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New insights on the impact of Transthyretin in neuroprotection and neurodegeneration

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SEDE ADMINISTRATIVA **INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**
FACULDADE DE CIÊNCIAS

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NEW INSIGHTS ON THE IMPACT OF TRANSTHYRETIN IN NEUROPROTECTION AND NEURODEGENERATION

Tese de Candidatura ao grau de Doutor em Biologia Molecular e Celular
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Eigentlich weiß man nur, wenn man wenig weiß.
Mit dem Wissen wächst der Zweifel.

(We know accurately only when we know little.
With knowledge, doubt increases.)

Johann Wolfgang Goethe

À minha família,

In this thesis the following published and submitted manuscripts were used:

Eira J, Silva CS, Sousa MM and Liz MA. *The cytoskeleton as a novel therapeutic target for old neurodegenerative disorders*. Progress in Neurobiology, 141, 61-82. (2016) doi:10.1016/j.pneurobio.2016.04.007

Magalhães J*, **Eira J**, Liz MA*. *The role of transthyretin on cell biology: a critical point to prevent pathology. (Submitted)*

Silva CS*, **Eira J***, Ribeiro CA, Oliveira A, Sousa MM, Cardoso I and Liz MA. *Transthyretin neuroprotection in Alzheimer's disease is dependent on proteolysis*. Neurobiology of Aging, 59, 10-14. (2017)

Eira J, Macedo N, Pero ME, Sousa MM, Bartolini F and Liz MA. *Transthyretin impact on nerve biology is linked to regulation of microtubule stability by tubulin acetylation. (Submitted).*

Eira J*, Magalhães J*, Macedo N, Socodato R, Hsieh S, Liz MA. *Dissecting neuronal cytoskeleton alterations in Familial Amyloid Neuropathy. (in preparation)*

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SUMMARY

Transthyretin (TTR), a protein mainly synthesized in the liver and choroid plexus of the brain, has been implicated in the regulation of nerve biology. TTR has neuritogenic activity, being able to increase axon regeneration *in vivo* and neurite outgrowth *in vitro*. In pathology, mutations in TTR lead to Familial Amyloid Polyneuropathy (FAP), a neurodegenerative disease characterized by the extracellular deposition of oligomers, aggregates and amyloid fibres of mutant TTR, particularly in the peripheral nervous system (PNS). As a consequence of TTR deposition, a dying-back axon degeneration occurs ultimately leading to neuronal death. These findings point out the intriguing characteristics of TTR, which physiologically is a neuritogenic protein but under pathological conditions induces neurodegeneration. Nevertheless, the cellular and molecular details underlying this dual role remain unravelled.

TTR was also shown to be a metalloprotease with an impact on amyloid β (Aβ) peptide generating Aβ peptides with lower amyloidogenic potential than the full-length counterpart. By using a proteolytically inactive mutant of TTR, we demonstrated that TTR proteolysis is required to reduce Aβ fibrillogenesis, to degrade neuronal-secreted Aβ, and to reduce Aβ-induced toxicity in hippocampal neurons. These results clearly show that TTR proteolytic activity is required for its neuroprotective role in Alzheimer's disease (AD), constituting a novel therapeutic target for the disease.

Abnormalities in cytoskeletal organization are a common feature of many neurodegenerative disorders and, in FAP, it is pertinent to consider cytoskeleton damage as a consequence of TTR deposition, since alterations in microtubule stability and axonal transport are emerging as common features of dying-back axonopathies. Thus, we investigated the hypothesis whether neuronal cytoskeleton modulation underlies the dual role of TTR on nerve biology. Using FAP and TTR KO animal models we show that TTR orchestrates the neuronal cytoskeleton organization. In pathologic conditions TTR was shown to promote Rac1 mediated impairment of the neuronal actin cytoskeleton organization, as well as alterations in microtubular cytoskeleton and axonal transport defects which need to be further dissected. Physiologically, TTR was shown to modulate MT stability by regulating α-tubulin acetylation levels.

To further unravel the means of this dual function of TTR in nerve biology, we performed a quantitative proteomics assay to pinpoint molecular targets that are differentially expressed in neuronal cells in the presence of either WT soluble TTR or TTR L55P aggregates. Several proteins were identified as being modulated by TTR aggregates including proteins involved in pathways implicated in neuroinflammatory

diseases such as STAT2, and proteins with cytoskeleton remodelling functions such as BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3.

Altogether, our findings highlight the neuronal cytoskeleton as a novel player in TTR impact in nerve pathophysiology, with an interesting potential for the development of new therapeutic strategies for FAP.

SUMÁRIO

A transtirretina (TTR), uma proteína sintetizada no fígado e no plexo coróide do cérebro, tem sido implicada na regulação da biologia do sistema nervoso. A TTR tem actividade neuritogénica, sendo capaz de aumentar a regeneração axonal in vivo e o crescimento neuronal in vitro. Patologicamente, mutações na TTR despoletam a Polineuropatia amiloidótica familiar (PAF), uma doença neurodegenerativa caracterizada pela deposição extracelular de oligómeros, agregados e fibras de TTR mutada, em particular no sistema nervoso periférico (SNP). Como consequência da deposição da TTR, ocorre uma degeneração neuronal do tipo "dying-back" que conduz, ultimamente, à morte neuronal. Estas observações realçam uma característica intrigante da TTR, que fisiologicamente é neuritogénica, mas em condições patológicas, induz neurodegeneração. Contudo, os mecanismos celulares e moleculares que motivam esta dupla função da TTR continuam por clarificar.

A TTR também foi descrita como tendo uma função de metaloprotease com um impacto sobre o péptido β-amilóide (Aβ) gerando péptidos Aβ com menor potencial amiloidogénico que a sua versão inteira. Usando um mutante da TTR proteoliticamente inactivo, nós demonstrámos que a proteólise da TTR é necessária para reduzir a fibrilogénese do Aβ, degradar o Aβ secretado por neurónios e reduzir a toxicidade provocada pelo Aβ em neurónios do hipocampo. Estes resultados mostram, claramente, que a actividade proteolítica da TTR é necessária para a neuroprotecção na doença de Alzheimer (DA), constituindo um novo alvo terapêutico para o tratamento da doença.

Alterações na organização do citoesqueleto neuronal são um factor comum em muitas doenças neurodegenerativas e, em PAF, é pertinente considerar danos no citoesqueleto como consequência da deposição da TTR uma vez que, alterações na estabilidade dos microtúbulos e no transporte axonal estão a emergir como acontecimentos comuns em neuropatias do tipo "dying-back". Por isso, nós investigámos a hipótese de que a função dual da TTR passa por um papel na modulação do citoesqueleto neuronal. Recorrendo a modelos animais de PAF e TTR KO nós demonstramos que a TTR regula o citoesqueleto neuronal. Em condições patológicas, nós mostrámos que a TTR provoca defeitos no citoesqueleto da actina mediado por Rac1, e também alterações nos microtúbulos e transporte axonal, que necessitam de ser dissecados mais exaustivamente. Fisiologicamente, a TTR mostrou-se como sendo um modulador da estabilidade de microtúbulos através da regulação dos níveis de α-tubulina acetilada.

De forma a decifrar mecanismos adicionais da dupla função da TTR na biologia do sistema nervoso, fizemos um ensaio de proteómica quantitativa para revelar alvos

moleculares expressos de forma diferencial em células neuronais tanto na presença de TTR WT como na presença de agregados de TTR L55P. Várias proteínas foram identificadas como estando diferencialmente expressas na presença de agregados de TTR incluindo proteínas envolvidas em doenças neuroinflamatórias, tal como a STAT2, e proteínas com funções de remodelação do citoesqueleto, tal como a proteína "BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3".

Em conclusão, as nossas observações realçam o citoesqueleto neuronal como um novo alvo no impacto da TTR na patofisiologia do nervo, com um potencial interessante no desenvolvimento de novas estratégias terapêuticas em PAF.

