native preparative Prosieve agarose (Lonza) gel electrophoresis. After electrophoresis, the TTR band was excised and eluted in 38 mM glycine, 5 mM Tris pH 8,3 overnight (O/N) at 4ºC. The protein was then detoxified using a high capacity endotoxin removal resin (Thermo Scientific) and quantified by Lowry's method using BioRad DC protein kit.

TTR WT was labeled with Alexa 488 using Alexa Fluor 488 Protein Labeling Kit (Life Technologies). Briefly, TTR was diluted 1:10 in 1M bicarbonate pH 8,3 and stirred for 1h at RT in the reactive dye vial. Labelled TTR (referred as TTR-488) was then purified through size exclusion purification BioGel P-30 Fine resin (Bio-Rad).

### TTR proteolysis assay

The enzymatic activity was measured as previously described. TTR (5 µM) was added to Abz-VHHQKL-EDDnp (5µM; Genscript) in 50 mM Tris pH 7,5. The fluorescence at $\lambda_{em}$ = 420 nm and $\lambda_{ex}$ = 320 nm was measured in a *f*max plate reader (Molecular Devices) at 37ºC for 30 min.

### Production of Aβ oligomers

Aβ peptide (Genscript) was dissolved in hexafluoroisopropanol (HFIP, Sigma) and incubated O/N at room temperature (RT). The HFIP was removed under a stream of nitrogen and the residue was then dissolved in dimethyl sulfoxide (DMSO, Sigma) at 2 mM. For oligomers formation, Aβ was diluted at a final concentration of 10 µM in F12 medium (Lonza) and incubated for 5 days at 4ºC, pH 7. To assess the assembly state of the Aβ, transmission electron microscopy using negative staining was performed at 50000x magnification.

## N2A cell culture

Neuro-2 A neuroblastoma cells stably expressing human APP carrying a double mutation at codons 670 and 671 (N2A-APPswe) were kindly provided by Dr. Huaxi Xu (Sanford-Burnham Medical Research Institute, California, USA).

N2A-APPswe cells (at a density of 50000 per well) were plated on a 24 well plate and were maintained in 1:1 Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; Sigma) / Opti-Minimum Essential Medium (Opti-MEM; Gibco) mixture supplemented with 5% FBS and 0,4% geneticine (Gibco). When 90% confluence was reached, the medium was substituted by a starvation medium constituted by 1:1 Opti-MEM/DMEM medium enriched with 0,4% geneticine (Gibco) containing 5 μM of either TTR WT or TTR H90A. Cells were maintained for 24h and the medium was collected and kept at -80ºC till use.

## Hippocampal neurons culture

Hippocampi were isolated from WT embryos (NMRI mice) at E17.5 and maintained in Hank's Balanced Salt Solution (HBSS, Sigma). Cells were digested with 0,06% trypsin (Sigma) in HBSS for 15 min at 37ºC, followed by addition of HBSS with 10% fetal bovine serum (FBS, Invitrogen) to stop digestion. After the washing steps, hippocampi were ressuspended in Neurobasal medium (Gibco) supplemented with 1% Pen/Strep (P/S, Invitrogen), B27 (Gibco) and L-Glutamine (Gibco) and were dissociated with two different diameter tips (P200 and P1000). The cell suspension was filtered and plated at a density of 150000 cells per well in coverslips previously coated with 20 μg/ml poly-L-lysine (Sigma).

## Caspase 3 fluorimetric assay

After 5 days *in vitro* (DIV), cells were treated with 10 μM Aβ oligomers plus 5 μM of either TTR WT or TTR H90A and maintained for 24h at 37ºC. Cells were then lysed with lysis buffer (25 mM HEPES pH 7.4, with 2,5 mM CHAPS and 2,5 mM DTT), centrifuged at 13000 rpm for 20 min at 4ºC and supernatants were kept at -80ºC till use. To assess apoptotic cell death, caspase 3 assay was performed using Caspase 3 Fluorimetric Assay Kit (Sigma). This assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin moiety. Samples were incubated with Ac-DEVD-AMC previously diluted in Assay Buffer for 90 min and subsequently the fluorescence at $\lambda_{em}$ = 460 nm and $\lambda_{ex}$ = 360 nm was measured in a plate reader (Biotek Synergy MX).

## *In vivo* experiments

### *Transgenic mouse model and intracerebral administration*

APPswe/PS1A246E/TTR$^{+/+}$ (referred as AD/TTR$^{+/+}$) and APPswe/PS1A246E/TTR$^{-/-}$ (referred as AD/TTR $^{-/-}$) transgenic mice, kindly provided by Isabel Cardoso (MIND group, IBMC), were used to perform TTR intracerebral injections. These mice were generated by crossing APPswe/PS1A246E transgenic mice with TTR null mice (TTR$^{-/-}$), generating APPswe/PS1A246E/TTR$^{+/+}$, APPswe/PS1A246E/TTR$^{+/-}$ and APPswe/PS1A246E/TTR$^{-/-}$ mice (Oliveira, Ribeiro et al. 2011). Animals were maintained in a controlled environment (12h light/dark cycle; temperature, 22 ± 2ºC; humidity, 45-65%) with food and water *ad libitum*. All the procedures followed National and European Union Guidelines for the care and use of laboratory animals.

In the pilot experiments, 15 and 16 months old female AD/TTR$^{-/-}$ mice (n=2 for each condition) were anesthetized with a mixture of ketamine (75 mg/kg) and medetomidine (1 mg/kg) administrated by intraperitoneal injection. For continuous intracerebral administration of TTR, mini osmotic pumps (rate: 0.5 µl/hr; Alzet) were placed unilaterally in the right hippocampus (AP -2,3; ML -1,5; DV -1,7). Approximately 24h prior to implantation, the pumps were filled either with 63 µg of TTR WT diluted in 100 µl of Phosphate-buffered saline (PBS) (to achieve a delivery of approximately 9 µg per day) or only with PBS, and were kept in PBS at RT until the time of surgery. To assess the intracerebral localization of TTR, a female AD mouse was injected with TTR-488 diluted in PBS using the same conditions.

In the following experiments, mini osmotic pumps were filled either with 63 µg of TTR WT or TTR H90A diluted in PBS. Female AD/TTR$^{+/+}$ mice of 9 months of age were anesthetized and the pumps were placed as described above. As a control, two non-injected female AD/TTR$^{+/+}$ mice were used.

### *Tissue processing*

#### Pilot experiments

Tissue processing was performed as previously described (Ribeiro, Oliveira et al. 2014), with some modifications depending on the purpose.

8 days post administration, the animals were anesthetized and the brain was removed. For the determination of Aβ levels in the test animals, the ipsilateral side of the brain was homogenized in lysis buffer (2 mM ethylenediamine tetraacetic acid (EDTA), 50 mM Tris buffered saline (TBS) (pH 7.4),0.1% Triton X-100 with protease inhibitors cocktail) and centrifuged at 13000 rpm for 15 min at 4ºC. The supernatant was collected, aliquoted, and frozen at −80ºC for further analysis (detergent-soluble fraction of brain Aβ). To obtain

the formic acid soluble fraction of brain Aβ, the pellet was dissolved in 70% formic acid in distilled water and centrifuged as stated above. The supernatant was collected and neutralized with 1 M Tris (pH 11.0) (1:20 dilution), aliquoted, and frozen at −80◦C till use. In the contralateral side, immunohistochemistry was performed. The contralateral side of the brain, used for Aβ immunohistochemistry, was fixed for 24h at 4ºC in 10% neutral buffered formalin and then transferred to a 30% sucrose solution for cryoprotection before cryostat sectioning.

In order to verify the localization of administrated TTR, animals injected using the same methodology with TTR-488 were anesthetized and then perfused with 4% paraformaldehyde (PFA). The brain was removed and postfixed with 4% PFA for 2-4h at 4ºC and then incubated with 30% sucrose solution at 4ºC until equilibrated. The brains were frozen using liquid nitrogen and then kept at -20ºC till use. Sequential 20 μm coronal cryosections were taken on a cryostat and mounted with Vectashield with DAPI. Images were captured at 10x magnification in a fluorescence microscope (Leica DMI 6000B).

### Treated animals

8 days post-TTR administration, the animals were anesthetized and then sacrificed. The hippocampus and the superior portion of the cortex (figure 12) of each side of the brain was dissected and treated independently. The detergent soluble and formic acid soluble Aβ fractions of both the hippocampus and cortex were homogenized as described above.

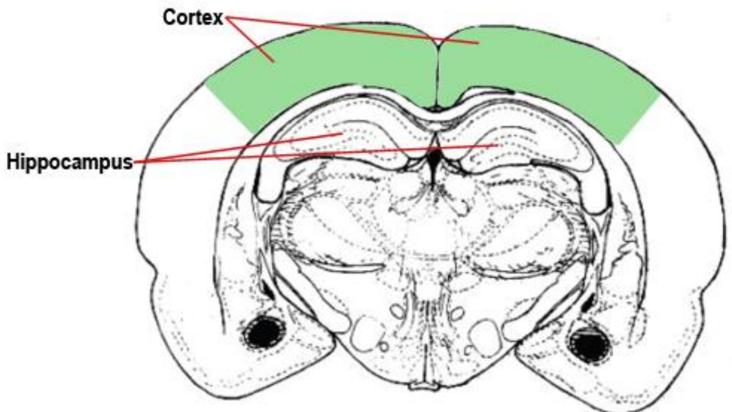


**Figure 12 – Representative image of the superior region of the cortex used for Aβ levels determination (green).**

### *Immunohistochemistry*

Aβ immunohistochemistry was performed as previously described (Ribeiro, Oliveira et al. 2014). Briefly, free floating 30 μm-thick half-brain cryosections were

incubated with 70% formic acid for 15 min at RT in slow agitation. The endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 min. Sections were then incubated with blocking buffer composed by 10% FBS and 0.5% Triton X-100 for 1h at RT, followed by incubation with biotinylated 6E10 primary antibody (1:750 in blocking buffer; Covance Research Products, Inc.) O/N at 4ºC in slow agitation. After the incubation with Vectastain® Elite ABC Reagent (Vector Laboratories), sections were developed with diaminobenzidine (DAB;Sigma) and mounted on slides coated with 0.1% gelatin. An Olympus BX50 light microscope was used in the analysis of the sections.

### $A\beta_{42}$ Enzyme-Linked Immunosorbent Assay (ELISA)

Aβ levels were evaluated by ELISA assay using Human $A\beta_{42}$ chromogenic-based kit (Invitrogen). Firstly, $A\beta_{42}$ standards were reconstituted with Standard Reconstitution buffer to 1 µg/ml. Cell supernatants from N2A-APPswe cells and brain samples from AD mice were then diluted in standard diluent buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) to avoid proteolysis of Aβ peptides. All samples were incubated with the detection antibody solution for 3h at RT or O/N at 4ºC. After the washing steps, samples were incubated with an anti-rabbit IgG horseradish peroxidase (HRP) working solution for 30 min, which was replaced by the stabilized chromogen (30 min at RT and in the dark). Finally, stop solution was added and the absorbance at 450 nm was read using a plaque reader (Thermo Scientific). Total protein quantification was performed using Lowry's method (DC Protein Assay Kit, BioRad).

### Zebrafish experiments

#### *Zebrafish models*

The transgenic zebrafish line, which expresses GFP in the endothelial cells (referred as Veins), and WT zebrafish were kindly provided by José Bessa (VDR group, IBMC). The transgenic line Veins is an unpublished reporter line that was generated by a random mobilized transposon, described by Bessa and colleagues, containing an enhancer trap cassette (Bessa, Luengo et al. 2014).

Zebrafish embryos were generated by synchronized natural pair-wise mating of adult fish and were used at single cell stage, 12 somites (14 hpf) and 24 hpf (Table 2), depending on the experimental procedures as described below. Embryos were grown at 28ºC in 0.003% 3-phenyl-thiourea diluted in E3 medium (PTU medium; used to inhibit pigmentation). Veins were generated by out-crossing female transgenic animals with WT males.

**Table 2 – Zebrafish stages of development at 28,5ºC.**

| PERIOD | STAGES | HOURS POST-FERTILIZATION | REPRESENTATIVE IMAGE |
|---|---|---|---|
| Blastula | Single cell | 0 | |
| Segmentation | 12 somites | 14 | |
| Pharyngula | Prism 5 | 24 | |
| Larval | Protruding-mouth | 72 | |

### *Cell and yolk injections*

Single cell stage WT embryos were microinjected in the yolk and in the cell with 5nl of 5, 10, 25, 33, 80, 200 and 500 μM Aβ peptide diluted in injection solution (10% phenol red 0.01 μg/μl IgG and in PBS). 10% and 25% DMSO diluted in PBS were used as control for the higher Aβ concentrations (200 and 500 μM, respectively). 10 μM TTR WT was also injected in the yolk of single cell stage WT embryos. Embryo viability was assessed after 24h.

### *Western Blot*

To detect Aβ by Western Blot, two protocols were tested for the preparation of tissue extracts. In the first protocol, 24h post-injection, embryos were de-chorionated and their yolk disrupted by pipetting up and down in Ginsburg Fish Ringers solution (111 mM NaCl, 3,6 mM KCl, 2,7 mM $CaCl_2$ and 1,9 mM $NaHCO_3$), followed by slow agitation (1100 rpm) for 5 min at RT and centrifugation (500 g) for 5 min at 4ºC. After removing the

supernatant, embryos were washed with ice-cold PBS and centrifuged at the same conditions. The pellet was then resuspended in sample loading buffer (SLB) (40 μl of SLB per 50 embryos) and kept at -20ºC till use. Samples were thawed and heated at 100ºC for 5 min and centrifuged at 13000 rpm for 5 min.

In the second protocol, the above procedure was performed with slight variations: i) the addition of 0,3 mM PMSF to the Ginsburg Fish Ringers solution and ii) the homogenization of the embryos with extraction buffer (10 mM Tris pH 7.4, 2% Triton X-100, 1 mM PMSF and protease inhibitors cocktail in water; 1,5 μL per embryo). Then, the samples were frozen in liquid nitrogen, centrifuged at 13000 rpm for 20 min at 4ºC and the supernatants kept at -80ºC till use.

Samples (corresponding to 25 and 30 embryos in the first and second protocols, respectively) were run in a 15% SDS-PAGE gel and then transferred to a nitrocellulose membrane (0.45 mm; GE HealthCare). The transference was performed in a wet system using Tris-Glicine buffer with 20% methanol at 25 V, O/N at 4ºC with magnetic agitation. The membrane was washed with deionized water, followed by a wash with TBS and then with TBS-T (TBS with 0,05% Tween). Subsequently, the membrane was incubated with blocking solution (5% milk (Sigma) in TBS-Tween) for 1h at RT. The membrane was incubated with anti-Aβ (1:1000; Sigma) or anti-TTR primary antibodies (1:1000, DAKO) in 5% milk for 1h at RT. The membrane was washed 3 x 10 min in TBS with 0,05% Tween and then incubated with HRP conjugated anti-mouse (1:3000, Santa Cruz Biotechnology) or anti-rabbit secondary antibodies (1:3000, Santa Cruz Biotechnology) diluted in 3% milk in TBS-T for 1h at RT. Immunodetection was performed by chemiluminescence using ECL reagent (Bio-Rad).