ABBREVIATIONS

ABP	Actin-binding protein
AD	Alzheimer's disease
ADF	Actin depolymerizing factor
ADP	Adenosine diphosphate
AIS	Axonal initial segment
ALS	Amyotrophic lateral sclerosis
ANS	Autonomic nervous system
AP-1	Activating protein 1
APC	Adenomatous polyposis coli
ApoA-I	Apolipoprotein A-I
apoE	Apolipoprotein E
apoJ	Apolipoprotein J
APP	Amyloid β precursor protein
Arp2/3	Actin related protein 2/3 complex
ASO	Antisense oligonucleotides
ATAT1	α-tubulin N-acetyltransferase 1
ATP	Adenosine triphosphate
ATTR	Amyloid TTR
Aβ	Amyloid β peptide
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CAMSAP	Calmodulin regulated spectrin-associated proteins
CCP	Cytosolic carboxypeptidase
Cdc42	Cell division control protein 42 homolog
cDNA	complementary DNA
CLASP	CLIP-associating proteins
CMT	Charcot-Marie-Tooth disease
CNS	Central nervous system
CO_2	Carbon Dioxide
CRMP2	Collapsing response mediator protein 2
CSF	Cerebrospinal fluid
DCTN1	Dynactin
DEAE	Diethylaminoethyl cellulose
DIV	Days *in vitro*
DLK-1	Dual leucine zipper kinase
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DNCHC1	Dynein heavy chain
DRG	Dorsal root ganglion
DTT	Dithiothreitol
EB	End binding protein
EB3	End binding protein 3
EB3-GFP	End binding protein 3 fused to green fluorescent protein
EB3-YFP	End binding protein 3 fused to yellow fluorescent protein
ECL	Enhanced chemiluminescent reagent
ECM	Extracellular matrix
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
F-actin	Filamentous actin
FAC	Familial Amyloid Cardiomyopathy
FAP	Familial amyloid polyneuropathy
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FluoU	Fluorodeoxyuridine
FRET	Fluorescence resonance energy transfer
G-actin	Globular actin
GAP	GTPase-activating proteins
GDI	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GIT	Gastrointestinal tract
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HD	Huntington’s disease
HDAC6	Histone deacetylase 6
HDL	High-density lipoproteins
HepG2	Human hepatoma cells
HNF	Hepatocyte nuclear factor
HRP	Horseradish peroxidase
HSF1	Heat shock transcription factor 1
HSPB1	Small heat-shock protein 1
HSPBAP1	HSPB1-associated protein 1
IF	Intermediate filaments
IGF-1R	Insulin-like growth factor type 1 receptor
JAK/STAT	Janus kinase/signal transducers and activators of transcription

kDa	Kilodalton
KIF	Kinesin superfamily proteins
KO	Knock-out
LAMP-1	Lysosomal-associated membrane protein 1
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LIMK	LIM kinase
LRP1	Low density lipoprotein receptor-related protein 1
MAP	Microtubule associated protein
MCAK	Mitotic centromere-associated kinesin
mNIS+7	Modified Neuropathy Impairment Score + 7 neurophysiologic tests
MPS	Membrane-associated periodic skeleton
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimide
mRNA	Messenger ribonucleic acid
MT	Microtubule
MTOC	Microtubule organizing centre
NDS	Normal donkey serum
NE	Norepinephrine
NF	Neurofilament
NF-H	Neurofilament heavy
NF-L	Neurofilament light
NF-M	Neurofilament medium
NF-κB	Nuclear factor κ B
NGF	Nerve growth factor
NPY	Neuropeptide Y
OCT	Optimum Cutting Temperature
ORF	Open reading frame
PAK	p21-activated kinase
PBS	Phosphate buffer saline
PD	Parkinson’s disease
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PFN1	Profilin 1
PI3K	Phosphatidylinositol 3-kinase
PIPES	Piperazinediethanesulfonic acid
PLL	Poly-L-lysine
pMCAO	Permanent middle cerebral artery occlusion
PNS	Peripheral nervous system
PTM	Post translational modification
QOL	Quality of life

Rac1	Ras-related C3 botulinum toxin substrate 1
RAG	Regeneration-associated genes
RAGE	Receptor for advanced glycation end products
RAP	Receptor-associated protein
RBP	Retinol binding protein
RFU	Relative fluorescence units
RhoA	Ras homologous member A
ROCK	RhoA kinase
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RT	Room temperature
rt-PCR	Reverse transcription polymerase chain reaction
RTK	Receptor tyrosine kinase
SAP	Serum amyloid P
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIRT2	Sirtuin-2
SOD1	Superoxide dismutase 1
SSA	Senile Systemic Amyloidosis
SSH1	Protein phosphatase Slingshot homolog 1
STAT	Signal transducer and activator of transcription
T4	Thyroxine
TBG	Thyroxine binding globulin
TBS-T	Tris-buffered saline, 0,1% Tween
TEM	Transmission electron microscopy
Th-T	Thioflavin-T
TMT	Tandem mass tagged
TTL	Tubulin tyrosine ligase
TTLL	Tubulin tyrosine ligase-like
TTR	Transthyretin
UF	Ubiquitous factor
WASP	Wiskott-Aldrich syndrome protein
WT	wild-type

INTRODUCTION

TRANSTHYRETIN

Transthyretin (TTR), previously known as pre-albumin, was firstly described in 1942 in human cerebrospinal fluid (CSF) by Kabat and colleagues (Kabat, Moore, & Landow, 1942) and originally named after its ability to migrate ahead of albumin on standard protein electrophoresis. Later, TTR was also described in the human serum (Seibert & Nelson, 1942). This protein is mainly synthesized in the liver (Felding & Fex, 1982; Raz & Goodman, 1969) and the choroid plexus of the brain (Aleshire, Bradley, Richardson, & Parl, 1983), which contributes to the pools of the protein in the plasma and CSF, respectively. TTR owes its actual name to its mainly described physiological functions. Transthyretin comes, thus, from the fact that this protein is mainly characterized as being a **trans**porter of **thy**roxine and **retin**ol. Nevertheless, deeper knowledge regarding this protein unveils a plethora of other physiological functions that, although described more recently, can in no way be overseen. The next sections will focus on dissecting these functions to, ultimately, pinpoint TTR as an admirable study case in health and disease.

GENE STRUCTURE

The *TTR* gene is a single copy-gene (Tsuzuki, Mita, Maeda, Araki, & Shimada, 1985) located in the long arm of chromosome 18 (Whitehead et al., 1984), namely in the 18q11.2-q12.1 region (Sparkes et al., 1987). It is a 7,6 kilobase (kb) long gene which contains four exons of 95, 131, 136 and 253 base pairs (respectively, exon 1, 2 3 and 4) and three introns of 934, 2090 and 3308 base pairs (Sasaki, Yoshioka, Takagi, & Sakaki, 1985; Tsuzuki et al., 1985). The first exon encodes a signal peptide of 20 aminoacids (which is removed post-translationally) and 3 aminoacids of the mature protein, exons 2, 3 and 4 encode the aminoacid residue sequences 4-47, 48-92, 93-127, respectively, of mature TTR (Sasaki et al., 1985; Tsuzuki et al., 1985). One of the particular features of this gene is the presence of two independent open reading frames (ORF), in the first and third introns, with the same transcription direction as *TTR* and putative consensus regulatory sequences for transcription but their function is not clear (Soares, Centola, Chae, Saraiva, & Kastner, 2003; Tsuzuki et al., 1985). In the 5'-flanking region, upstream from the transcription initiation site, several consensus sequences were found: two overlapping sequences with extensive homology to the glucocorticoid-responsive element at positions -224 and -212 base pairs, a CAAT box from -101 to -96 base pairs, a TATA box from -30 to -24 base pairs and a GC-rich region of about 20 base pairs. In the 3'-

untranslated region, downstream the coding sequence, a polyadenylation signal (AATAAA) was identified at position 123 (Sasaki et al., 1985) (Fig. 1).