***Brain intraventricular TTR injection***

At 24hpf, WT embryos had their chorion manually removed. Embryos were anesthetized with tricaine (Sigma) and placed in an agar coated petri dish. Under the stereomicroscope, the embryos were injected in the ventricle using a micromanipulator attached to an injector pump with 5 nl of: i) TTR-488 (0,63 μg/μl); or ii) Aβ peptide at either 200 μM or 500 μM. Images were captured after 24h in a fluorescence stereomicroscope at 4x magnification.

***Angiogenesis assay and Aβ peptide treatment***

The assay was performed according to a previous study (Lu, Liu et al. 2014). 5 μM Aβ peptide diluted in PTU medium was administrated to Veins embryos at the developmental stage of 12 somites until 72hpf. The solution was renewed every 24h. Control embryos were maintained in 0.25% dimethyl sulfoxide (DMSO) in PTU. 72hpf

treated larvae (Table 2) were anesthetized with tricaine (stock solution 4g/l) and observed at the fluorescent stereomicroscope at 9x magnification. The area of the subintestinal vessels that extend into the yolk region of 3 embryos per condition was examined using Fiji software.

#### *Acridine orange immunohistochemistry*

Embryos were dechorionated at 72hpf and placed in 5 μg/ml of the vital dye acridine orange (Molecular Probes) for 5 min. For visualization in the fluorescent stereomicroscope at 9x magnification, embryos were anesthetized with tricaine.

### Statistical Analysis

Results are presented as means ± SEM or means ± SD. All analyses were calculated with Student's t-test test by using GraphPad Prism Software: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

### TTR proteolytic activity is required for Aβ clearance in a cell based system

In order to assess the proteolytic activity of the TTR forms used in the following experiments, the fluorogenic Abz-VHHQKL-EDDnp peptide was incubated either with TTR WT or TTR H90A. As seen in figure 13, while TTR WT is able to cleave Aβ, TTR H90A is not, demonstrating that TTR H90A, that will be used in the following experiments, is proteolytic inactive.

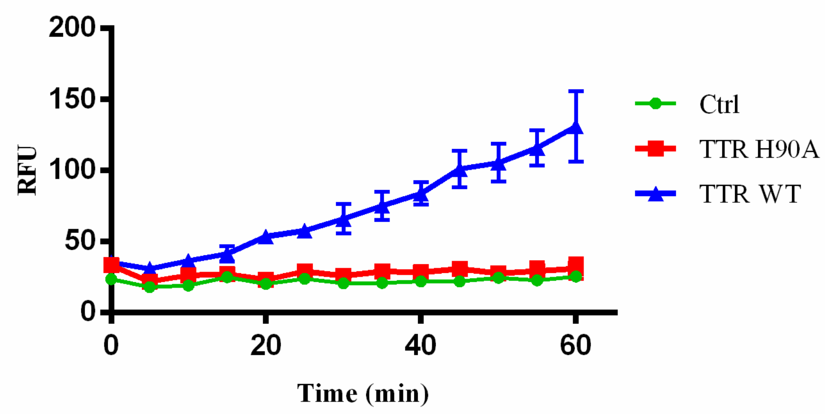


**Figure 13 – Assessment of TTR proteolytic cleavage of the fluorogenic peptide Abz-VHHQKL-EDDnp.** TTR WT is able to cleave the fuorogenic peptide while TTR H90A is not. (Ctrl - Abz-VHHQKL-EDDnp peptide; TTR H90A - Abz-VHHQKL-EDDnp incubated with TTR H90A; TTR WT - Abz-VHHQKL-EDDnp peptide incubated with TTR WT)

TTR was previously shown to be able to cleave Aβ *in vitro* (Costa, Ferreira-da-Silva et al. 2008). To investigate if this effect was maintained in a cell-based system, N2A-APPswe cells that carry the Swedish mutation of APP and secrete high levels of Aβ peptide were incubated with either TTR WT or TTR H90A.

N2A-APPswe cells treated with TTR WT showed 2,4-fold reduction Aβ levels (figure 14). Addition of TTR H90A had no effect on Aβ levels when compared to control cells, showing that the decreased Aβ levels after treatment with TTR WT are related with proteolysis (figure 14). These results show that TTR proteolytic activity is maintained in a physiological environment and impacts on Aβ clearance.

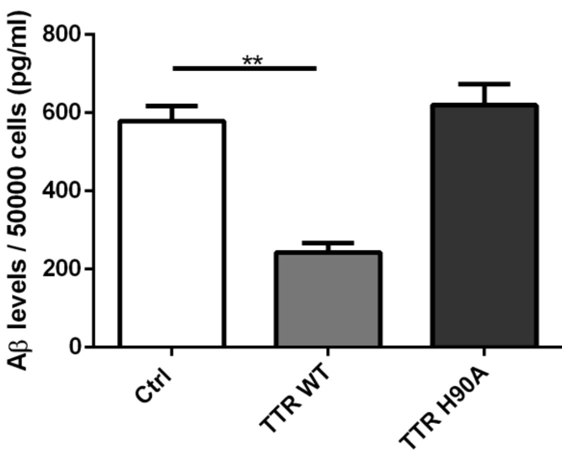


**Figure 14- Analysis of Aβ clearance induced by either TTR WT or TTR H90A using N2A-APPswe cells.** The incubation of N2A-APPswe cells with TTR WT lead to decreased Aβ levels while the incubation with TTR H90A had no effect. Statistical analysis: Results showed as means ± SEM. Student's t-test - *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

## Aβ-induced death of hippocampal neurons is decreased by TTR proteolysis

Hippocampal neurons have been widely used as a cellular model of Aβ-induced neurotoxicity, since these neurons are the most affected in AD (Hu, He et al. 2015). To evaluate the effect of TTR proteolytic activity on neuronal cell death induced by Aβ oligomers, mouse hippocampal neurons were incubated with 10 μM Aβ oligomers plus either TTR WT or TTR H90A.

To assure that the production of Aβ oligomers was achieved, electronic microscopy analysis was performed and, in fact, the incubation of Aβ at the referred above conditions successfully produced Aβ oligomers (figure 15A). The cytotoxic effect was evaluated using caspase-3 assay.

In hippocampal neurons, Aβ oligomers induced neurotoxicity as suggested by the increased levels of caspase-3 activity (figure 15B). TTR WT was able to decrease in 28% the toxic effect induced by Aβ oligomers. However the TTR-induced neuroprotective effect was not reproduced with the addition of TTR H90A (figure 15B). These results show that only proteolytically active TTR is able to reduce Aβ-induced cell death, suggesting that TTR proteolytic activity is required for its neuroprotective effect in AD. Although the two TTR forms increased caspase-3 activation when comparing with the control, the levels were still below the ones of Aβ oligomers. The induction of cell death might be justified by the partial aggregation ability retained by these variants (figure 15B).

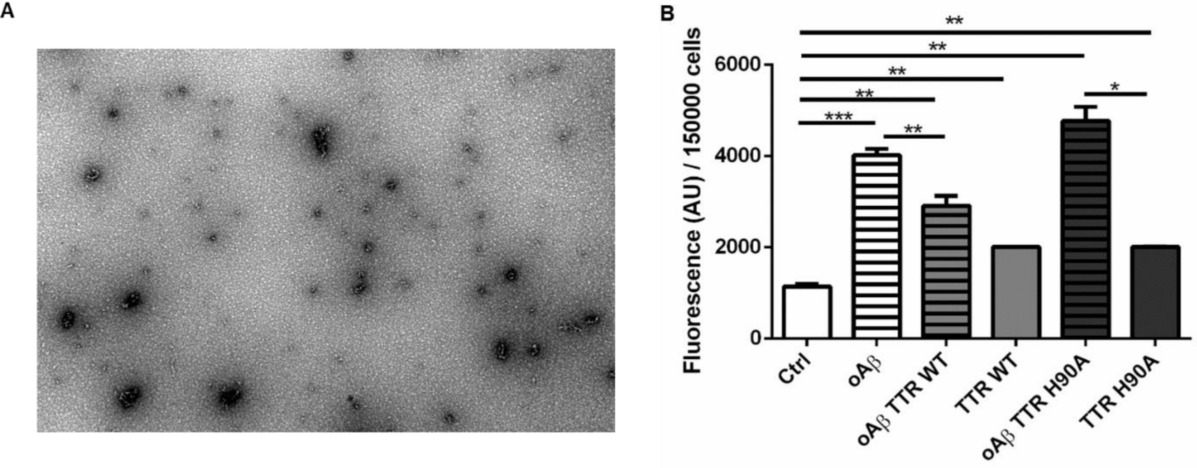


**Figure 15 - TTR neuroprotective effect on Aβ oligomers induced neurotoxicity is dependent on TTR proteolytic activity. (A)** TEM analysis of Aβ oligomers. **(B)** Caspase-3 assay showed that TTR WT was able to decrease the neurotoxic effect caused by Aβ oligomers on hippocampal neurons while TTR H90A had no effect. Statistical analysis: Results showed as means ± SEM. Student's t-test - $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

## TTR intracerebral administration has no effect on Aβ levels of AD mice

AD/TTR mice were intracerebraly administrated with TTR variants in order to test TTR proteolytic activity *in vivo*. In this animal model, Aβ deposition starts at 6 months of age and females present higher brain Aβ levels (Oliveira, Ribeiro et al. 2011). We started by confirming the brain localization of injected TTR-488. As represented in figure 16A, TTR was localized in the hippocampus only in the ipsilateral side. However, accumulation of TTR-488 close to the injection site was also verified (figure 16B).

In the pilot tests, 15 and 16 months old AD/TTR$^{-/-}$ female mice were injected in the right hippocampus using an osmotic pump previously filled with either PBS or TTR WT. After 8 days, animals were sacrificed and both detergent soluble and formic acid soluble Aβ levels of the ipsilateral hemisphere were analyzed. As one PBS injected animal died, we were able to compare only one control with two TTR-treated females. Both TTR-treated animals presented reduced Aβ levels when compared to the control, being the effect more striking in the detergent soluble fractions (figure 17A). However, a high variability within the TTR treated group was observed, what could be related to the one month of age difference between the two animals. To clarify whether differences were related to TTR administration we performed Aβ immunohistochemistry in the contralateral hemispheres to visualize the basal levels of Aβ plaques. We observed similar differences between animals in the contralateral side as the ones observed in the ipsilateral side (figure 17B). As demonstrated in figure 16A, administrated TTR has no access to the contralateral side what shows that the results determined in the ipsilateral side were not related to TTR effect

but to an intrinsic variability in Aβ deposition between animals, which was observed even in animals with the same age.

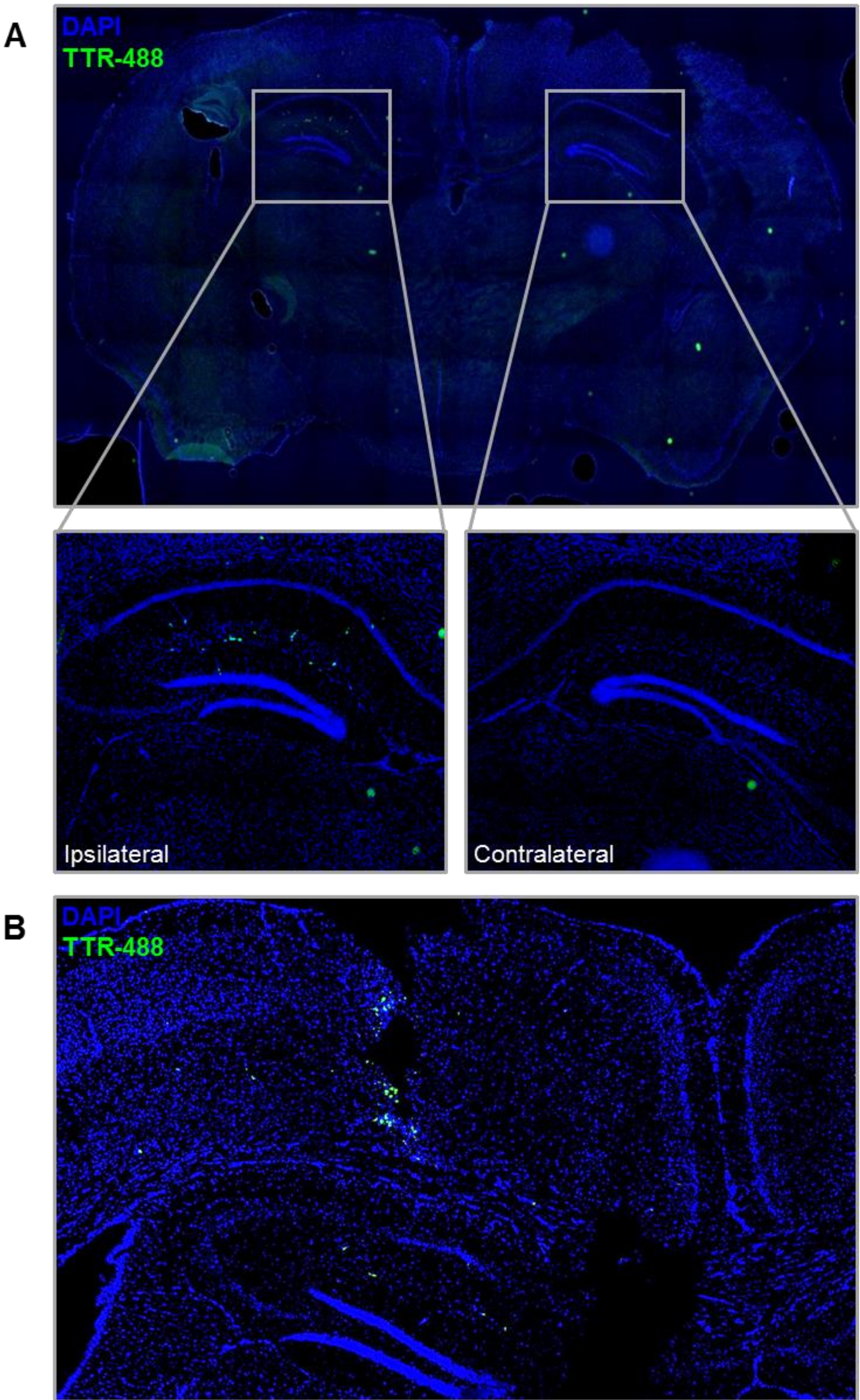


**Figure 16 – Administrated TTR localizes in the hippocampus but is also retained at the site of injection.** After intracerebral injection, labeled TTR was found in the ipsilateral hemisphere but not in the contralateral one **(A)** and also at the site of injection **(B).**