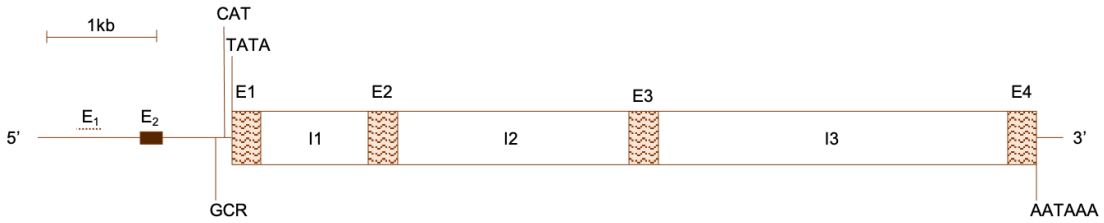

Figure 1. Schematic representation of the human *TTR* gene structure. Exons 1 to 4 (E1 to E4) and introns 1 to 3 (I1 to I3); E_1: sequence homologous to mouse enhancer; E_2: human specific enhancer; GCR: glucocorticoid responsive element; CAT: CAAT box; TATA: TATA box; AATAAA: polyadenylation signal.

The *TTR* gene is highly conserved throughout evolution. The gene was extensively characterized in the 80s in humans (Sasaki et al., 1985), rat (Fung et al., 1988), and mouse (R. H. Costa, Lai, & Darnell, 1986). The mouse and rat genes are also composed by four exons and show a high degree of homology when compared to the human *TTR* gene sequence. The DNA sequence of the coding region of the mouse *TTR* gene shows 82% and 90% homology with the human and rat gene, respectively (the aminoacid sequence homology is higher, with 91% homology to the human and 96% to the rat aminoacid sequences) (R. H. Costa et al., 1986).

GENE REGULATION

Cell-type-specific transcription is the first event, and perhaps one of the most crucial events, leading to expression of specific genes in differentiated cell types. TTR is a protein subjected to that type of regulatory mechanism which leads to its distinct expression profile in the liver and the choroid plexus (P. W. Dickson, Aldred, et al., 1985; P. W. Dickson, Howlett, & Schreiber, 1985).

Two major regulatory regions are present in the 5' flanking region of the *TTR* gene: a proximal promoter sequence at -90 to -150 base pairs and a distal enhancer sequence at -1860 to -1960 base pairs (R. H. Costa et al., 1986; R. H. Costa, Lai, Grayson, & Darnell, 1988). Studies performed in mice identified several possible regulatory sites in the aforementioned promoter and enhancer regions such as the members of four different liver-enriched transcription factor families (HNF-1, HNF-3, HNF-4, and C/EBP proteins) (R. H. Costa, Grayson, & Darnell, 1989; R. H. Costa, Grayson, Xanthopoulos, & Darnell, 1988), HNF-6 (Samadani & Costa, 1996), and the growth factor-inducible AP-1 protein

(Qian, Samadani, Porcella, & Costa, 1995). The regulation of the human gene is less known, but homology studies revealed that several regulatory signals exist in the 5' flanking region of the gene, including binding sites for the nuclear factors HNF-1, 3, 4 and C/EBP (Sakaki, Yoshioka, Tanahashi, Furuya, & Sasaki, 1989). The TTR enhancer is also recognized by an uncharacterized ubiquitous factor and contains an HNF-3 binding site specific for the HNF-3β isoform as depicted in Figure 2 (Samadani, Qian, & Costa, 1996). These regions are sufficient for directing TTR expression in the liver (Samadani & Costa, 1996). However, *TTR* gene expression also occurs in the choroid plexus, but little is known about the regulatory mechanisms besides the fact that the expression of mouse TTR in the choroid plexus must involve different DNA regulatory elements and factors to stimulate transcription from the identical initiation site that is used in the liver (Nagata et al., 1995; Yan, Costa, Darnell, Chen, & Van Dyke, 1990). While a shorter sequence of approximately 0.7 kb single mRNA upstream the mRNA cap site is sufficient to drive TTR expression in the liver, choroid plexus expression requires the presence of a further upstream sequence (over 3 kilobases) (Nagata et al., 1995; Yan et al., 1990). The TTR mRNA encodes a pro-TTR monomer, which contains a 20- amino acid signal peptide located in the N-terminal region. Pro-TTR is processed during its migration through membrane of the endoplasmic reticulum to yield the native TTR monomer (Soprano, Herbert, Soprano, Schon, & Goodman, 1985).

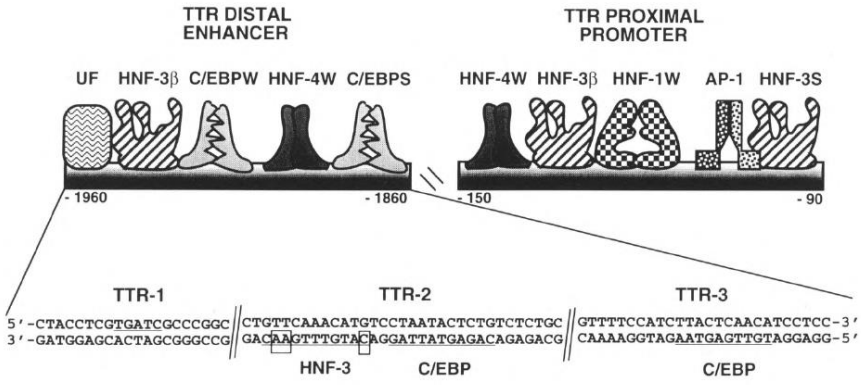

Figure 2. Schematic representation of TTR regulatory regions with its transcription factors. On the TTR promoter and enhancer regions are the weak affinity protein binding sites for the hepatocyte nuclear factor-1 (HNF-1W), HNF-4W, activating protein-1 (AP-1), and ubiquitous factor (UF). The sequences of three oligonucleotides in the TTR enhancer are shown (TTR-1, TTR-2, and TTR-3) and indicated on the sequences are the positions of the C/EBP and HNF-3 binding sites (boxed residues indicate mismatches with the HNF-3 consensus). The TTR-1 and TTR-2 oligonucleotide sequences are separated by two nucleotides whereas the TTR-2 and TTR-3 oligonucleotide sequences are separated by 14 nucleotides that contain the HNF-4 binding site. Adapted from Samadani et al., 1996.

PROTEIN STRUCTURE

TTR is a 55kDa homotetrameric protein with each monomer composed by 127 aminoacids (Kanda, Goodman, Canfield, & Morgan, 1974) and approximately 14kDa of weight. The X-ray crystal structure was resolved at 1.8Å by Blake and colleagues in 1978 (Blake, Geisow, Oatley, Rerat, & Rerat, 1978) where they showed a β-sheet-dominant secondary structure. The polypeptide chain is arranged in 8 antiparallel β-strands, termed A through H, forming the monomer that contains two β-sheets composed by strands DAGH and CBEF (Fig. 3A). The β-strands are 6 to 9 residues long, excepting strand D that has only 3 residues. The β-sheets are linked by seven loops and a small helical region (α-helix), involving amino acids 75 to 83, located at the end of strand E. The N- and C-terminal regions of each monomer are, respectively, composed by 10 and 5 unorganized residues. To form the dimer, two monomers associate through hydrogen bonds between strands H and F of each monomer, leading to the extension of the two 4-stranded-sheets (in each monomer) to two 8-stranded sheets (in the dimer). The resulting strand arrangement in the dimer is DAGHH'G'A'D' and CBEFF'E'B'C' where the prime symbol refers to a strand from a different monomer (Fig. 3A). To form the tetramer, the interactions between the two dimers are established by hydrogen bonds between main chain atoms belonging to loop AB of one monomer and strand H' from the other monomer as well as by hydrophobic contacts. These latter interactions are weaker than the ones established by the monomers in a dimer, which is of significant importance when discussing tetramer disassembly kinetics and amyloid formation (subject further discussed in the next chapters).

The assembly of the tetramer with their inner eight-stranded sheets DAGHH'G'A'D' face-to-face leads to the formation of a narrow cylindrical hydrophobic channel running through the centre of the molecule (Fig. 3B) (Blake et al., 1978; Blake, Geisow, Swan, Rerat, & Rerat, 1974). This channel carries two symmetrical binding sites, being able to accommodate two T_4 molecules, one of the major TTR physiological ligands. Besides thyroxine (T4), TTR is also the main carrier of retinol (vitamin A), and establishes a 1:1 molar complex with retinol binding protein (RBP), which binding is limited to a maximum of two RBP molecules per TTR tetramer (Monaco, Rizzi, & Coda, 1995).

On an evolutionary standpoint, it is noteworthy to mention that the three-dimensional structure of TTR is conserved among vertebrates. This observation is supported by the similar crystal structures determined for human (Blake et al., 1978), chicken (Sunde et al., 1996), rat (Wojtczak, 1997), mouse (Reixach et al., 2008) and sea bream (Eneqvist et al., 2004) TTR. Vital parts of the protein such as the hydrophobic core and areas involved in the quaternary structure interactions or ligand binding sites are maintained. An exception

in this latter scenario would be chicken TTR which, when compared to wild-type human TTR displays differences in the region known to be involved in binding to RBP, has a shorter helical component than human TTR and also some of the monomer-monomer interactions are different (Sunde et al., 1996). Regarding the primary structure, the TTR sequence region that changed the most during vertebrate evolution was the N-terminal region. In lower vertebrates this region is longer and more hydrophobic, while in eutherians the N-terminal region is shorter and more hydrophilic (Prapunpoj, Richardson, Fumagalli, & Schreiber, 2000; Schreiber & Richardson, 1997).

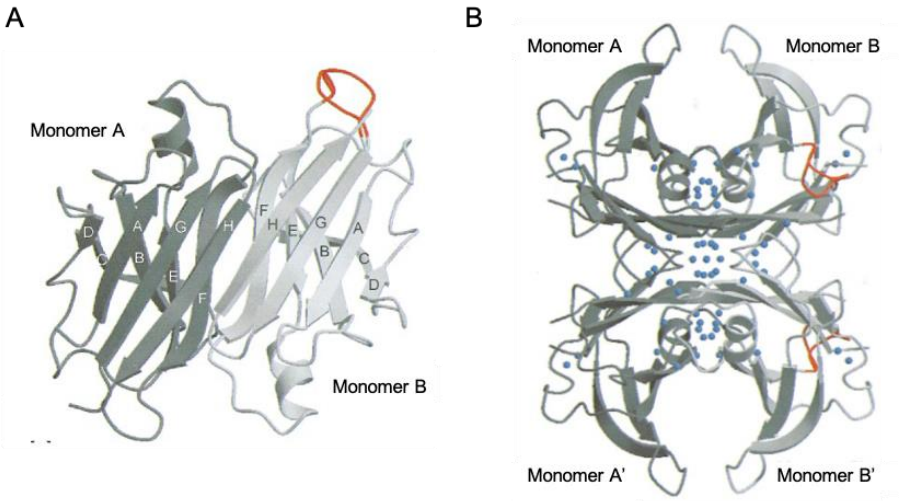

Figure 3. Ribbon drawing of the transthyretin structure. (A) Structure of the dimer with monomer A coloured in dark gray and monomer B in light gray. The β-strands from each monomer are denoted A-H as suggested by Blake *et al.* (1978). Two β-sheets (D-A-G-H and C-B-E-F) in each monomer form a β-barrel. Two monomers dimerize through an intermolecular mainchain interaction involving the H-strands from each monomer to form a continuous eight-stranded β-sheet. The two paths of the FG-loop in the B monomer are shown in red. (B) Structure of the tetramer generated by applying 2-fold crystallographic symmetry operator on the dimer in the asymmetric unit. The two dimers interact through hydrophobic contacts involving the loop regions between β-strands G and H and β-strands A and B. The thyroxine-binding sites are situated in one large hydrophobic channel formed between the two dimers. The positions of 36 buried water molecules are indicated as blue spheres. Adapted from Hornberg, Eneqvist, Olofsson, Lundgren, & Sauer-Eriksson, 2000.

PROTEIN EXPRESSION

TTR is expressed at an early stage in embryonic development. In mice, for example, TTR mRNA is detected at the 10th day of gestation (Murakami et al., 1987). The two major sites of TTR expression are the liver and the epithelial cells of the choroid plexus in the brain. This gene expression pattern is already extensively described throughout different species, namely rat (P. W. Dickson, Howlett, et al., 1985; Fung et al., 1988), human (P. W. Dickson & Schreiber, 1986), sheep (Schreiber et al., 1990), chicken (Southwell, Duan,

Tu, & Schreiber, 1991), and pig (Duan, Richardson, Kohrle, et al., 1995). Curiously, it was shown that, when normalized by the tissue mass, the choroid plexus expresses 100 times larger amounts of TTR mRNA than the liver (P. W. Dickson, Aldred, et al., 1985). Also, secretion of TTR is regulated independently in the liver and choroid plexus (P. W. Dickson, Aldred, Marley, Bannister, & Schreiber, 1986). Transthyretin was also shown to be expressed in the endothelial cells of Islets of Langerhans (G. T. Westermark & Westermark, 2008), in the ciliary pigment epithelia (Kawaji et al., 2005) and, although in less amounts, in the retinal pigment epithelium (RPE) (Cavallaro, Martone, Dwork, Schon, & Herbert, 1990; Mizuno, Cavallaro, & Herbert, 1992), which acts as blood barrier for the retina (Pfeffer, Becerra, Borst, & Wong, 2004). In the eye, TTR and RBP are synthesized in RPE cells and secreted together from the interphotoreceptor matrix, located at the apical side of these cells (Cavallaro et al., 1990). There are some suggestions for the role of TTR as a carrier of T4 during eye development (Ong, Davis, O'Day, & Bok, 1994). Expression of TTR in other tissues were also suggested such as the pancreas (Kato, Kato, Blaner, Chertow, & Goodman, 1985), intestine (Loughna, Bennett, & Moore, 1995), visceral yolk sac during fetal development in rats (Soprano, Soprano, & Goodman, 1986), and, in fairly minor amounts, stomach, heart, skeletal muscle and spleen from rats (Soprano et al., 1985). One study also reported expression of TTR in the meninges (Blay, Nilsson, Owman, Aldred, & Schreiber, 1993) and, more recently, TTR synthesis has also been demonstrated in the human placenta (McKinnon, Li, Richard, & Mortimer, 2005). Importantly, TTR expression has also been suggested to occur in dorsal root ganglia (DRG) neurons (Murakami, Ohsawa, & Sunada, 2008), however these observations are a target of controversy due to claims clarifying that TTR expression in DRG is rather the product of artefacts (M. M. Sousa & Saraiva, 2008). Nonetheless, the latest reports in this regard clarified, using rt-PCR, that Schwann cells and DRG of peripheral nerves in mice, indeed, express TTR (Murakami, Ohsawa, Zhenghua, Yamamura, & Sunada, 2010).