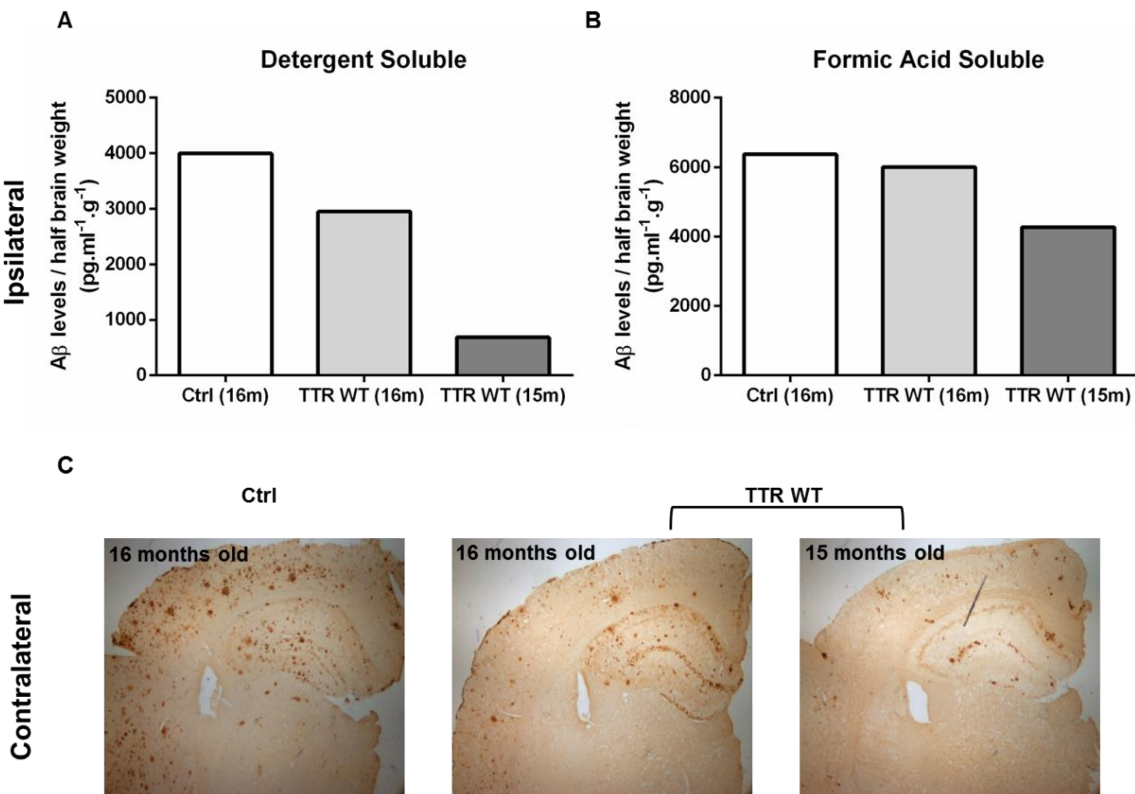


**Figure 17 –AD/TTR$^{-/-}$ female mice present a high variability on Aβ levels and plaque burden.** Half brain detergent soluble **(A)** and formic acid soluble Aβ levels **(B)** and representative images of Aβ plaque burden **(C)** of AD/TTR$^{-/-}$ female mice at different ages (15 and 16 months old) after intracerebral injection of either PBS (control) or TTR WT.

Although no results were obtained in the pilot studies, we followed by performing the experiments to compare the effect between WT TTR and TTRH90A but with some modifications. Since the animals used in the pilot studies presented a very high amount of plaques especially localized in the superior cortex and hippocampal regions (figure 17C) which would interfere with the proper analysis of the results (slight variations could be lost), younger animals of 9 months of age were used in the following experiments. AD/TTR$^{+/+}$ mice were also used in order to mimic the natural occurring genotype of AD patients. Furthermore, the hippocampus and the superior cortex were dissected for the determination of Aβ levels in order not to lose slight variants that might have occurred in these regions.

9 months old AD/TTR$^{+/+}$ female mice were injected with either TTR WT or TTR H90A, and Aβ levels for both detergent soluble and formic acid soluble fractions were analyzed by ELISA. Although the contralateral side was considered as an intrinsic control, non-injected mice were also used as controls. The results obtained for both brain regions clearly showed an enormous variation between animals within the same experimental group (figure 18) and also between the two hemispheres of the same animal for the control group (data not shown). Regarding the hippocampus, although both TTR WT and TTRH90A administration seemed to induce a tendency to a decrease in the detergent

soluble Aβ levels comparing with the controls, similar levels were observed between the ipsilateral and contralateral sides discarding the hypothesis of being a TTR-induced effect (figure 18A). For the formic acid soluble Aβ levels, variations were observed between the ipsilateral and contralateral sides of the control animals. Moreover, neither TTR WT nor TTRH90A showed an effect (figure 18B).

Regarding the cortex, both the detergent and formic acid soluble fractions of TTR WT and TTR H90A treated mice showed no differences on Aβ levels between the contralateral and the ipsilateral side (figure 18C and 18D). Moreover, no differences were observed when compared to the control group.

Both in the hippocampus and in the cortex we observed increased levels of the formic acid soluble Aβ fractions when compared to the detergent soluble ones, in all the experimental conditions. These results suggest that the brains of these animals are richer in plaques than in less aggregated forms.

Since no conclusions can be taken out of TTR WT and TTR H90A administration, one cannot infer with certainty if TTR neuroprotective effect in AD is related to its proteolytic activity *in vivo* using this methodology.

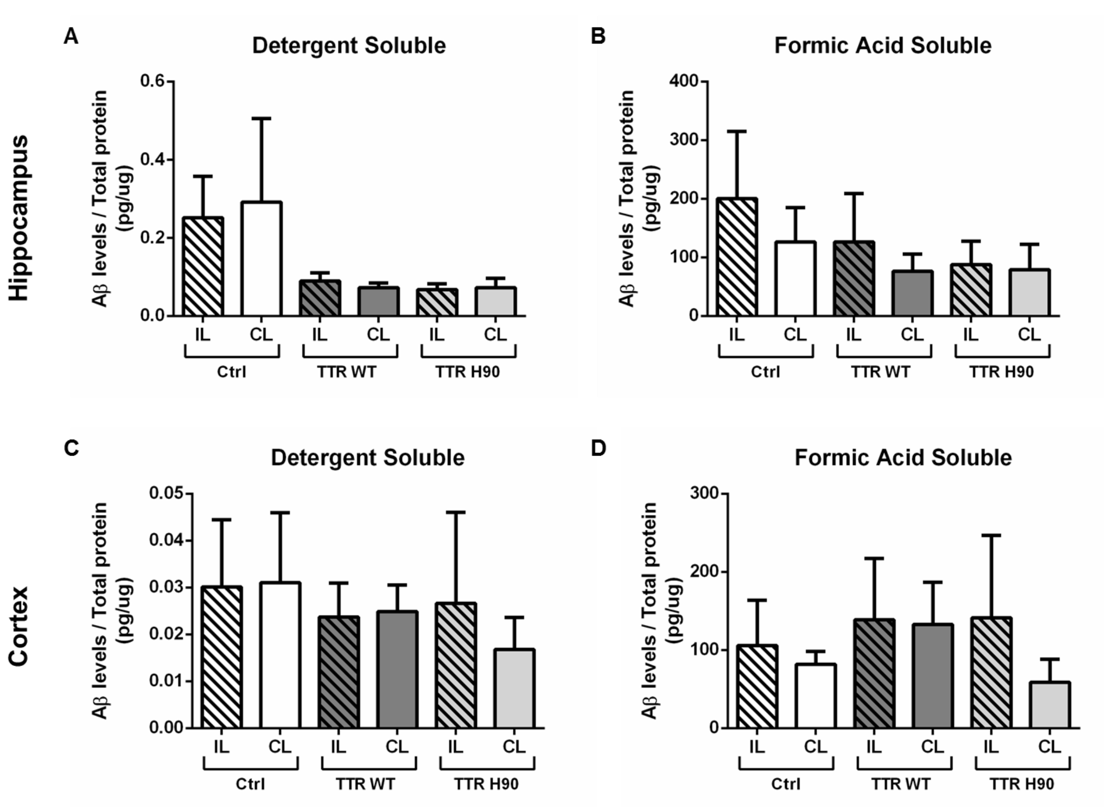


**Figure 18 – 9 months old AD TTR$^{+/+}$ female mice show a high variability on both hippocampus and cortex Aβ levels of after TTR treatment.** Aβ levels of 9 months old AD TTR$^{+/+}$ females that suffered intracerebral injections of either TTR WT or TTR H90A (ctrl – non-injected animals). Both the cortex and hippocampal Aβ levels for both detergent soluble (A and C) and formic acid soluble (B and D) fractions showed to be highly different between animals. Results showed as means ± SD. (Ctrl: n=2; TTR WT: n=4; TTR H90A: n=3)

### Evaluation of Zebrafish as a valuable tool to test TTR proteolytic activity *in vivo*

In order to further evaluate if TTR proteolytic activity is maintained in an *in vivo* system, 10 μM Aβ and/or 10 μM TTR WT injections in the yolk or the cell of single cell stage WT zebrafish embryos were performed. Two different protocols for protein extraction were tested but, although TTR was successfully identified (figure 19A), Aβ was not detected (data not shown). ELISA assay and immunohistochemistry against Aβ were also tested and neither of these methodologies worked (data not shown). To verify which Aβ concentration could be reached (in order to assure Aβ detection) without inducing an increase in embryo death, a viability curve was also performed, demonstrating that embryos were still viable at 200 μM Aβ (figure 19B).

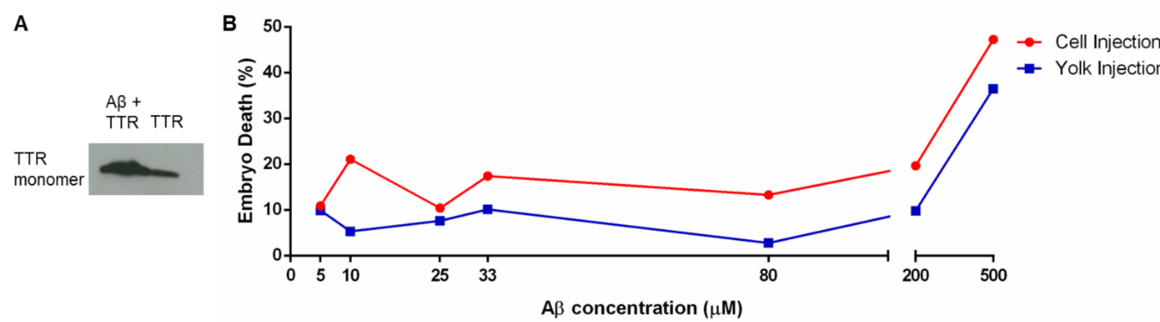


**Figure 19 – TTR WT is detectable through Western blot and WT zebrafish embryos remain viable until 200μM Aβ injection. (A)** TTR is detectable through Western blot analysis after injection in the yolk of WT zebrafish embryos; **(B)** WT zebrafish embryos were injected with different Aβ concentrations both in the yolk (blue line) and in the cell (red line) and 500 μM Aβ showed to be the most toxic to WT zebrafish embryos via both type of injections.

In the following experiments, we decided to move to a more relevant model that better mimic the natural occurring situation: the existence of Aβ and TTR in the brain. Recently, Nery et al described that intraventricular injections of Aβ were able to induce cognitive defects in zebrafish, proposing this model as a good platform for the study of AD pathology in zebrafish (Nery, Eltz et al. 2014). In order to overcome the limitations found regarding Aβ detection, we used the reported methodology to inject the ventricle of 24hpf WT zebrafish embryos with 200 μM Aβ. Although the concentration used was twenty times higher than the one reported in the referred paper, after western blot analysis, Aβ was still not detected (data not shown). Since we are able to detect TTR by western blot, in parallel, 24hpf WT zebrafish embryos were injected in the brain ventricle with TTR-488 in order to verify where TTR localizes when injected in this animal model. TTR seemed to travel inside the embryo and accumulated in the endothelial cells (figure 20). Furthermore, no morphological changes were observed after intraventricular injection in any of the above mentioned experiments.

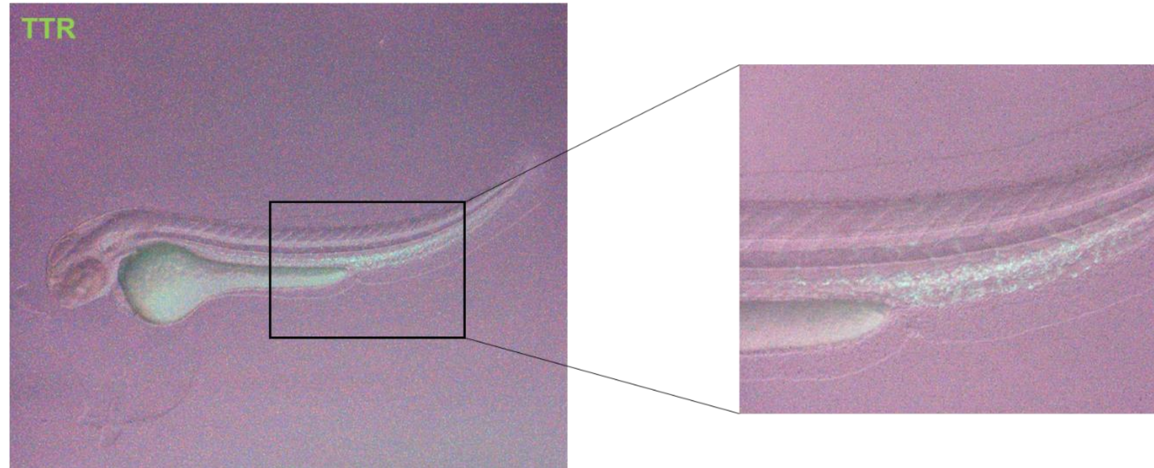


**Figure 20 - TTR injected in the ventricle is mislocated into the endothelial cells.** WT zebrafish embryos were intraventricularly injected with TTR-488 and presented a mislocation of TTR into the endothelial cells.

We followed by using a second model where Aβ was shown to induce a striking effect in zebrafish. We reproduced a previous reported methodology in which zebrafish embryos that express GFP in the vasculature (named Veins) are maintained in PTU medium supplemented with 5 μM Aβ (Aβ medium) every 24h. In this study, the vasculature of 72hpf embryos maintained in Aβ medium is reduced (Lu, Liu et al. 2014). As seen in figures 21A and 21B, Aβ exposed Veins embryos showed a 2,55 fold decrease in the vasculature area. Furthermore, the induction of apoptosis was also tested using acridine orange staining but no differences between the control and the Aβ exposed Veins embryos were found (figure 21C). Besides the reduction of the vasculature, no other morphological alterations were visible. Nevertheless, these results support the use of this procedure as a platform for the study of TTR proteolytic activity *in vivo* and should be used in the future.