The expression of TTR is modulated in developmentally-specific and species-specific manners since the levels of TTR vary in some species and according to its developmental stage. Some of the examples were studied by Richardson and colleagues where they showed that TTR is detected in the plasma of fetal and adult eutherians, and of some marsupials and birds, but not in the plasma of adult reptiles, amphibians and fish (Richardson et al., 2005). TTR has been identified in all classes of vertebrates including fish, where some species express TTR during development throughout life such as the case of *Sparus aurata* (Funkenstein, Perrot, & Brown, 1999; C. R. Santos & Power, 1999). In the case of Australian polyprotodont marsupials TTR is synthesized by the liver only during development and by the choroid plexus throughout life (Duan, Richardson, Babon, et al., 1995; Richardson et al., 2005), while in diprotodont marsupials, and eutherians,

TTR is synthesized by both, the liver and the choroid plexus, throughout life (Richardson et al., 2002; Richardson et al., 2005). The synthesis of TTR by the liver and choroid plexus was detected in several species of birds during development and in adult stage (Southwell et al., 1991). In the amphibian species studied, the expression of TTR was shown in the liver during development (Prapunpoj, Yamauchi, Nishiyama, Richardson, & Schreiber, 2000). Some species of reptiles (eg. *S. punctatus*) detected the expression of TTR by the liver during development (Richardson et al., 2005), others in the choroid plexus (eg. *T. rugosa*) (Achen et al., 1993), and others in the eye (*C. porosus*) (Prapunpoj, Richardson, & Schreiber, 2002). In non-vertebrates, proteins with an aminoacid sequence similar to human TTR have been described and named TTR-like proteins. The TTR-like protein genes were identified in bacteria (*E. coli*), in invertebrates (*C. elegans*), in plants (*A. thaliana*). Similar to TTR, these homologue proteins present a tetrameric structure although they present different localizations and functions, as they do not bind hormones or similar ligands (Hennebry, Wright, Likic, & Richardson, 2006). Searching for TTR sequence homologues in *D. Melanogaster* there is a protein that exhibits some level of homology with TTR (CG30016). It belongs to the TTR/hydroxyisourate hydrolase superfamily, however there is no description if the protein is a homotetramer and if exerts the same functions as in mammals.

The conservation of TTR expression pattern in choroid plexus from reptiles to mammals throughout life led to the hypothesis that the expression of the TTR gene first arose in the brain of reptiles which overlapped with an increase in size of the brain, as the reptiles were the first species possessing traces of a cortex (Schreiber & Richardson, 1997). On the other hand, the existence of TTR in fish suggested that TTR evolved from a common fish ancestor of the tetrapod evolutionary line (C. R. Santos & Power, 1999). During human embryonic development, TTR is first expressed in the tela choroidea, the precursor of the choroid plexus, followed by expression in the liver (Harms et al., 1991; Richardson et al., 1994). This TTR expression behaviour in the choroid plexus, conserved throughout evolution and starting early in embryonic development, points to a convincing role of TTR in the nervous system.

PROTEIN METABOLISM

The total body TTR turnover in humans is 250-300 mg/m^2/day (Vahlquist, Peterson, & Wibell, 1973). The biological half-life of TTR is about 2-3 days in humans (Socolow, Woeber, Purdy, Holloway, & Ingbar, 1965; Vahlquist et al., 1973), 22-23 hours in monkeys (Vahlquist & Peterson, 1972) and 29 hours in rats (P. W. Dickson, Howlett, & Schreiber,

1982). As aforementioned, TTR is mainly synthesized in the liver and the choroid plexus being the liver the main source of the plasma-circulating TTR. Plasma concentration of TTR ranges from 170 to 420 μg/ml (Vatassery, Quach, Smith, Benson, & Eckfeldt, 1991). The protein synthesized in the epithelial cells of the choroid plexus is secreted into the CSF (P. W. Dickson et al., 1987), where TTR concentration varies from 5 to 20 μg/mL (Vatassery et al., 1991) and represents 20% of the total CSF proteins (Weisner & Roethig, 1983). TTR degradation occurs in its majority in the liver (36-38% of total body TTR degradation), followed by the muscle (12-15%) and skin (8-10%). In minor amounts (1-8%), TTR is also catabolized in tissues including kidneys, adipose tissue, testes, and the gastrointestinal tract (GIT). The same degradation sites were determined for both TTR synthesized in the liver and in the choroid plexus; no TTR degradation was observed in the nervous system (Makover et al., 1988).

Not all metabolic mechanisms of TTR are fully understood, particularly its cellular uptake. Studies using human hepatoma cells (HepG2) suggest TTR internalization is receptor-mediated (Divino & Schussler, 1990). This work described a saturable high affinity binding of TTR to human hepatomas, which corroborates the existence of a TTR-receptor. Sousa M. and colleagues described, a decade later, a mechanism involving Megalin, a multiligand receptor, member of the low-density lipoprotein receptor (LDLr) family, for renal uptake of TTR (M. M. Sousa, Norden, et al., 2000) (Fig. 4). Megalin is a member of the low-density lipoprotein (LDL) receptor family and is involved in the receptor-mediated endocytosis of a wide range of ligands. The ability of TTR to bind megalin was shown to be similar in the presence or absence of its ligands, T4 and RBP. As RBP is also a megalin ligand, it was proposed that this renal receptor had a role in TTR-mediated renal uptake of T4 (M. M. Sousa, Norden, et al., 2000). Besides the kidney, megalin is also expressed in the nervous system, in oligodendrocytes (Wicher et al., 2006), astrocytes (Bento-Abreu et al., 2008) and neurons, including retinal ganglion cells (Fitzgerald et al., 2007), cerebellar granule neurons (Ambjorn et al., 2008), cortical neurons (Chung et al., 2008) and sensory dorsal root ganglion (DRG) neurons (Fleming, Mar, Franquinho, Saraiva, & Sousa, 2009).

In the liver, a yet unidentified receptor-associated protein (RAP)-sensitive receptor was shown to also mediate TTR internalization (Fig. 4) (M. M. Sousa & Saraiva, 2001) and, more recently, was also shown to bind and regulate the expression of the insulin-like growth factor 1 receptor (IGF-1R) (Vieira, Gomes, & Saraiva, 2015).

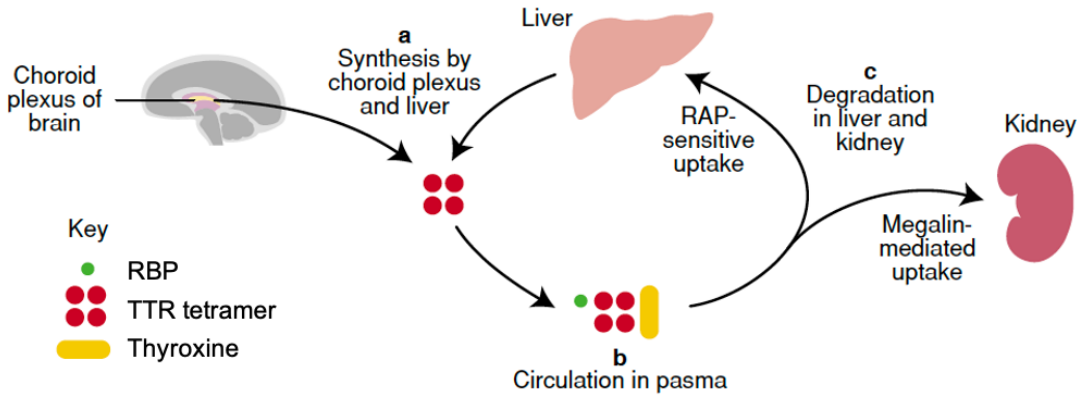

Figure 4. Transthyretin synthesis and metabolism. The TTR tetramer (red circles) is synthesized in the liver and choroid plexus of the brain (a), circulating in plasma bound to RBP (green circle) and T4 (yellow bar) (b). TTR internalization, in the liver, is mediated by RAP-sensitive uptake, and, in the kidney, through megalin receptor (c). Adapted from Saraiva, 2002.

TRANSTHYRETIN IN PATHOPHYSIOLOGY

Transthyretin is a case of study not only regarding its role in pathology (discussed in the next section) but also regarding its physiologic roles. TTR is described mainly as a transporter protein but has a flagrant importance as a protease and in nerve biology and repair. All functions will be dissected in this section.

TRANSTHYRETIN AS A TRANSPORTER

As mentioned above, the most recognised physiological role for TTR is the transport of thyroid hormones, like T_4, and retinol, in the latter case by association with RBP. Thyroid hormones are transported in blood circulation and delivered to the target tissues mostly due to binding to serum proteins. Regarding T_4 transport, TTR is not an exclusive transporter since other plasma proteins are capable of binding T_4 such as thyroxine binding globulin (TBG), albumin and lipoproteins. Although TBG is much less concentrated in the plasma than TTR, it presents the highest affinity constant for T_4 and transports about 70% of the plasma T_4. TTR has an intermediate affinity for T_4 and transports about 15% of the hormone, and finally, albumin, which presents the lowest binding affinity is accountable for 10% of T_4 transport (Loun & Hage, 1992). In rodents, the amount of TTR-bound T_4 is approximately 50% (Hagen & Solberg, 1974). In contrast, in the CSF, TTR is the major T_4 carrier, transporting 80% of this hormone, both in humans and mice (Robbins, 2000).

Retinol, or vitamin A, circulates in the plasma bound to the RBP. RBP is synthesized and secreted primarily by hepatocytes and is the sole specific transport protein for retinol in the circulation. In the plasma, RBP-retinol is found in a complex with TTR and TTR is responsible for the binding of almost all RBP (95%), in blood plasma (Saraiva, Magalhaes, Ferreira, & Almeida, 2012). This association is proposed to facilitate RBP release from its site of synthesis in the endoplasmic reticulum and to prevent renal filtration of RBP (Goodman, 1984; Wolf, 1984). The presence of retinol bound to RBP is essential for the formation of a stable complex with TTR (Noy, Slosberg, & Scarlata, 1992). The location of the TTR binding sites for RBP was initially revealed by the crystal structure of human TTR complexed with chicken RBP (Monaco et al., 1995). Later, the structure of the human complex was also described (Naylor & Newcomer, 1999). *In vitro* one tetramer of TTR can bind two molecules of RBP. However, the concentration of RBP in the plasma is limiting, and the complex isolated from serum is composed of TTR and RBP in a 1:1 molar complex (Monaco et al., 1995).

In accordance with the function of TTR as a transporter, TTR knockout (KO) mice exhibited decreased plasma levels of both T4 and retinol (Episkopou et al., 1993). Nevertheless, the levels of T4 were unaltered in tissues, in the absence of TTR, and the thyroid hormone function appeared normal, confirming a normal thyroid function in these animals (Palha et al., 1994). Furthermore, these mice presented unaltered levels of retinol in tissues, as compared to wild type (WT) mice, and did not display any symptoms of vitamin A deficiency (Episkopou et al., 1993; van Bennekum et al., 2001) suggesting that despite being important for the transport of T4 and RBP-bound retinol, TTR does not play a critical role on their metabolism. Additionally, RBP levels in the liver of TTR KO mice are 60% higher than those of wild-type mice, suggesting that the absence of TTR may reduce secretion of RBP-retinol from the liver (Wolf, 1995).

In addition to thyroid hormones and vitamin A, other substances were proposed to interact physiologically with TTR, such as metallothioneins 1/2 and 3 (Goncalves et al., 2008; Martinho et al., 2010), norepinephrine (NE) oxidation products (Boomsma, Man in 't Veld, & Schalekamp, 1991), pterins (Ernstrom, Pettersson, & Jornvall, 1995), lysosome-associated membrane protein (LAMP-1) (Chang et al., 2004) and perlecan (Liz, Mar, Franquinho, & Sousa, 2010; Smeland, Kolset, Lyon, Norum, & Blomhoff, 1997). More recently, studies provided evidence for specific interactions of resveratrol (Klabunde et al., 2000) and some flavonoids (Radovic, Mentrup, & Kohrle, 2006) with the T4 binding sites of TTR and for their ability to stabilize the TTR tetramer and to inhibit fibrils formation (Green, Foss, & Kelly, 2005).

TRANSTHYRETIN AS A PROTEASE

In the last two decades a considerate breakthrough was achieved concerning the discovery of novel ligands and functions for TTR. One of those ligands is apolipoprotein A-I (ApoA-I). Although TTR was previously described being present in lipoproteins (Tanaka et al., 1994), Sousa M and colleagues described that 1-2% of total plasma TTR was circulating in high density lipoproteins (HDL) and that this interaction was dependent of ApoA-I (M. M. Sousa, Berglund, & Saraiva, 2000). These findings were fortified by crosslinking experiments that showed the formation of a complex of TTR with HDL and ApoA-I (M. M. Sousa, Berglund, et al., 2000) and this TTR-ApoA-I interaction is, in fact, believed to influence deposition o mutant ApoA-I amyloid deposition (de Sousa et al., 2000).

This interaction was further investigated by Liz and colleagues, which found that TTR functions as a novel protease, being able to cleave the C-terminus of ApoA-I (Liz,

Faro, Saraiva, & Sousa, 2004). The relevance of the proteolytic effect of TTR on ApoA-I was further addressed and it was found that TTR-cleaved ApoA-I displays reduced ability to promote cholesterol efflux and presents increased amyloidogenic potential, possibly affecting HDL biology and the development of atherosclerosis (Liz, Gomes, Saraiva, & Sousa, 2007). An additional substrate of TTR was unveiled in 2009 when TTR was shown to be responsible for the cleavage of neuropeptide Y (NPY) *in vitro*, suggesting a possible relevance of TTR in neurobiology (Liz et al., 2009).

The catalytic machinery behind the proteolytic activity of TTR was revealed for the first time by Liz and colleagues. 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 His88, His90 and Glu92 being the residues constituting the active site (Fig. 5) (Gouvea et al., 2013; Liz et al., 2012).

TTR is strongly linked to neuroprotection against amyloid Aβ peptide (Aβ) deposition in the context of Alzheimer's Disease (AD). In this context, Aβ was identified as a novel TTR substrate. TTR was shown to cleave not only soluble but also aggregated forms of Aβ *in vitro* (R. Costa, Ferreira-da-Silva, Saraiva, & Cardoso, 2008), diminishing its fibrillogenesis and toxicity (R. Costa, Goncalves, Saraiva, & Cardoso, 2008) and suggesting a relevant role for TTR in AD, further dissected in the next section.

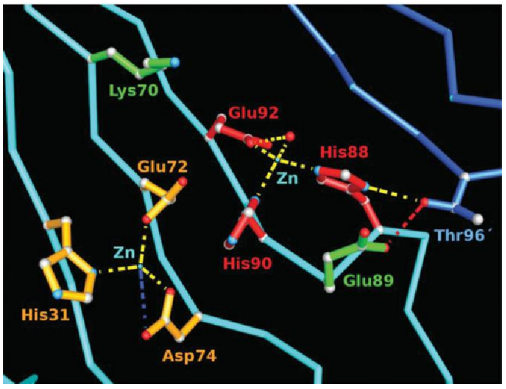

Figure 5. Structure of TTR active site. In cyan and dark blue are represented two different TTR monomers. The metallic ion zinc is linked by His88 and His90 and Glu92 is contacting with a water molecule. A second Zn^{2+} binding site consists of Glu72, His31 and Asp74. Glu89 and Thr96 of a second monomer contact by a hydrogen bond which is also in contact with His88. Attractive forces are showed in dashed lines. Adopted from Liz et al., 2012.

TRANSTHYRETIN AS A PROTECTIVE AGENT IN THE NERVOUS SYSTEM

Peripheral Nervous System

The role of TTR on the biology of the nervous system, particularly on nerve physiology and repair, has been a focus of investigation due to the involvement of this protein in a peripheral neuropathy and the fact that its preferential deposition is in the peripheral nervous system (PNS) of the patients. In fact, the exploration of the role of TTR in nerve biology was enabled by the development of the TTR KO mouse model. These mice, generated in 1993 by Episkopou and colleagues (Episkopou et al., 1993), are viable, appear phenotypically normal, remain fertile and have a normal life span. As mentioned above, TTR KO mice display, however, reduced levels of serum retinol, RBP, and thyroid hormone indicating that TTR has a role in maintaining normal levels of these metabolites in plasma (Episkopou et al., 1993).