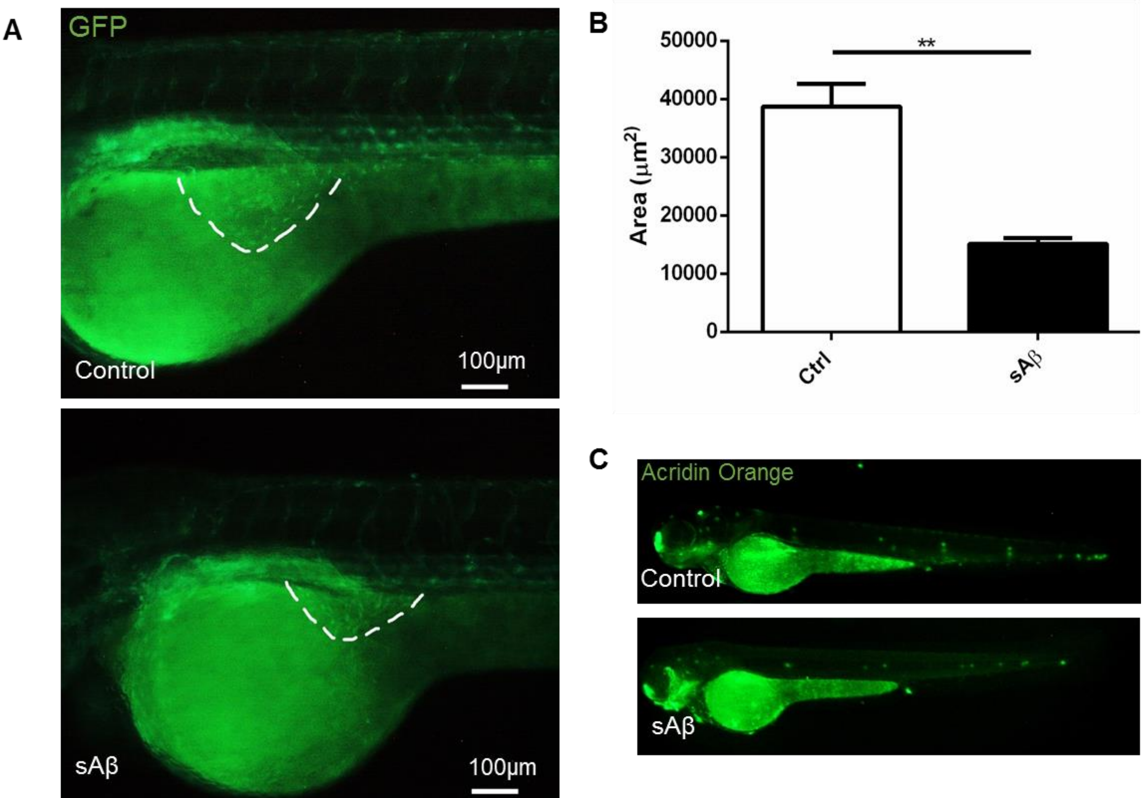


**Figure 21 - Aβ exposure leads to a reduction in the area of the vasculature of Veins zebrafish embyros, but does not induce cell death. A and B-** 72hpf Veins zebrafish embryos exposed to 5 μM soluble Aβ showed a 2,55 fold decrease in the area of the vasculature (p=0.0042). **C -** 72hpf Veins zebrafish embryos exposed to 5 μM soluble Aβ didn't present apoptotic staining when comparing with the control situation. Statistics: Results showed as means ± SEM. Student t-test - *p < 0.05, **p < 0.01, ***p < 0.001

## DISCUSSION

Aβ accumulation is considered the major pathological change in AD progression. In this respect, therapeutic strategies targeting Aβ degradation, by delivery of Aβ-degrading enzymes to the brain, are emerging as a valuable option for treating AD. TTR is a metalloprotease described as being able to cleave the Aβ peptide (Costa, Ferreira-da-Silva et al. 2008). Moreover, *in vitro* data from our group demonstrated that TTR WT, but not the proteolytically inactive form of the protein, is capable of interfering with Aβ fibrillization by both inhibiting and disrupting fibril formation (unpublished data). These results suggested TTR proteolysis of Aβ as a protective mechanism in AD. However this hypothesis based on *in vitro* results was lacking support from *in vivo* data. In this work, we evaluated the role of TTR proteolytic activity in AD in cell based assays, using both cell lines and primary neuronal cultures, and *in vivo* using mice and zebrafish as animal models.

We started by confirming TTR cleavage of Aβ in physiological conditions by using N2A-APPswe cells, a CNS cell line that stably expresses APP Swedish mutation producing high levels of Aβ peptide. Besides further confirming TTR cleavage of Aβ, this cell based assay also demonstrated that TTR proteolysis contributes to Aβ clearance. Ongoing experiments are being currently performed to analyze the effect of TTR WT and TTR H90A in a reported model in which differentiated THP-1 cells incubated with Aβ induce the formation of congo-red positive Aβ plaques (Friedrich, Tepper et al. 2010). This experiment will confirm if TTR proteolytic activity is required for the clearance of higher aggregated Aβ species under physiological conditions. In order to access the effect of TTR proteolysis on Aβ-induced cytotoxicity, we used primary cultures of hippocampal neurons, a clinically relevant cell type in AD. We demonstrated that TTR WT was able to decrease Aβ oligomers-induced caspase-3 activation, while TTR H90A had no effect. Nevertheless, TTR WT did not promote a complete rescue of cytotoxicity. These results might be related with the reported low activity of the protein (Liz, Leite et al. 2012). Moreover, we determined that both TTR forms induced neurotoxicity in the absence of Aβ oligomers. These results that might result from TTR aggregation might be affecting the effect in the presence of Aβ oligomers. In summary, the cell based assays further confirmed TTR cleavage of Aβ in physiological conditions demonstrating that TTR proteolytic activity is required for its neuroprotective effect in AD, by degrading Aβ and decreasing Aβ-induced neurotoxicity. These results prompted us to analyze the effect of TTR proteolysis *in vivo*.

To dissect the effect of TTR proteolytic activity in AD pathology *in vivo*, intracerebral unilateral administration of either TTR WT or TTR H90A were performed in

an AD mice model. Although we were unable to detect any effect of either TTR WT or TTR H90A in the levels of $A\beta_{42}$ in our model, we observed a high variability between animals of the same experimental group and even between the two brain hemispheres of the same animal (in control animals). This variability was previously observed in the reported characterization of the model used (Oliveira, Ribeiro et al. 2011) and we consider it might have affected our results. Therefore, no conclusions about TTR proteolytic activity in AD pathology *in vivo* can be taken out of these experiments. Nevertheless, the lack of an effect induced by TTR might by related with several facts: i) low activity of the protease *in vivo*; ii) reduced amount of enzyme in affected regions; iii) a putative aggregation of TTR *in vivo* blocking its effect; and iv) high Aβ content that hamper the detection of slight variations.

Previously reported studies showed that the overexpression of human TTR in AD mice had beneficial effects both at behavioral and biochemical levels (Buxbaum, Ye et al. 2008). To overcome the limitations above described, AD mice overexpressig both TTR variants could be developed in order to study the effect of TTR proteolysis in AD pathology *in vivo*. In this sense, the use of adeno-associated virus serotype 9 (AAV9) would be advised since this system was demonstrated to be successful in the transduction of the hippocampal tissue (Klein, Dayton et al. 2008) and has several advantages regarding longer term expression and safety (Simonato, Bennett et al. 2013). In these mice, the determination of Aβ levels and plaque burden and behavioral tests should be performed in order to understand if the TTR proteolytic activity influences the development of the disease. The use of this methodology is supported by previously a reported study for NEP in which NEP treated animals presented a significant decrease in congophilic-stained plaques in the hippocampus (Carty, Nash et al. 2013).

Since we couldn't take any reliable conclusions from AD mice experiments, we decided to use zebrafish as an *in vivo* system for the study of TTR proteolytic activity due to its unequaled advantages (Martin-Jimenez, Campanella et al. 2015). Injections of Aβ with or without TTR WT were performed in the yolk and cell of WT single cell stage zebrafish embryos. Since Aβ detection was not achieved by neither of the methods used (immunohistochemistry, western blot and ELISA), we decided to use a published and more relevant methodology in which a brain intraventricular injection of Aβ was shown to induce cognitive defects on zebrafish WT embryos and, although the concentration used was twenty times higher than the one reported, we were still unable to detect Aβ. As such, we did not pursue with the analysis of TTR proteolysis in the referred model. Nevertheless, in the referred paper, Aβ detection was not performed, being the most relevant result the induction of cognitive dysfunction by Aβ injection. Therefore, further studies should verify the induction of cognitive defects under the conditions here tested. Additionally, in order

to verify TTR localization after intraventricular injection, 24hpf WT zebrafish embryos were injected with TTR-488 using the same methodology. Labelled TTR diffused inside the animal and localized in the endothelial cells, what might be related to the lack of a functional blood-brain barrier at this stage (Fleming, Diekmann et al. 2013).

In order to overcome the constraints faced, Veins zebrafish embryos were incubated with Aβ and the gut vasculature was analyzed, a model previously described for Aβ detrimental effects (Lu, Liu et al. 2014). Treated animals showed a significant decrease in the area covered by the gut vasculature when comparing with controls but no differences were found in the cell viability assay. These results show that the reporter line Veins might be a valuable tool in the study of TTR proteolytic activity *in vivo*. Being that, further studies in which this line is injected in the brain ventricle either with TTR WT or TTR H90A and is then submitted to Aβ exposure are currently ongoing.

Additionaly, the development of an AD zebrafish model overexpressing either human TTR WT or TTRH90A would be a valuable option. Comparing with mice, zebrafish has great advantages: it allows generation of a sufficient number of subjects in less time and with less associated-costs and since it is a simpler animal model, less variants would interfere with the expected outcome. Since zebrafish WT PS1 is able to cleave APPswe producing high amounts of $A\beta_{42}$ (Leimer, Lun et al. 1999), it would be interesting to generate an APPswe transgenic zebrafish that expressed the mutant in the nervous system throughout life. To do so, the APPswe gene sequence governed by the expression of the HuC promoter which drives gene expression to the CNS (Higashijima, Hotta et al. 2000) could be inserted into a Tol2 transposable element (Kawakami, Shima et al. 2000). After characterization of the model, this line could be crossed with TTR transgenic zebrafish, a line that could be generated by the same methodology, but using the FABP10 promoter to direct gene expression into the hepatocytes (Mudbhary, Hoshida et al. 2014). Aβ levels, cytotoxicity and cognitive defects in this model could be addressed to further reinforce the data here presented for the role of TTR proteolytic activity *in vivo*.

In summary, the results here presented support the importance of TTR proteolytic activity in a neuroprotective context. If TTR activity will be relevant for preventing the development of AD *in vivo*, the modulation of TTR proteolysis might constitute a valuable therapeutic strategy.

# CHAPTER 3

## NEUROCYTOSKELETON REMODELING AS A CONSEQUENCE OF PROTEIN AGGREGATION

## THEORETICAL BACKGROUND

Several neurodegenerative diseases occur as a consequence of the deposition protein aggregates. Although being a hallmark in neurodegeneration, the actual role of protein aggregation in disease progression remains unclear. Alzheimer's disease (AD) and Parkinson's disease (PD) are two major examples of central nervous system (CNS) disorders resultant from protein aggregation. AD is associated with extracellular deposits of amyloid-β peptide (Aβ) in senile plaques and accumulation of hyperphosphorylated tau proteins in neurofibrillary tangles leading to progressive cognitive decline (Castellani, Rolston et al. 2010). PD is typically characterized by motor impairment, in which the key pathological hallmarks are the formation of cytoplasmic inclusions composed of α-synuclein, known as Lewy Bodies, and the loss of dopaminergic neurons (Galloway, Mulvihill et al. 1992). In the peripheral nervous system (PNS), familial amyloid polyneuropathy (FAP) is a typical Portuguese case of protein aggregate-induced neuropathy. FAP is characterized by the deposition of insoluble mutated transthyretin (TTR), in the form of oligomers, aggregates and amyloid fibrils, particularly in the PNS (Plante-Bordeneuve and Said 2011).

Recent studies have revealed that defects on the neuronal cytoskeleton and axonal transport are associated with several neurodegenerative disorders. AD represents the leading disease where cytoskeleton damage has been addressed mainly because of the involvement of tau, a microtubule stabilizer. Moreover, Aβ oligomers have been shown to mediate neurotoxicity by inducing major alterations in both microtubules and actin dynamics (Mendoza-Naranjo, Gonzalez-Billault et al. 2007, Ramser, Gan et al. 2013, Pianu, Lefort et al. 2014).

Considering the effect of Aβ oligomers in the neuronal cytoskeleton, it would be important to determine whether cytoskeleton alterations induced by protein aggregates constitute a common pathogenic mechanism among different neurodegenerative disorders. In fact, cytoskeleton defects have been also suggested in PD (Cartelli, Casagrande et al. 2013, Hunn, Cragg et al. 2015). Although dopaminergic neurons are the main neuronal population affected in PD, brain immunohistochemical analyses from PD patients showed a reduction in hippocampal cholinergic activity and also Lewy bodies deposition in the basal forebrain and hippocampus (Hall, Reyes et al. 2014). In this respect, both monomeric and aggregated forms of α-synuclein were shown to be secreted by neurons (Marques and Outeiro 2012). α-synuclein secretion could explain the referred pathology spreading in PD, and suggest an extracellular pathologic role for α-synuclein, which might be related to cytoskeleton remodeling. Nevertheless, the effect of α-synuclein oligomers on the cytoskeleton of hippocampal neurons was not previously assessed.

Concerning FAP, unpublished data from our group showed that there is a disruption of the normal growth cone morphology of the peripheral dorsal root ganglia neurons induced by TTR oligomers. A recently published paper has also suggested that there is a CNS involvement in FAP pathology (Maia, Magalhaes et al. 2015). The report disclosed that patients who carry the TTR V30M mutation and had liver transplant show clinical CNS disturbances that might be related to TTR V30M accumulation in the CNS. However, the role of TTR variants in CNS neurons was still not addressed.

In this work we aimed to characterize the effect of different prone-to-aggregate proteins in the neuronal cytoskeleton of a common cell type. We used as read-out the growth cone morphology of hippocampal neurons and the effect of the following species was analyzed: i) Aβ oligomers, untreated or treated with TTR, to determine whether TTR reverts cytoskeleton defects induced by Aβ oligomers; ii) α-synuclein in different aggregations stages and iii) TTR amyloidogenic mutants.

## MATERIALS AND METHODS

### Preparation of protein aggregates

Aβ oligomers were produced as referred in chapter 2, section 2. For this purpose, 10 µM Aβ diluted in F12 medium (Lonza) was incubated for 3 days at 4ºC, pH 7. The three different TTR forms (WT, V30M, L55P) were produced as stated in chapter 2, section 2 and were incubated for 2-4h at RT, pH 7 to produce aggregated forms. α-synuclein monomers, oligomers and fibrils were kindly provided by Dr. Hugo Vicente Miranda from The Chronic Diseases Research Center (Lisbon, Portugal).

### Hippocampal neurons culture

The hippocampal neurons culture were performed as referred in chapter 2, section 2. For the analysis of growth cone morphology, cells were plated at a density of 10000 cells per well in coverslips previously coated with 20 µg/ml poly-L-lysine (Sigma). After 3 DIV, cells were incubated O/N, 37ºC with the referred protein species: 10 µM Aβ oligomers; 5 µM of each TTR variant; 500 nM α-synuclein of each aggregated forms.

### Immunocytochemistry

After fixation with Super Phem buffer (4% PFA, 4% sucrose (Merck), 300 mM PIPES (Merck), 125 mM HEPES (Sigma), 50 mM EGTA (Sigma), 10 mM magnesium chloride (Merck) in water) for 15min, cells were permeabilized with 0,2% Triton X-100 (Sigma) for 5 min at RT and were then incubated with 200 mM ammonium chloride (Merck) for 5 min to quench the endogenous fluorescence. Blocking buffer solution, composed of 2% FBS (Invitrogen), 2% bovine serum albumin (Sigma) and 0,2% fish gelatin in PBS, was added and cells were incubated for 1h at RT. Cells were then incubated for 1h at RT with primary antibody mouse βIII tubulin (1:2000, Promega) in 10% blocking buffer. Cells were incubated with the secondary antibody anti-mouse alexa 488 (1:1000, Life Technologies) and phalloidin conjugated with alexa 568 (1:100, Life Technologies) in 10% blocking buffer. Finally, the samples were mounted in Vectashield with DAPI and images were captured by In Cell microscope (GE Healthcare) at 40x magnification.

### Growth cone morphology analysis

Growth cone morphology was analyzed in over 100 polarized cells (neurons with a long, well-defined axon). Growth cones were divided into two classes: normal and dystrophic (Table 3). Also, the relative percentage of polarized cells was assessed.

**Table 3 - Growth cone morphology is divided into two classes: normal and dystrophic.** Axons are double labelled with phalloidin that stains F-actin (red) and with a monoclonal antibody against βIII tubulin (green).

| TYPE | REPRESENTATIVE IMAGE | DESCRIPTION |
| --- | --- | --- |
| Normal | | actin presented both filopodia and lamellipodia;<br>MTs presented a splayed or bundled conformation |
| Dystrophic | | total disorganization of actin and MTs pattern |

## Statistical Analysis

Results are presented as means ± SEM. All analyses were calculated with 1-way ANOVA test, followed by Tukey's multiple comparison tests using GraphPad Prism Software: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

## TTR WT is unable to rescue Aβ oligomers-induced cytoskeletal alterations in the growth cone of hippocampal neurons

The neurotoxic effect of Aβ has been linked to alterations in the neuronal cytoskeleton (see chapter 1, section 3.2.1). Since dystrophic neurites are found in the surroundings of Aβ plaques, we decided to evaluate if Aβ oligomers (the main toxic form in AD) induce alterations in the growth cone morphology of hippocampal neurons. As TTR was suggested as a neuroprotector in AD, we also assessed if TTR WT is able to rescue Aβ oligomers-induced phenotype. The different categories in which growth cones of polarized hippocampal neurons were divided are represented in table 3.

The percentage of polarized hippocampal neurons was unchanged after treatment with either Aβ oligomers or Aβ oligomers plus TTR WT, suggesting that the neurons maintained their natural cellular architecture (figure 22A). As expected, due to the described impact of Aβ oligomers on the neuronal cytoskeleton, analysis of the growth cone morphology of hippocampal neurons showed that Aβ oligomers promoted a 15% increase in the percentage of dystrophic growth cones when comparing with the control neurons (figure 22B). In this phenotype, the growth cone morphology was remarkably altered, with MTs confined to the central zone with little extension into the periphery and lacking the typical lamellipodial actin structures (table 3). The addition of TTR WT did not rescue this phenotype, showing only a 4% decrease in the percentage of dystrophic growth cones when comparing with Aβ oligomers condition (figure 22B).

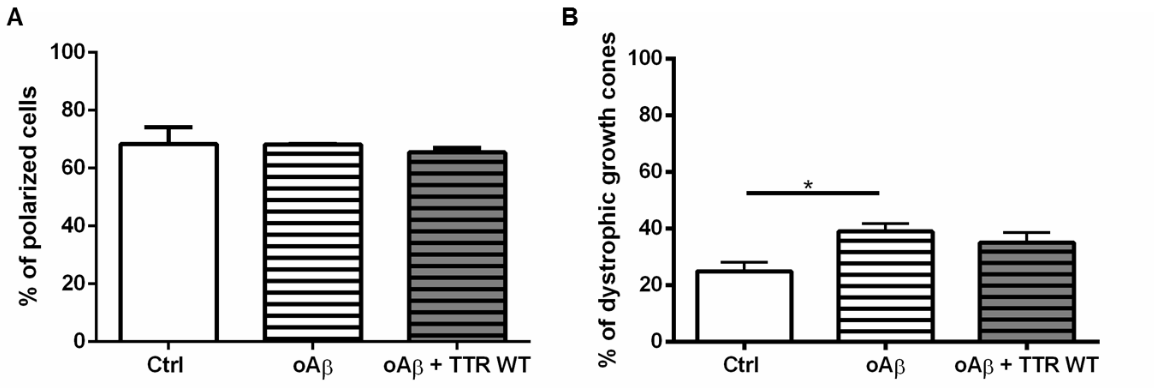


**Figure 22 – Aβ oligomers alter the growth cone morphology of polarized hippocampal neurons, a phenotype that is not rescued by the addition of TTR WT. A -** Hippocampal neurons incubated with 10µM Aβ oligomers alone or in the presence of 5µM TTR WT showed a similar percentage of polarized cells. **B -** Hippocampal neurons incubated with 10µM Aβ oligomers presented an increased number of dystrophic growth cones. However, the addition of 5µM TTR WT did not rescue this phenotype. Statistics: number of replicates: n=2; 1-way ANOVA test; data are mean ± SEM; *$p < 0.05$.

## α-synuclein does not impact in the neuronal cytoskeleton remodeling

α-synuclein has been shown to impact on the neurocytoskeleton, altering axonal transport and MT dynamics (see chapter 1, section 3.2.2). α-synuclein was detected in the extracellular environment, accumulating as LB also in the hippocampus of PD patients. In order to assess if α-synuclein induces the same morphological alterations as Aβ in the neuronal growth cone, mice hippocampal neurons were incubated either with 500 nM of α-synuclein monomers, oligomers or fibrils. The percentage of polarized cells was maintained in the three experimental conditions (figure 23A), showing that α-synuclein aggregated forms did not alter the overall morphology of the neurons. Concerning alterations in the growth cone morphology, in the three experimental conditions a slight increase in the percentage of dystrophic growth cones was observed, although not statistically significant, being that α-synuclein monomers, oligomers and fibrils increased the number of dystrophic growth cones by 12%, 9% and 11%, respectively, when comparing with control neurons (figure 23B). To further sustain the hypothesis that α-synuclein is able to induce alterations in the growth cone morphology of hippocampal neurons, the number of replicates should be increased.

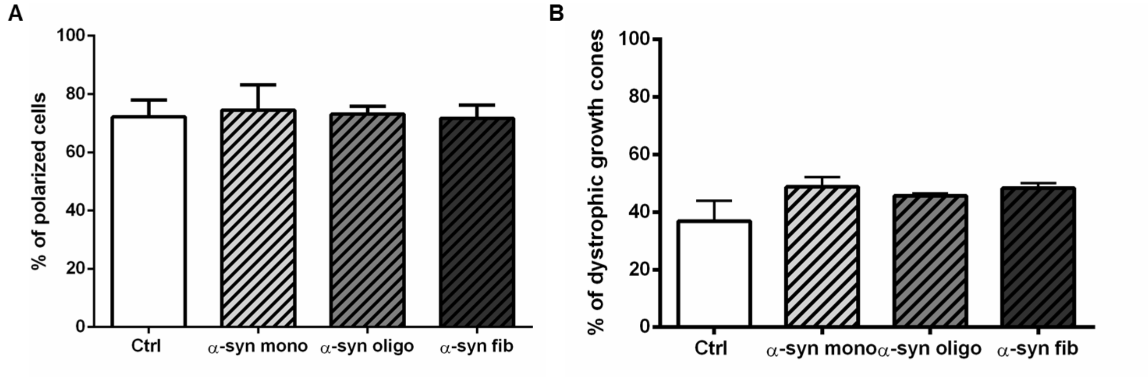


**Figure 23 – Growth cone morphology polarized hippocampal neurons is not altered with the addition of different α-synuclein species. A -** The percentage of polarized cells was maintained between the different experimental conditions. **B -** Hippocampal neurons incubated with 500nM of either α-synuclein monomers, oligomers or fibrils presented no statistical differences in the number of dystrophic growth cones when comparing with the control situation. Statistics: number of replicates: n=1; 1-way ANOVA test; data are mean ± SEM; *p < 0.05.

## Cytoskeleton organization of hippocampal neurons is not altered in the presence of TTR amyloidogenic mutants

Unpublished data from our group showed that there is an alteration of the neuronal cytoskeleton of PNS neurons in the presence of TTR oligomers (see chapter 1, section 3.2.3). To verify if the phenotype is maintained in CNS neurons cultures, we incubated mouse hippocampal neurons with either TTR WT or the amyloidogenic mutants TTR

V30M and TTR L55P. Although the incubation of hippocampal neurons with the different TTR variants did not alter the polarized structure characteristic of these neurons in culture (figure 24A), we hypothesized that, as previously reported for DRG neurons, TTR was able to induce alterations in the growth cone morphology of hippocampal neurons. We observed that TTR WT induced a 9% decrease, although not statistically different, in the number of dystrophic growth cones when comparing with the control, a phenotype that was not verified with the incubation with TTR V30M. The incubation with TTR L55P resulted in 8% decrease in the number of dystrophic growth cones showing a similar phenotype to TTR WT treated neurons (figure 24B). To further sustain the hypothesis that TTR amyloidogenic mutants are able to induce alterations in the growth cone morphology of hippocampal neurons, the number of replicates should be increased.

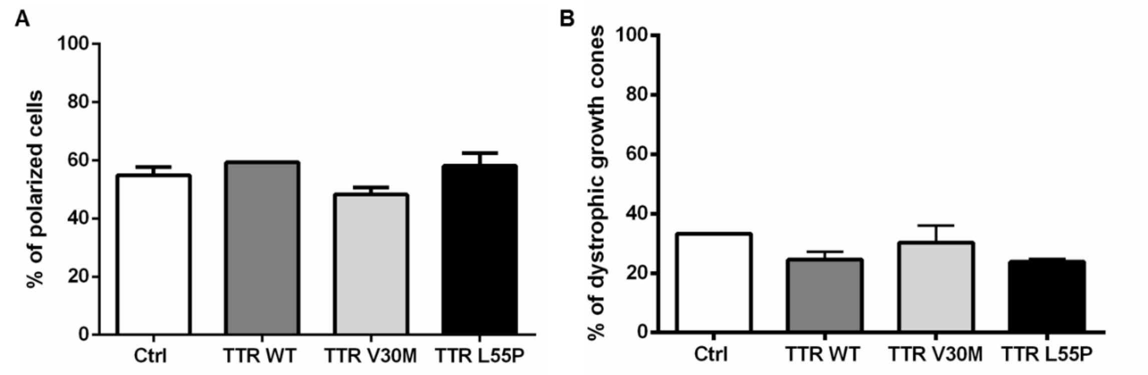


**Figure 24 – TTR WT and TTR mutants do not alter the growth cone morphology of polarized hippocampal neurons. A -** Hippocampal neurons incubated with 5μM of either TTR WT, TTR M30 or TTR P55 maintained cell polarization. **B –** TTR WT and TTR mutants (V30M and L55P) didn't increase the number of dystrophic growth cones when comparing with the control. Statistics: number of replicates: n=1; 1-way ANOVA test; data are mean ± SEM; *p < 0.05.

## DISCUSSION

Several neurodegenerative diseases occur as a consequence of the deposition of protein aggregates. Although being a hallmark in neurodegeneration, the actual role of protein aggregation in disease progression remains unclear. The neuronal cytoskeleton has been suggested as one of the key cellular components affected in the neurodegenerative processes. In this work, the effect of distinct prone-to-aggregation proteins, namely Aβ, α-synuclein, and TTR (the hallmarks of AD, PD and FAP, respectively) in the neuronal cytoskeleton of hippocampal neurons was assessed. Cultured hippocampal neurons have a well-organized axonal cytoskeleton organization presenting a growth cone with a characteristic distribution of both microtubules and actin filaments, what constitutes a valuable tool to analyze alterations in those cytoskeleton components. As such, in this work we used the growth cone morphology, by quantifying the percentage of dystrophic growth cones which present a complete disorganization of both microtubules and actin filaments, as a read-out for the effect of distinct aggregated species on cytoskeleton damage. We also assessed the polarization status of neurons under the presence of these toxic species. The percentage of polarized neurons was maintained in all the experimental conditions, demonstrating that the neuron preserves its normal architecture (the somatodendritic and axonal compartments) in the presence of aggregated proteins.

The incubation of hippocampal neurons with Aβ oligomers induced an increase in the number of dystrophic growth cones, an expected phenotype considering the reported effects of Aβ oligomers in microtubule stability and on actin dynamics (Mendoza-Naranjo, Gonzalez-Billault et al. 2007, Ramser, Gan et al. 2013, Pianu, Lefort et al. 2014). However, the Aβ oligomers-induced cytoskeleton alterations were not rescued by the addition of TTR WT. These results in combination with the results obtained in the previous chapter of this thesis, suggest that TTR neuroprotective effect on AD is related to a decrease in Abeta-induced cytotoxicity without affecting the Aβ oligomers-induced cytoskeleton damage.

We also performed preliminary experiments to address whether, similarly to Aβ oligomers, aggregates of α-synuclein and TTR impact on the cytoskeleton organization of hippocampal neurons. During the process of protein fibrillization there is the formation of intermediate species (oligomers and protofibrils), which are now regarded as the predominant neurotoxic species in both AD (see Chapter 1 section 2.4), PD (Roberts and Brown 2015) and FAP (Sousa, Cardoso et al. 2001). Moreover, oligomers of different proteins were suggested to share a common structure despite the different amino acid composition (Kayed, Head et al. 2003), suggesting a common mode of pathogenic activity.

Regarding the role of α-synuclein aggregation in the neuronal cytoskeleton, we observed a slight, but not statistical, increase in the number of dystrophic growth cones in the presence of the different species of α-synuclein, monomers, oligomers and fibrils. Although additional replicates of this experiment are required, the present results suggest that α-synuclein might impact on the cytoskeleton organization of hippocampal neurons, what could underlie the suggested spreading of PD pathology from dopaminergic neurons to the hippocampus induced by the action of secreted extracellular α-synuclein (Marques and Outeiro 2012). Additionally, the results with α-synuclein showed no differences between the different protein aggregation states what is not in accordance with the reported increased toxicity of the non-fibrillar species (Roberts and Brown 2015).

Hippocampal neurons incubated either with TTR WT, TTR V30M and TTR L55P had no significant morphological alterations in the growth cones. Yet, a slight decrease in the percentage of dystrophic growth cones was visible in the presence of TTR WT, which was not induced by TTR V30M suggesting a loss of function of the amyloidogenic variant. Surprisingly, TTR L55P treated neurons showed a slight decrease in the percentage of dystrophic growth cones similarly to the effect induced by TTR WT. In this experiments TTR proteins were incubated at room temperature to induce aggregation as previously described (Gasperini, Hou et al. 2011). Nevertheless, the aggregation state of TTR in our experimental setup should be confirmed by dynamic light scattering (DLS). Although replicate experiments are still required, the obtained results in hippocampal neurons were different from the ones obtained with DRG neurons, where TTR oligomers completed alter the growth cone morphology of the PNS neurons. As such, it might be the case that the impact of TTR aggregates in the neuronal cytoskeleton might be restricted to DRG neurons, which are in fact the clinically relevant cell type in FAP.