Further exploration of the phenotypic features of TTR KO mice disclosed an avenue of functions for TTR that are more related to the nervous system. These mice were shown to exhibit increased exploratory activity and reduced depressive-like behaviour, perhaps due to the modulation of the noradrenergic system by the increase of noradrenaline in the limbic forebrain (J. C. Sousa et al., 2004). Substantiating this role of TTR in modulating depressive behaviour are reports of NPY, known as an anti-depressant neurotransmitter, to be increased in dorsal root ganglia (DRG), sciatic nerve, spinal cord, hippocampus, cortex and CSF of TTR KO mice (Nunes, Saraiva, & Sousa, 2006).

Additionally, TTR KO mice exhibit sensorimotor impairment (Fleming, Saraiva, & Sousa, 2007) and, in 2007, Fleming and colleagues revealed for the first time that TTR acts as an enhancer of nerve regeneration. After sciatic nerve crush, TTR KO mice presented delayed functional recovery when compared to WT littermates, as well as slower recovery of locomotor activity, slower nerve conduction velocity and decreased number of myelinated and unmyelinated axons. This phenotype was rescued when TTR was locally expressed in the nerve demonstrating that TTR has an important role in peripheral nerve function and repair (Fleming et al., 2007). TTR contribution to nerve regeneration was suggested to be correlated with its ability to increase neurite outgrowth, *in vitro*, when it was shown that, not only WT neurons grew more than TTR KO neurons, but also that, when added to TTR KO DRG neurons in culture, WT TTR was able to increment neuronal growth (Fleming et al., 2007). Later, TTR neuritogenic activity was shown to be dependent on its megalin-mediated internalization through the formation of clathrin-coated vesicles (Fleming et al., 2009). These studies also showed that TTR KO mice exhibit impaired retrograde axonal transport both, *in vitro* and *in vivo*, as

demonstrated by the reduced amount of transferrin (which functions as a tracer for retrograde transport when conjugated with Texas red) in cell bodies of TTR KO DRG neurons, when comparing with WT neurons and by the decreased accumulation of $p75^{NTR}$ in chronically constricted injured TTR KO sciatic nerves distally to the ligation side (Fleming et al., 2009). In fact, the transmission of signals to the cell body is a key process in nerve regeneration. These findings raised the hypothesis that the compromised retrograde transport of TTR KO axons could be, in part, responsible for the delayed regenerative capacity of TTR KO mice and decreased neurite outgrowth in the absence of TTR. However, molecular and mechanistical details underlying TTR neuritogenic effect remain obscure and still need to be unravelled.

Central Nervous System

Apart from the PNS, TTR also presents critical neuroprotective features in the CNS. In fact, studies have pointed TTR as a transcription inducer of IGF-IR, known as a protective receptor against apoptosis. This work described that TTR induces increased levels of IGF-IR, showing that TTR triggers IGF-IR nuclear translocation in cultured hippocampal neurons (Vieira et al., 2015). The hippocampus is acknowledged as the major regulator of memory and cognition and, studies have shown that TTR KO mice exhibit memory impairment indicating that the absence of TTR accelerates cognitive deficits usually associated with ageing (J. C. Sousa, Cardoso, Marques, Saraiva, & Palha, 2007). Additionally, TTR expression was shown to be decreased in aged memory-impaired rats compared with aged memory-unimpaired rats (Brouillette & Quirion, 2008). In this study, TTR KO mice were also used and the age-related memory deficits they exhibited were rescued with the administration of retinoic acid (Brouillette & Quirion, 2008).

Moreover, TTR was also shown to be neuroprotective in a cerebral ischemia context, one of the major causes of brain injuries in the world. Using a mouse model in which brain ischemia was surgically induced by permanent middle cerebral artery occlusion (pMCAO), TTR KO mice heterozygous for heat shock factor 1 (compromised heat shock response) displayed a significant increase in cortical infarction, cerebral edema, and microglial/leukocyte response. Additionally, WT animals presented CSF derived TTR localized throughout the infarct area (S. D. Santos, Lambertsen, et al., 2010). Using the same ischemia model Gomes and colleagues showed that the larger infarcts seen in TTR KO mice subjected to pMCAO were related to a downregulation of TTR and megalin in neurons (Gomes et al., 2016). Also, in this work TTR was shown to provide

trophic support via megalin by promoting neurite outgrowth in cultured hippocampal neurons from TTR KO and WT mice incubated with exogenous recombinant TTR. TTR promoted increased neurite outgrowth in both TTR KO and WT neurons, under physiological conditions and under conditions of excitotoxic insults. This neuritogenic effect in CNS neurons was shown to be mediated by megalin, similarly to what was seen in the PNS, pinpointing this receptor as a major player in the TTR neuroprotective function (Gomes et al., 2016).

In a Parkinson's disease context, TTR was shown to be involved in alpha-synuclein clearance, and CSF TTR levels were shown to be significantly higher in dementia patients with Lewy bodies, compared to controls (Maetzler et al., 2012).

Alzheimer's Disease

Still in the context of CNS neurodegenerative disorders, compelling suggestions implicate TTR as neuroprotective in AD. Studies performed in the early 90s showed that Aβ40 fibril formation was inhibited upon incubation with human CSF (Wisniewski, Castano, Ghiso, & Frangione, 1993) which, at that time, was explained by the sequestration of Aβ by extracellular proteins circulating in CSF such as apoE and apoJ (Ghiso et al., 1993; Goldgaber et al., 1993; Wisniewski et al., 1993). However, in 1994, Schwarzman and colleagues discovered that, in CSF and contrarily to the expectations, apoE was not the major protein binding to Aβ, but TTR was (Schwarzman et al., 1994), and proposed later the sequestration hypothesis as a possible explanation for the peptide aggregation and consequent progression of AD. In fact, TTR and Amyloid-β (Aβ) peptide interaction has been addressed by many groups due to the fact that CSF and plasma TTR levels of Alzheimer's disease (AD) patients are decreased, proposing a neuroprotective action of TTR in AD (Hansson et al., 2009; Ribeiro et al., 2012; Riisoen, 1988). The controversial hypothesis that the choroid plexus/CSF derived TTR interacts with Aβ levels has been under strong scrutiny given that Aβ is mainly present in the hippocampus and cortex. Thus, the presence of TTR in brain areas other than its site of synthesis and secretion has been subject of study and although TTR expression was demonstrated in the hippocampus of both AD patients and AD mice models (Li, Masliah, Reixach, & Buxbaum, 2011; Schwarzman & Goldgaber, 1996; Stein et al., 2004), 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 (S. D. Santos, Fernandes, & Saraiva, 2010). Altogether, the referred studies highlight the importance of TTR in AD and reinforce the need for a better understanding of this interaction. In this respect, Alemi and colleagues have shown, using a human cerebral

microvascular endothelial cell line (hCMEC/D3), that TTR promotes a LRP1-mediated Aβ clearance and that this process is dependent on the TTR tetramer stability (Alemi et al., 2016; Alemi, Silva, Santana, & Cardoso, 2017). Additional findings suggested that the protective capacity of TTR was related to its binding to toxic/pre-toxic Aβ aggregates in both intracellular and extracellular environment in a chaperone-like manner (Buxbaum 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 (R. Costa, Goncalves, et al., 2008). TTR was described as being a pro survival gene when studies demonstrated that in the Tg2576 transgenic mice overexpressing the mutant form of human amyloid precursor protein with the Swedish mutation (APPSw), TTR was overexpressed (Stein & Johnson, 2002). TTR up-regulation was also reported in situations of exposure of AD transgenic mice to an "enriched environment", also resulting in pronounced reductions in cerebral Aβ levels and amyloid deposits, compared to animals raised under "standard housing" conditions (Lazarov et al., 2005). Later on, further studies using transgenic models for AD fortified the role of human TTR on Aβ aggregation and toxicity (Buxbaum et al., 2008). While the overexpression of human TTR ameliorated AD features in APP transgenic mice (Buxbaum et al., 2008), the ablation of the mouse TTR gene resulted in accelerated amyloid deposition and increased Aβ brain levels (Buxbaum et al., 2008; Choi et al., 2007; Oliveira, Ribeiro, Cardoso, & Saraiva, 2011).