In order to be able to compare the effect of different prone-to-aggregate proteins in the neuronal cytoskeleton and explore the existence of common pathological mechanisms, hippocampal neurons should be incubated with the different protein species in a similar aggregation stage as assessed by DLS. After analyzing the impact of these proteins in the growth cone morphology, the consequences to axonal degeneration should also be assessed. Several parameters could be tested such as the formation of axonal swellings, indicators of axonal degeneration, and the impact of protein aggregates on microtubule stability, axonal transport and actin dynamics. These studies would help in the determination of common therapeutic approaches targeting the cytoskeleton that would be a valuable strategy for unrelated neurodegenerative disorders caused by protein aggregation.

## GENERAL CONCLUSION

In this work, we aimed to unravel the role of TTR proteolytic activity in AD both *in vitro* and *in vivo*. Using N2A-APPswe cell line, TTR proteolytic activity was demonstrated to be maintained in a physiological environment and to be involved in Aβ clearance. By studying Aβ oligomers-induced neurotoxicity using hippocampal neurons, we also verified that TTR proteolytic activity is required for its neuroprotective effect in AD. Regarding the *in vivo* studies, no conclusions could be taken out of the intracerebral injections of TTR performed in AD mice, since there was a high variability between the subjects. Also, using Veins zebrafish, that express GFP in the gut vasculate, we were able to reproduce a methodology previously reported that will serve in the future as a valuable tool for the study of TTR proteolysis *in vivo*.

Regarding the second chapter of this thesis, hippocampal neurons were incubated with different prone-to-aggregate proteins and growth cone morphology was assessed. By assessing the percentage of polarized cells, we verified that neurons preserve their polarized architecture after treatment. Aβ oligomers increased the number of dystrophic growth cones, however TTR WT didn't rescue the phenotype. We also tested α-synuclein and TTR amyloidogenic variants at different aggregation states and no differences were found between the different species and the control. Nevertheless, further studies should be conducted in order to unravel the precise mechanisms by which these proteins induce alterations in the neurocytoskeleton. The discovery of these mechanisms might open new insights in the development of new therapies that target the neuronal cytoskeleton in neurodegeneration diseases.

# REFERENCES

Achen, M. G., W. Duan, T. M. Pettersson, P. J. Harms, S. J. Richardson, M. C. Lawrence, R. E. Wettenhall, A. R. Aldred and G. Schreiber (1993). "Transthyretin gene expression in choroid plexus first evolved in reptiles." Am J Physiol **265**(5 Pt 2): R982-989.

Agorogiannis, E. I., G. I. Agorogiannis, A. Papadimitriou and G. M. Hadjigeorgiou (2004). "Protein misfolding in neurodegenerative diseases." Neuropathol Appl Neurobiol **30**(3): 215-224.

Akhmanova, A. and M. O. Steinmetz (2008). "Tracking the ends: a dynamic protein network controls the fate of microtubule tips." Nat Rev Mol Cell Biol **9**(4): 309-322.

Alim, M. A., Q. L. Ma, K. Takeda, T. Aizawa, M. Matsubara, M. Nakamura, A. Asada, T. Saito, H. Kaji, M. Yoshii, S. Hisanaga and K. Ueda (2004). "Demonstration of a role for alpha-synuclein as a functional microtubule-associated protein." J Alzheimers Dis **6**(4): 435-442; discussion 443-439.

Alves, I. L., K. Altland, M. R. Almeida, P. Winter and M. J. Saraiva (1997). "Screening and biochemical characterization of transthyretin variants in the Portuguese population." Hum Mutat **9**(3): 226-233.

Arnold, D. B. and G. Gallo (2014). "Structure meets function: actin filaments and myosin motors in the axon." J Neurochem **129**(2): 213-220.

Bamburg, J. R. and G. S. Bloom (2009). "Cytoskeletal pathologies of Alzheimer disease." Cell Motil Cytoskeleton **66**(8): 635-649.

Bessa, J., M. Luengo, S. Rivero-Gil, A. Ariza-Cosano, A. H. Maia, F. J. Ruiz-Ruano, P. Caballero, S. Naranjo, J. J. Carvajal and J. L. Gomez-Skarmeta (2014). "A mobile insulator system to detect and disrupt cis-regulatory landscapes in vertebrates." Genome Res **24**(3): 487-495.

Bradke, F., J. W. Fawcett and M. E. Spira (2012). "Assembly of a new growth cone after axotomy: the precursor to axon regeneration." Nat Rev Neurosci **13**(3): 183-193.

Buxbaum, J. N., Z. Ye, N. Reixach, L. Friske, C. Levy, P. Das, T. Golde, E. Masliah, A. R. Roberts and T. Bartfai (2008). "Transthyretin protects Alzheimer's mice from the behavioral and biochemical effects of Abeta toxicity." Proc Natl Acad Sci U S A **105**(7): 2681-2686.

Cartelli, D., F. Casagrande, C. L. Busceti, D. Bucci, G. Molinaro, A. Traficante, D. Passarella, E. Giavini, G. Pezzoli, G. Battaglia and G. Cappelletti (2013). "Microtubule alterations occur early in experimental parkinsonism and the microtubule stabilizer epothilone D is neuroprotective." Sci Rep **3**: 1837.

Carty, N., K. R. Nash, M. Brownlow, D. Cruite, D. Wilcock, M. L. Selenica, D. C. Lee, M. N. Gordon and D. Morgan (2013). "Intracranial injection of AAV expressing NEP but not IDE reduces amyloid pathology in APP+PS1 transgenic mice." PLoS One **8**(3): e59626.

Castellani, R. J., R. K. Rolston and M. A. Smith (2010). "Alzheimer disease." Dis Mon **56**(9): 484-546.

Cheng, F., G. Vivacqua and S. Yu (2011). "The role of alpha-synuclein in neurotransmission and synaptic plasticity." J Chem Neuroanat **42**(4): 242-248.

Cooper, G. M. H., R.E. (2013). The cell: a molecular approach, Sinauer Associates, Inc.

Costa, R., F. Ferreira-da-Silva, M. J. Saraiva and I. Cardoso (2008). "Transthyretin protects against A-beta peptide toxicity by proteolytic cleavage of the peptide: a mechanism sensitive to the Kunitz protease inhibitor." PLoS One **3**(8): e2899.

Costa, R., A. Goncalves, M. J. Saraiva and I. Cardoso (2008). "Transthyretin binding to A-Beta peptide--impact on A-Beta fibrillogenesis and toxicity." FEBS Lett **582**(6): 936-942.

Decker, H., K. Y. Lo, S. M. Unger, S. T. Ferreira and M. A. Silverman (2010). "Amyloid-beta peptide oligomers disrupt axonal transport through an NMDA receptor-dependent mechanism that is mediated by glycogen synthase kinase 3beta in primary cultured hippocampal neurons." J Neurosci **30**(27): 9166-9171.

Dickson, P. W., A. R. Aldred, P. D. Marley, G. F. Tu, G. J. Howlett and G. Schreiber (1985). "High prealbumin and transferrin mRNA levels in the choroid plexus of rat brain." Biochem Biophys Res Commun **127**(3): 890-895.

Dickson, P. W., G. J. Howlett and G. Schreiber (1982). "Metabolism of prealbumin in rats and changes induced by acute inflammation." Eur J Biochem **129**(2): 289-293.

Dickson, P. W., G. J. Howlett and G. Schreiber (1985). "Rat transthyretin (prealbumin). Molecular cloning, nucleotide sequence, and gene expression in liver and brain." J Biol Chem **260**(13): 8214-8219.

Dickson, P. W. and G. Schreiber (1986). "High levels of messenger RNA for transthyretin (prealbumin) in human choroid plexus." Neurosci Lett **66**(3): 311-315.

Ding, H., P. J. Dolan and G. V. Johnson (2008). "Histone deacetylase 6 interacts with the microtubule-associated protein tau." J Neurochem **106**(5): 2119-2130.

Doggui, S., J. Brouillette, J. G. Chabot, M. Farso and R. Quirion (2010). "Possible involvement of transthyretin in hippocampal beta-amyloid burden and learning behaviors in a mouse model of Alzheimer's disease (TgCRND8)." Neurodegener Dis **7**(1-3): 88-95.

Duan, W., S. J. Richardson, J. Kohrle, L. Chang, B. R. Southwell, P. J. Harms, C. M. Brack, T. M. Pettersson and G. Schreiber (1995). "Binding of thyroxine to pig transthyretin, its cDNA structure, and other properties." Eur J Biochem **230**(3): 977-986.

Duthey, B. (2013). Alzheimer Disease and other Dementias, World Health Organization.

Edwards, M., A. Zwolak, D. A. Schafer, D. Sept, R. Dominguez and J. A. Cooper (2014). "Capping protein regulators fine-tune actin assembly dynamics." Nat Rev Mol Cell Biol **15**(10): 677-689.

Episkopou, V., S. Maeda, S. Nishiguchi, K. Shimada, G. A. Gaitanaris, M. E. Gottesman and E. J. Robertson (1993). "Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone." Proc Natl Acad Sci U S A **90**(6): 2375-2379.

Fleming, A., H. Diekmann and P. Goldsmith (2013). "Functional characterisation of the maturation of the blood-brain barrier in larval zebrafish." PLoS One **8**(10): e77548.

Fleming, C. E., F. M. Mar, F. Franquinho, M. J. Saraiva and M. M. Sousa (2009). "Transthyretin internalization by sensory neurons is megalin mediated and necessary for its neuritogenic activity." J Neurosci **29**(10): 3220-3232.

Fleming, C. E., M. J. Saraiva and M. M. Sousa (2007). "Transthyretin enhances nerve regeneration." J Neurochem **103**(2): 831-839.

Friedrich, R. P., K. Tepper, R. Ronicke, M. Soom, M. Westermann, K. Reymann, C. Kaether and M. Fandrich (2010). "Mechanism of amyloid plaque formation suggests an intracellular basis of Abeta pathogenicity." Proc Natl Acad Sci U S A **107**(5): 1942-1947.

Furuya, H., M. Nakazato, M. J. Saraiva, S. P. Costa, H. Sasaki, H. Matsuo, I. Goto and Y. Sakaki (1989). "Tetramer formation of a variant type human transthyretin (prealbumin) produced by Escherichia coli expression system." Biochem Biophys Res Commun **163**(2): 851-859.

Galloway, P. G., P. Mulvihill and G. Perry (1992). "Filaments of Lewy bodies contain insoluble cytoskeletal elements." Am J Pathol **140**(4): 809-822.

Galloway, P. G., G. Perry and P. Gambetti (1987). "Hirano body filaments contain actin and actin-associated proteins." J Neuropathol Exp Neurol **46**(2): 185-199.

Gasperini, R. J., X. Hou, H. Parkington, H. Coleman, D. W. Klaver, A. J. Vincent, L. C. Foa and D. H. Small (2011). "TRPM8 and Nav1.8 sodium channels are required for transthyretin-induced calcium influx in growth cones of small-diameter TrkA-positive sensory neurons." Mol Neurodegener **6**(1): 19.

George, J. M. (2002). "The synucleins." Genome Biol **3**(1): REVIEWS3002.

Getz, R. K., B. G. Kennedy and N. J. Mangini (1999). "Transthyretin localization in cultured and native human retinal pigment epithelium." Exp Eye Res **68**(5): 629-636.

Glenner, G. G. and C. W. Wong (1984). "Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein." Biochem Biophys Res Commun **120**(3): 885-890.

Goodman, D. (1987). "Retinoids and retinol-binding proteins. ." The Harvey Lectures Series **81**: 22.

Haass, C., C. A. Lemere, A. Capell, M. Citron, P. Seubert, D. Schenk, L. Lannfelt and D. J. Selkoe (1995). "The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway." Nat Med **1**(12): 1291-1296.

Haass, C. and D. J. Selkoe (2007). "Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide." Nat Rev Mol Cell Biol **8**(2): 101-112.

Hagen, G. A. and L. A. Solberg, Jr. (1974). "Brain and cerebrospinal fluid permeability to intravenous thyroid hormones." Endocrinology **95**(5): 1398-1410.

Hall, H., S. Reyes, N. Landeck, C. Bye, G. Leanza, K. Double, L. Thompson, G. Halliday and D. Kirik (2014). "Hippocampal Lewy pathology and cholinergic dysfunction are associated with dementia in Parkinson's disease." Brain **137**(Pt 9): 2493-2508.

Hansson, S. F., U. Andreasson, M. Wall, I. Skoog, N. Andreasen, A. Wallin, H. Zetterberg and K. Blennow (2009). "Reduced levels of amyloid-beta-binding proteins in cerebrospinal fluid from Alzheimer's disease patients." J Alzheimers Dis **16**(2): 389-397.

Harms, P. J., G. F. Tu, S. J. Richardson, A. R. Aldred, A. Jaworowski and G. Schreiber (1991). "Transthyretin (prealbumin) gene expression in choroid plexus is strongly conserved during evolution of vertebrates." Comp Biochem Physiol B **99**(1): 239-249.

Henriques, A. G., J. M. Oliveira, L. P. Carvalho and E. S. O. A. da Cruz (2014). "Abeta Influences Cytoskeletal Signaling Cascades with Consequences to Alzheimer's Disease." Mol Neurobiol.

Henriques, A. G., S. I. Vieira, E. S. E. F. da Cruz and E. S. O. A. da Cruz (2010). "Abeta promotes Alzheimer's disease-like cytoskeleton abnormalities with consequences to APP processing in neurons." J Neurochem **113**(3): 761-771.

Higashijima, S., Y. Hotta and H. Okamoto (2000). "Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer." J Neurosci **20**(1): 206-218.

Hirokawa, N., S. Niwa and Y. Tanaka (2010). "Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease." Neuron **68**(4): 610-638.

Hirokawa, N. and Y. Noda (2008). "Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics." Physiol Rev **88**(3): 1089-1118.

Hu, W. Y., Z. Y. He, L. J. Yang, M. Zhang, D. Xing and Z. C. Xiao (2015). "The Ca(2+) channel inhibitor 2-APB reverses beta-amyloid-induced LTP deficit in hippocampus by blocking BAX and caspase-3 hyperactivation." Br J Pharmacol **172**(9): 2273-2285.

Hunn, B. H., S. J. Cragg, J. P. Bolam, M. G. Spillantini and R. Wade-Martins (2015). "Impaired intracellular trafficking defines early Parkinson's disease." Trends Neurosci **38**(3): 178-188.

Izumoto, S., D. Younger, A. P. Hays, R. L. Martone, R. T. Smith and J. Herbert (1992). "Familial amyloidotic polyneuropathy presenting with carpal tunnel syndrome and a new transthyretin mutation, asparagine 70." Neurology **42**(11): 2094-2102.

Janke, C. and J. C. Bulinski (2011). "Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions." Nat Rev Mol Cell Biol **12**(12): 773-786.

Jankowsky, J. L., D. J. Fadale, J. Anderson, G. M. Xu, V. Gonzales, N. A. Jenkins, N. G. Copeland, M. K. Lee, L. H. Younkin, S. L. Wagner, S. G. Younkin and D. R. Borchelt (2004). "Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase." Hum Mol Genet **13**(2): 159-170.

Jarrett, J. T., E. P. Berger and P. T. Lansbury, Jr. (1993). "The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease." Biochemistry **32**(18): 4693-4697.

Johnson, S. M., S. Connelly, C. Fearns, E. T. Powers and J. W. Kelly (2012). "The transthyretin amyloidoses: from delineating the molecular mechanism of aggregation linked to pathology to a regulatory-agency-approved drug." J Mol Biol **421**(2-3): 185-203.

Kanda, Y., D. S. Goodman, R. E. Canfield and F. J. Morgan (1974). "The amino acid sequence of human plasma prealbumin." J Biol Chem **249**(21): 6796-6805.

Kawakami, K., A. Shima and N. Kawakami (2000). "Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage." Proc Natl Acad Sci U S A **97**(21): 11403-11408.

Kayed, R., E. Head, J. L. Thompson, T. M. McIntire, S. C. Milton, C. W. Cotman and C. G. Glabe (2003). "Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis." Science **300**(5618): 486-489.

Kerridge, C., N. D. Belyaev, N. N. Nalivaeva and A. J. Turner (2014). "The Abeta-clearance protein transthyretin, like neprilysin, is epigenetically regulated by the amyloid precursor protein intracellular domain." J Neurochem **130**(3): 419-431.

Klein, R. L., R. D. Dayton, J. B. Tatom, K. M. Henderson and P. P. Henning (2008). "AAV8, 9, Rh10, Rh43 vector gene transfer in the rat brain: effects of serotype, promoter and purification method." Mol Ther **16**(1): 89-96.

Kruger, R., W. Kuhn, T. Muller, D. Woitalla, M. Graeber, S. Kosel, H. Przuntek, J. T. Epplen, L. Schols and O. Riess (1998). "Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease." Nat Genet **18**(2): 106-108.

Kumar, A., C. M. Nisha, C. Silakari, I. Sharma, K. Anusha, N. Gupta, P. Nair, T. Tripathi and A. Kumar (2015). "Current and novel therapeutic molecules and targets in Alzheimer's disease." J Formos Med Assoc.

LaFerla, F. M., K. N. Green and S. Oddo (2007). "Intracellular amyloid-beta in Alzheimer's disease." Nat Rev Neurosci **8**(7): 499-509.

Lambert, M. P., A. K. Barlow, B. A. Chromy, C. Edwards, R. Freed, M. Liosatos, T. E. Morgan, I. Rozovsky, B. Trommer, K. L. Viola, P. Wals, C. Zhang, C. E. Finch, G. A. Krafft and W. L. Klein (1998). "Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins." Proc Natl Acad Sci U S A **95**(11): 6448-6453.

Lashuel, H. A., Z. Lai and J. W. Kelly (1998). "Characterization of the transthyretin acid denaturation pathways by analytical ultracentrifugation: implications for wild-type, V30M, and L55P amyloid fibril formation." Biochemistry **37**(51): 17851-17864.

Leimer, U., K. Lun, H. Romig, J. Walter, J. Grunberg, M. Brand and C. Haass (1999). "Zebrafish (Danio rerio) presenilin promotes aberrant amyloid beta-peptide production and requires a critical aspartate residue for its function in amyloidogenesis." Biochemistry **38**(41): 13602-13609.

Leissring, M. A., W. Farris, A. Y. Chang, D. M. Walsh, X. Wu, X. Sun, M. P. Frosch and D. J. Selkoe (2003). "Enhanced proteolysis of beta-amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death." Neuron **40**(6): 1087-1093.

Lepinoux-Chambaud, C. and J. Eyer (2013). "Review on intermediate filaments of the nervous system and their pathological alterations." Histochem Cell Biol **140**(1): 13-22.

Li, X. and J. N. Buxbaum (2011). "Transthyretin and the brain re-visited: is neuronal synthesis of transthyretin protective in Alzheimer's disease?" Mol Neurodegener **6**: 79.

Li, X., E. Masliah, N. Reixach and J. N. Buxbaum (2011). "Neuronal production of transthyretin in human and murine Alzheimer's disease: is it protective?" J Neurosci **31**(35): 12483-12490.

Liz, M. A., C. J. Faro, M. J. Saraiva and M. M. Sousa (2004). "Transthyretin, a new cryptic protease." J Biol Chem **279**(20): 21431-21438.

Liz, M. A., C. E. Fleming, A. F. Nunes, M. R. Almeida, F. M. Mar, Y. Choe, C. S. Craik, J. C. Powers, M. Bogyo and M. M. Sousa (2009). "Substrate specificity of transthyretin: identification of natural substrates in the nervous system." Biochem J **419**(2): 467-474.

Liz, M. A., C. M. Gomes, M. J. Saraiva and M. M. Sousa (2007). "ApoA-I cleaved by transthyretin has reduced ability to promote cholesterol efflux and increased amyloidogenicity." J Lipid Res **48**(11): 2385-2395.

Liz, M. A., S. C. Leite, L. Juliano, M. J. Saraiva, A. M. Damas, D. Bur and M. M. Sousa (2012). "Transthyretin is a metallopeptidase with an inducible active site." Biochem J **443**(3): 769-778.

Loughna, S., P. Bennett and G. Moore (1995). "Molecular analysis of the expression of transthyretin in intestine and liver from trisomy 18 fetuses." Hum Genet **95**(1): 89-95.

Lowery, L. A. and D. Van Vactor (2009). "The trip of the tip: understanding the growth cone machinery." Nat Rev Mol Cell Biol **10**(5): 332-343.

Lu, X. L., J. X. Liu, Q. Wu, S. M. Long, M. Y. Zheng, X. L. Yao, H. Ren, Y. G. Wang, W. W. Su, R. T. Fai Cheung, J. S. Zeng, H. Su and Z. Pei (2014). "Protective effects of puerarin against Abeta40-induced vascular dysfunction in zebrafish and human endothelial cells." Eur J Pharmacol **732**: 76-85.

Maia, L. F., R. Magalhaes, J. Freitas, R. Taipa, M. M. Pires, H. Osorio, D. Dias, H. Pessegueiro, M. Correia and T. Coelho (2015). "CNS involvement in V30M transthyretin amyloidosis: clinical, neuropathological and biochemical findings." J Neurol Neurosurg Psychiatry **86**(2): 159-167.

Makover, A., H. Moriwaki, R. Ramakrishnan, M. J. Saraiva, W. S. Blaner and D. S. Goodman (1988). "Plasma transthyretin. Tissue sites of degradation and turnover in the rat." J Biol Chem **263**(18): 8598-8603.

Maloney, M. T., L. S. Minamide, A. W. Kinley, J. A. Boyle and J. R. Bamburg (2005). "Beta-secretase-cleaved amyloid precursor protein accumulates at actin inclusions induced in neurons by stress or amyloid beta: a feedforward mechanism for Alzheimer's disease." J Neurosci **25**(49): 11313-11321.

Marques, O. and T. F. Outeiro (2012). "Alpha-synuclein: from secretion to dysfunction and death." Cell Death Dis **3**: e350.

Marr, R. A., E. Rockenstein, A. Mukherjee, M. S. Kindy, L. B. Hersh, F. H. Gage, I. M. Verma and E. Masliah (2003). "Neprilysin gene transfer reduces human amyloid pathology in transgenic mice." J Neurosci **23**(6): 1992-1996.

Martin-Jimenez, R., M. Campanella and C. Russell (2015). "New zebrafish models of neurodegeneration." Curr Neurol Neurosci Rep **15**(6): 33.

McCutchen, S. L., W. Colon and J. W. Kelly (1993). "Transthyretin mutation Leu-55-Pro significantly alters tetramer stability and increases amyloidogenicity." Biochemistry **32**(45): 12119-12127.

McLean, C. A., R. A. Cherny, F. W. Fraser, S. J. Fuller, M. J. Smith, K. Beyreuther, A. I. Bush and C. L. Masters (1999). "Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease." Ann Neurol **46**(6): 860-866.

Mendoza-Naranjo, A., C. Gonzalez-Billault and R. B. Maccioni (2007). "Abeta1-42 stimulates actin polymerization in hippocampal neurons through Rac1 and Cdc42 Rho GTPases." J Cell Sci **120**(Pt 2): 279-288.

Minamide, L. S., A. M. Striegl, J. A. Boyle, P. J. Meberg and J. R. Bamburg (2000). "Neurodegenerative stimuli induce persistent ADF/cofilin-actin rods that disrupt distal neurite function." Nat Cell Biol **2**(9): 628-636.

Mudbhary, R., Y. Hoshida, Y. Chernyavskaya, V. Jacob, A. Villanueva, M. I. Fiel, X. Chen, K. Kojima, S. Thung, R. T. Bronson, A. Lachenmayer, K. Revill, C. Alsinet, R. Sachidanandam, A. Desai, S. SenBanerjee, C. Ukomadu, J. M. Llovet and K. C. Sadler (2014). "UHRF1 overexpression drives DNA hypomethylation and hepatocellular carcinoma." Cancer Cell **25**(2): 196-209.

Nalivaeva, N. N., C. Beckett, N. D. Belyaev and A. J. Turner (2012). "Are amyloid-degrading enzymes viable therapeutic targets in Alzheimer's disease?" J Neurochem **120 Suppl 1**: 167-185.

Naslund, J., V. Haroutunian, R. Mohs, K. L. Davis, P. Davies, P. Greengard and J. D. Buxbaum (2000). "Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline." JAMA **283**(12): 1571-1577.

Nery, L. R., N. S. Eltz, C. Hackman, R. Fonseca, S. Altenhofen, H. N. Guerra, V. M. Freitas, C. D. Bonan and M. R. Vianna (2014). "Brain intraventricular injection of amyloid-beta in zebrafish embryo impairs cognition and increases tau phosphorylation, effects reversed by lithium." PLoS One **9**(9): e105862.

Neukirchen, D. and F. Bradke (2011). "Neuronal polarization and the cytoskeleton." Semin Cell Dev Biol **22**(8): 825-833.

Newman, M., G. Verdile, R. N. Martins and M. Lardelli (2011). "Zebrafish as a tool in Alzheimer's disease research." Biochim Biophys Acta **1812**(3): 346-352.

Oliveira, S. M., C. A. Ribeiro, I. Cardoso and M. J. Saraiva (2011). "Gender-dependent transthyretin modulation of brain amyloid-beta levels: evidence from a mouse model of Alzheimer's disease." J Alzheimers Dis **27**(2): 429-439.

Ono, K., M. M. Condron and D. B. Teplow (2009). "Structure-neurotoxicity relationships of amyloid beta-protein oligomers." Proc Natl Acad Sci U S A **106**(35): 14745-14750.

Pal, R., G. Alves, J. P. Larsen and S. G. Moller (2014). "New insight into neurodegeneration: the role of proteomics." Mol Neurobiol **49**(3): 1181-1199.

Palha, J. A., V. Episkopou, S. Maeda, K. Shimada, M. E. Gottesman and M. J. Saraiva (1994). "Thyroid hormone metabolism in a transthyretin-null mouse strain." J Biol Chem **269**(52): 33135-33139.

Palha, J. A., M. T. Hays, G. Morreale de Escobar, V. Episkopou, M. E. Gottesman and M. J. Saraiva (1997). "Transthyretin is not essential for thyroxine to reach the brain and other tissues in transthyretin-null mice." Am J Physiol **272**(3 Pt 1): E485-493.

Park, M. H., J. K. Lee, S. Choi, J. Ahn, H. K. Jin, J. S. Park and J. S. Bae (2013). "Recombinant soluble neprilysin reduces amyloid-beta accumulation and improves memory impairment in Alzheimer's disease mice." Brain Res **1529**: 113-124.

Paula, V. J. R. G., F. M.; Diniz, B. S.; Forlenza, O. V. (2009). "Neurobiological pathways to Alzheimer's disease: amyloid-beta, Tau protein or both?" Dementia & Neuropsychologia **3**(3): 188-194.

Perl, D. P. (2010). "Neuropathology of Alzheimer's disease." Mt Sinai J Med **77**(1): 32-42.

Petersen, R. B., H. Goren, M. Cohen, S. L. Richardson, N. Tresser, A. Lynn, M. Gali, M. Estes and P. Gambetti (1997). "Transthyretin amyloidosis: a new mutation associated with dementia." Ann Neurol **41**(3): 307-313.

Petratos, S., Q. X. Li, A. J. George, X. Hou, M. L. Kerr, S. E. Unabia, I. Hatzinisiriou, D. Maksel, M. I. Aguilar and D. H. Small (2008). "The beta-amyloid protein of Alzheimer's disease increases neuronal CRMP-2 phosphorylation by a Rho-GTP mechanism." Brain **131**(Pt 1): 90-108.

Piaceri, I., B. Nacmias and S. Sorbi (2013). "Genetics of familial and sporadic Alzheimer's disease." Front Biosci (Elite Ed) **5**: 167-177.

Pianu, B., R. Lefort, L. Thuiliere, E. Tabourier and F. Bartolini (2014). "The Abeta(1)(-)(4)(2) peptide regulates microtubule stability independently of tau." J Cell Sci **127**(Pt 5): 1117-1127.

Pike, C. J., B. J. Cummings and C. W. Cotman (1992). "beta-Amyloid induces neuritic dystrophy in vitro: similarities with Alzheimer pathology." Neuroreport **3**(9): 769-772.

Plante-Bordeneuve, V. and G. Said (2011). "Familial amyloid polyneuropathy." Lancet Neurol **10**(12): 1086-1097.

Polymeropoulos, M. H., C. Lavedan, E. Leroy, S. E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubenstein, R. Boyer, E. S. Stenroos, S. Chandrasekharappa, A. Athanassiadou, T. Papapetropoulos, W. G. Johnson, A. M. Lazzarini, R. C. Duvoisin, G. Di Iorio, L. I. Golbe and R. L. Nussbaum (1997). "Mutation in the alpha-synuclein gene identified in families with Parkinson's disease." Science **276**(5321): 2045-2047.

Power, D. M., N. P. Elias, S. J. Richardson, J. Mendes, C. M. Soares and C. R. Santos (2000). "Evolution of the thyroid hormone-binding protein, transthyretin." Gen Comp Endocrinol **119**(3): 241-255.

Qiu, H., K. Shimada and Z. Cheng (1992). "Chromosomal localization of the mouse prealbumin gene (Ttr) by in situ hybridization." Cytogenet Cell Genet **61**(3): 186-188.

Quintas, A., D. C. Vaz, I. Cardoso, M. J. Saraiva and R. M. Brito (2001). "Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants." J Biol Chem **276**(29): 27207-27213.

Rabins, P. V. and D. e. a. Blacker (2007). Practice Guideline for the Treatment of Patients With Alzheimer’s Disease and Other Dementias. A. P. Association.

Ramser, E. M., K. J. Gan, H. Decker, E. Y. Fan, M. M. Suzuki, S. T. Ferreira and M. A. Silverman (2013). "Amyloid-beta oligomers induce tau-independent disruption of BDNF axonal transport via calcineurin activation in cultured hippocampal neurons." Mol Biol Cell **24**(16): 2494-2505.

Rapezzi, C., C. C. Quarta, L. Riva, S. Longhi, I. Gallelli, M. Lorenzini, P. Ciliberti, E. Biagini, F. Salvi and A. Branzi (2010). "Transthyretin-related amyloidoses and the heart: a clinical overview." Nat Rev Cardiol **7**(7): 398-408.

Reed, N. A., D. Cai, T. L. Blasius, G. T. Jih, E. Meyhofer, J. Gaertig and K. J. Verhey (2006). "Microtubule acetylation promotes kinesin-1 binding and transport." Curr Biol **16**(21): 2166-2172.

Reitz, C. and R. Mayeux (2014). "Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers." Biochem Pharmacol **88**(4): 640-651.

Remmers, E. F., E. A. Goldmuntz, H. Zha, L. J. Crofford, J. M. Cash, P. Mathern, Y. Du and R. L. Wilder (1993). "Linkage map of seven polymorphic markers on rat chromosome 18." Mamm Genome **4**(5): 265-270.

Ribeiro, C. A., S. M. Oliveira, L. F. Guido, A. Magalhaes, G. Valencia, G. Arsequell, M. J. Saraiva and I. Cardoso (2014). "Transthyretin stabilization by iododiflunisal promotes amyloid-beta peptide clearance, decreases its deposition, and ameliorates cognitive deficits in an Alzheimer's disease mouse model." J Alzheimers Dis **39**(2): 357-370.

Ribeiro, C. A., I. Santana, C. Oliveira, I. Baldeiras, J. Moreira, M. J. Saraiva and I. Cardoso (2012). "Transthyretin decrease in plasma of MCI and AD patients: investigation of mechanisms for disease modulation." Curr Alzheimer Res **9**(8): 881-889.

Richardson, S. J., A. J. Bradley, W. Duan, R. E. Wettenhall, P. J. Harms, J. J. Babon, B. R. Southwell, S. Nicol, S. C. Donnellan and G. Schreiber (1994). "Evolution of marsupial and other vertebrate thyroxine-binding plasma proteins." Am J Physiol **266**(4 Pt 2): R1359-1370.

Richardson, S. J., J. A. Monk, C. A. Shepherdley, L. O. Ebbesson, F. Sin, D. M. Power, P. B. Frappell, J. Kohrle and M. B. Renfree (2005). "Developmentally regulated thyroid hormone distributor proteins in marsupials, a reptile, and fish." Am J Physiol Regul Integr Comp Physiol **288**(5): R1264-1272.

Riisoen, H. (1988). "Reduced prealbumin (transthyretin) in CSF of severely demented patients with Alzheimer's disease." Acta Neurol Scand **78**(6): 455-459.

Roberts, H. L. and D. R. Brown (2015). "Seeking a mechanism for the toxicity of oligomeric alpha-synuclein." Biomolecules **5**(2): 282-305.

Ross, C. A. P. M. A. (2005). "What is the role of protein aggregation in neurodegeneration?" Molecular Cell Biology **6**: 891-898.

Rowczenio, D. and A. Wechalekar. (2015). "Mutations in Hereditary Amyloidosis: Mutations in Transthyretin Gene." from http://www.amyloidosismutations.com/mut-attr.php.

Saha, A. R., J. Hill, M. A. Utton, A. A. Asuni, S. Ackerley, A. J. Grierson, C. C. Miller, A. M. Davies, V. L. Buchman, B. H. Anderton and D. P. Hanger (2004). "Parkinson's disease alpha-synuclein mutations exhibit defective axonal transport in cultured neurons." J Cell Sci **117**(Pt 7): 1017-1024.

Santos, S. D., R. Fernandes and M. J. Saraiva (2010). "The heat shock response modulates transthyretin deposition in the peripheral and autonomic nervous systems." Neurobiol Aging **31**(2): 280-289.

Saraiva, M. J. (2001). "Transthyretin mutations in hyperthyroxinemia and amyloid diseases." Hum Mutat **17**(6): 493-503.

Saraiva, M. J., S. Birken, P. P. Costa and D. S. Goodman (1984). "Amyloid fibril protein in familial amyloidotic polyneuropathy, Portuguese type. Definition of molecular abnormality in transthyretin (prealbumin)." J Clin Invest **74**(1): 104-119.

Saraiva, M. J., J. Magalhaes, N. Ferreira and M. R. Almeida (2012). "Transthyretin deposition in familial amyloidotic polyneuropathy." Curr Med Chem **19**(15): 2304-2311.

Saraiva, M. J., W. Sherman, C. Marboe, A. Figueira, P. Costa, A. F. de Freitas and M. A. Gawinowicz (1990). "Cardiac amyloidosis: report of a patient heterozygous for the transthyretin isoleucine 122 variant." Scand J Immunol **32**(4): 341-346.

Sasaki, H., N. Yoshioka, Y. Takagi and Y. Sakaki (1985). "Structure of the chromosomal gene for human serum prealbumin." Gene **37**(1-3): 191-197.

Schreiber, G., A. R. Aldred, A. Jaworowski, C. Nilsson, M. G. Achen and M. B. Segal (1990). "Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus." Am J Physiol **258**(2 Pt 2): R338-345.

Schreiber, G. and S. J. Richardson (1997). "The evolution of gene expression, structure and function of transthyretin." Comp Biochem Physiol B Biochem Mol Biol **116**(2): 137-160.

Schwarzman, A. L. and D. Goldgaber (1996). "Interaction of transthyretin with amyloid beta-protein: binding and inhibition of amyloid formation." Ciba Found Symp **199**: 146-160; discussion 160-144.

Schwarzman, A. L., L. Gregori, M. P. Vitek, S. Lyubski, W. J. Strittmatter, J. J. Enghilde, R. Bhasin, J. Silverman, K. H. Weisgraber, P. K. Coyle and et al. (1994). "Transthyretin sequesters amyloid beta protein and prevents amyloid formation." Proc Natl Acad Sci U S A **91**(18): 8368-8372.

Sebastiao, M. P., M. J. Saraiva and A. M. Damas (1998). "The crystal structure of amyloidogenic Leu55 --> Pro transthyretin variant reveals a possible pathway for transthyretin polymerization into amyloid fibrils." J Biol Chem **273**(38): 24715-24722.

Shirao, T. and C. Gonzalez-Billault (2013). "Actin filaments and microtubules in dendritic spines." J Neurochem **126**(2): 155-164.

Simonato, M., J. Bennett, N. M. Boulis, M. G. Castro, D. J. Fink, W. F. Goins, S. J. Gray, P. R. Lowenstein, L. H. Vandenberghe, T. J. Wilson, J. H. Wolfe and J. C. Glorioso (2013). "Progress in gene therapy for neurological disorders." Nat Rev Neurol **9**(5): 277-291.

Skare, J., H. Yazici, E. Erken, H. Dede, A. Cohen, A. Milunsky and M. Skinner (1990). "Homozygosity for the met30 transthyretin gene in a Turkish kindred with familial amyloidotic polyneuropathy." Hum Genet **86**(1): 89-90.

Socolow, E. L., K. A. Woeber, R. H. Purdy, M. T. Holloway and S. H. Ingbar (1965). "Preparation of I-131-labeled human serum prealbumin and its metabolism in normal and sick patients." J Clin Invest **44**(10): 1600-1609.

Soprano, D. R., J. Herbert, K. J. Soprano, E. A. Schon and D. S. Goodman (1985). "Demonstration of transthyretin mRNA in the brain and other extrahepatic tissues in the rat." J Biol Chem **260**(21): 11793-11798.

Sousa, M. M., I. Cardoso, R. Fernandes, A. Guimaraes and M. J. Saraiva (2001). "Deposition of transthyretin in early stages of familial amyloidotic polyneuropathy: evidence for toxicity of nonfibrillar aggregates." Am J Pathol **159**(6): 1993-2000.

Sousa, M. M., A. G. Norden, C. Jacobsen, T. E. Willnow, E. I. Christensen, R. V. Thakker, P. J. Verroust, S. K. Moestrup and M. J. Saraiva (2000). "Evidence for the role of megalin in renal uptake of transthyretin." J Biol Chem **275**(49): 38176-38181.

Sousa, M. M. and M. J. Saraiva (2001). "Internalization of transthyretin. Evidence of a novel yet unidentified receptor-associated protein (RAP)-sensitive receptor." J Biol Chem **276**(17): 14420-14425.

Sousa, V. L., S. Bellani, M. Giannandrea, M. Yousuf, F. Valtorta, J. Meldolesi and E. Chieregatti (2009). "{alpha}-synuclein and its A30P mutant affect actin cytoskeletal structure and dynamics." Mol Biol Cell **20**(16): 3725-3739.

Southwell, B. R., W. Duan, G. F. Tu and G. Schreiber (1991). "Ontogenesis of transthyretin gene expression in chicken choroid plexus and liver." Comp Biochem Physiol B **100**(2): 329-338.

Stefanis, L. (2012). "alpha-Synuclein in Parkinson's disease." Cold Spring Harb Perspect Med **2**(2): a009399.

Stein, T. D., N. J. Anders, C. DeCarli, S. L. Chan, M. P. Mattson and J. A. Johnson (2004). "Neutralization of transthyretin reverses the neuroprotective effects of secreted amyloid precursor protein (APP) in APPSW mice resulting in tau phosphorylation and loss of hippocampal neurons: support for the amyloid hypothesis." J Neurosci **24**(35): 7707-7717.

Suchowerska, A. and T. Fath (2014). "Cytoskeletal changes in diseases of the nervous system." Frontiers in Biology **9**(1): 5-17.

Szeverenyi, I., A. J. Cassidy, C. W. Chung, B. T. Lee, J. E. Common, S. C. Ogg, H. Chen, S. Y. Sim, W. L. Goh, K. W. Ng, J. A. Simpson, L. L. Chee, G. H. Eng, B. Li, D. P. Lunny, D. Chuon, A. Venkatesh, K. H. Khoo, W. H. McLean, Y. P. Lim and E. B. Lane (2008). "The Human Intermediate Filament Database: comprehensive information on a gene family involved in many human diseases." Hum Mutat **29**(3): 351-360.

Tanzi, R. E., P. H. St George-Hyslop and J. F. Gusella (1989). "Molecular genetic approaches to Alzheimer's disease." Trends Neurosci **12**(4): 152-158.

van Bennekum, A. M., S. Wei, M. V. Gamble, S. Vogel, R. Piantedosi, M. Gottesman, V. Episkopou and W. S. Blaner (2001). "Biochemical basis for depressed serum retinol levels in transthyretin-deficient mice." J Biol Chem **276**(2): 1107-1113.

Vatassery, G. T., H. T. Quach, W. E. Smith, B. A. Benson and J. H. Eckfeldt (1991). "A sensitive assay of transthyretin (prealbumin) in human cerebrospinal fluid in nanogram amounts by ELISA." Clin Chim Acta **197**(1): 19-25.

Vickers, J. C., A. E. King, A. Woodhouse, M. T. Kirkcaldie, J. A. Staal, G. H. McCormack, C. A. Blizzard, R. E. Musgrove, S. Mitew, Y. Liu, J. A. Chuckowree, O. Bibari and T. C. Dickson (2009). "Axonopathy and cytoskeletal disruption in degenerative diseases of the central nervous system." Brain Res Bull **80**(4-5): 217-223.

Walsh, D. M., B. P. Tseng, R. E. Rydel, M. B. Podlisny and D. J. Selkoe (2000). "The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain." Biochemistry **39**(35): 10831-10839.

Wang, J., D. W. Dickson, J. Q. Trojanowski and V. M. Lee (1999). "The levels of soluble versus insoluble brain Abeta distinguish Alzheimer's disease from normal and pathologic aging." Exp Neurol **158**(2): 328-337.

Wang, X., F. Cattaneo, L. Ryno, J. Hulleman, N. Reixach and J. N. Buxbaum (2014). "The systemic amyloid precursor transthyretin (TTR) behaves as a neuronal stress protein regulated by HSF1 in SH-SY5Y human neuroblastoma cells and APP23 Alzheimer's disease model mice." J Neurosci **34**(21): 7253-7265.

Weisner, B. and H. J. Roethig (1983). "The concentration of prealbumin in cerebrospinal fluid (CSF), indicator of CSF circulation disorders." Eur Neurol **22**(2): 96-105.

Westermark, P., K. Sletten, B. Johansson and G. G. Cornwell, 3rd (1990). "Fibril in senile systemic amyloidosis is derived from normal transthyretin." Proc Natl Acad Sci U S A **87**(7): 2843-2845.

Whitehead, A. S., M. Skinner, G. A. Bruns, W. Costello, M. D. Edge, A. S. Cohen and J. D. Sipe (1984). "Cloning of human prealbumin complementary DNA. Localization of the gene to chromosome 18 and detection of a variant prealbumin allele in a family with familial amyloid polyneuropathy." Mol Biol Med **2**(6): 411-423.

Woeber, K. A. and S. H. Ingbar (1968). "The contribution of thyroxine-binding prealbumin to the binding of thyroxine in human serum, as assessed by immunoadsorption." J Clin Invest **47**(7): 1710-1721.

Xu, K., G. Zhong and X. Zhuang (2013). "Actin, spectrin, and associated proteins form a periodic cytoskeletal structure in axons." Science **339**(6118): 452-456.

Yuan, A., M. V. Rao, Veeranna and R. A. Nixon (2012). "Neurofilaments at a glance." J Cell Sci **125**(Pt 14): 3257-3263.

Zempel, H., E. Thies, E. Mandelkow and E. M. Mandelkow (2010). "Abeta oligomers cause localized Ca(2+) elevation, missorting of endogenous Tau into dendrites, Tau phosphorylation, and destruction of microtubules and spines." J Neurosci **30**(36): 11938-11950.

Zhou, R. M., Y. X. Huang, X. L. Li, C. Chen, Q. Shi, G. R. Wang, C. Tian, Z. Y. Wang, Y. Y. Jing, C. Gao and X. P. Dong (2010). "Molecular interaction of alpha-synuclein with tubulin influences on the polymerization of microtubule in vitro and structure of microtubule in cells." Mol Biol Rep **37**(7): 3183-3192.

Zhou, Y., G. Gu, D. R. Goodlett, T. Zhang, C. Pan, T. J. Montine, K. S. Montine, R. H. Aebersold and J. Zhang (2004). "Analysis of alpha-synuclein-associated proteins by quantitative proteomics." J Biol Chem **279**(37): 39155-39164.

Zhu, X., A. K. Raina, H. Boux, Z. L. Simmons, A. Takeda and M. A. Smith (2000). "Activation of oncogenic pathways in degenerating neurons in Alzheimer disease." Int J Dev Neurosci **18**(4-5): 433-437.

Zolyomi, Z., M. D. Benson, K. Halasz, T. Uemichi and G. Fekete (1998). "Transthyretin mutation (serine 84) associated with familial amyloid polyneuropathy in a Hungarian family." Amyloid **5**(1): 30-34.