Further *in vitro* studies showed that a direct interaction between TTR and Aβ cancelled the deleterious properties of Aβ oligomers (R. 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 (R. 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. Altogether, these preliminary findings open a promising research avenue to address the possibility of a neuroprotective role of TTR in Aβ cleavage.

TRANSTHYRETIN AMYLOIDOSIS

Amyloidoses encompass a large group of acquired and hereditary diseases pathologically defined by the misfolding, aggregation and subsequent deposition of characteristic insoluble protein (amyloid), in the form of fibrils or plaques, in several tissues (Merlini & Bellotti, 2003). These fibrillar species are 7-10nm wide and are characterized by the typical cross-β architecture, with β-strands running perpendicular to the fibre axis, forming antiparallel β-sheets (Eanes & Glenner, 1968). Amyloid species present a distinctive optical behaviour and dye binding properties, such as apple-green birefringence upon binding Congo red dye (Dobson, 2003; Selkoe, 2003). The accumulation of amyloid ultimately leads to dysfunction of the affected organs and many different diseases characterized by amyloid formation share common pathways of protein aggregation and mechanisms of pathogenesis.

TTR is related to three different forms of amyloidosis, which can be either sporadic or hereditary. These lead to three different human disorders characterized by the extracellular deposition of TTR aggregates, Familial Amyloid Polyneuropathy (FAP), Familial Amyloid Cardiomyopathy (FAC) and Senile Systemic Amyloidosis (SSA). Due to its enrichment in β-strands – the most common feature of most amyloid proteins – TTR is a protein that displays an elevated amyloidogenic potential.

A sporadic form of TTR amyloidosis caused by deposition of WT TTR is SSA, which is characterized by the accumulation of non-mutated TTR amyloid deposits mainly in cardiac tissue, giving rise to complications, such as cardiomegaly and congestive cardiac failure (Cornwell, Sletten, Johansson, & Westermark, 1988; P. Westermark, Sletten, Johansson, & Cornwell, 1990). SSA is usually asymptomatic and has been shown to affect individuals in late stages of life affecting to some degree 25% of the population greater than 80 years old (P. Westermark et al., 1990). The development of polyneuropathy, caused by WT TTR amyloidosis has also been reported in some SSA patients (Lam, Margeta, & Layzer, 2015).

Although amyloidoses are typically modulated by numerous environmental factors, there are the hereditary forms of TTR amyloidosis which are triggered by mutations in TTR. Concerning the hereditary form of TTR amyloidosis, more than 100 mutations in the TTR gene have been described (Connors, Lim, Prokaeva, Roskens, & Costello, 2003). With the exception of one deletion, all of them are single amino acid substitutions, derived from single-point mutations (Saraiva, 2001b). TTR amyloidogenic variants present some clinical heterogeneity but are, in most cases, associated with neuropathies, especially with PNS involvement (Saraiva, 2001b). However, other conditions have been described such as cardiomyopathy (Saraiva et al., 1992), carpal tunnel syndrome (Izumoto et al., 1992),

predominant vitreous TTR deposition (Salvi et al., 1993) and leptomeningeal involvement (Petersen et al., 1997). The most common TTR mutation is a substitution of valine by methionine at the position 30 (V30M), resulting from a G to A nucleotide change in the exon 2 of the TTR gene, and it is associated with FAP (Saraiva, Birken, Costa, & Goodman, 1984; Saraiva, Costa, & Goodman, 1983). One of the most highly aggressive and amyloidogenic TTR mutations is the replacement of leucine by proline at position 55 (L55P), inducing an early onset type of progressive neuropathy (before 20 years of age) (Jacobson, McFarlin, Kane, & Buxbaum, 1992). It has been reported that this mutation decreases tetramer stability, due to impairment of dimer-dimer interactions (McCutchen, Colon, & Kelly, 1993; Sebastiao, Saraiva, & Damas, 1998).

There are also a few TTR mutations related predominantly to FAC. The most common is V122I, described in 3.9% of the African-American population and over 5% of the population in some areas of West Africa (Jacobson et al., 1997). Moreover, patients with TTR mutations such as L12P (Brett et al., 1999), D18G (Vidal et al., 1996) or A25T (Sekijima et al., 2003) display TTR leptomeningeal amyloidosis, which discloses scenarios of TTR amyloidosis with CNS involvement.

Nevertheless, TTR mutations are also reported to display protective features and guide TTR amyloidosis in the opposite direction. One example is the TTR T119M variant. This mutation is non-pathogenic and is considered protective over the effect of TTR V30M, since compound heterozygotic carriers of both TTR V30M and TTR T119M display a less severe form of the disease (Longo Alves, Hays, & Saraiva, 1997). Other mutations in TTR have also been described as protective such as Arg104His or Thr119Met, as they stabilize the tetrameric structure of TTR contributing to a delayed course of the disease in patients carrying V30M substitution (Almeida, Alves, Terazaki, Ando, & Saraiva, 2000).

MECHANISM OF TRANSTHYRETIN AGGREGATION

Understanding the TTR tetramer dissociation process is vital in understanding the TTR amyloidosis phenomenon. Although remarkable progress has been made over the last 2 decades in understanding the biology and structural basis of TTR amyloidogenesis in FAP some details of the mechanism are still to be revealed, which fuels the need to perform further research in TTR amyloidosis processes. Currently, it is accepted that amyloid formation might involve diverse molecular pathways, with formation of an amyloidogenic intermediate occurring prior to fibril formation, whether it results from a destabilization of the TTR tetramer leading to dissociation into monomers or from a proteolytic event.

As mentioned above, one hypothesis for amyloid fibrils formation is related to proteolytic cleavage. This hypothesis is supported by studies that showed the presence of TTR peptide fragments as well as the full-length protein in amyloid fibrils extracted from FAP patients and composed by TTR V30M. This hypothesis hasn't been, however, very much supported along the years since there are studies that didn't detect these TTR proteolytic fragments (Saraiva et al., 1984; Tawara, Nakazato, Kangawa, Matsuo, & Araki, 1983) suggesting the need to further explore the role of proteolysis in TTR amyloidogenesis. In this respect, a very recent study showed that a disruption of the TTR CD loop by enzymatic cleavage is, in fact, one of the factors behind the formation of toxic TTR oligomers (Dasari et al., 2020).

Nonetheless, today, the most accepted hypothesis of TTR amyloidosis is conformational change. Biophysical studies have provided strong evidence for this theory, particularly by structural studies using X-ray diffraction of TTR L55P, which led to the proposal of a model where monomers are the building blocks of amyloid (Sebastiao et al., 1998). Moreover, stability studies have shown a correlation between amyloidogenic potential and decrease in the tetrameric stability, thus leading to the hypothesis that amyloid fibril formation might be triggered by tetramer dissociation into natively folded monomer with low conformational stability which subsequently undergoes denaturation. The unfolded monomers with a high tendency for ordered oligomerization will aggregate very efficiently into a variety of aggregated morphologies, including oligomers, non-fibrillar aggregates and amyloid fibrils (Fig. 6) (Johnson, Connelly, Fearns, Powers, & Kelly, 2012; Saraiva, 2001a). Moreover, TTR tetramers in serum of FAP patients with various variants were shown to be less stable than in normal subjects (Tojo, Sekijima, Kelly, & Ikeda, 2006). Both amyloidogenic and non-amyloidogenic TTR variants may dissociate into non-native monomers, yet only the amyloidogenic variants produce large amounts of partially unfolded monomeric species as a consequence of the marginal conformational stability of the non-native monomer. The intermediate species, such as oligomers and early aggregates, were shown to be the most cytotoxic species in the TTR amyloidogenic cascade (Reixach, Deechongkit, Jiang, Kelly, & Buxbaum, 2004; M. M. Sousa, Cardoso, Fernandes, Guimaraes, & Saraiva, 2001). Moreover, binding of TTR to its ligands, such as T4, stabilizes the tetramer against dissociation (Miroy et al., 1996).

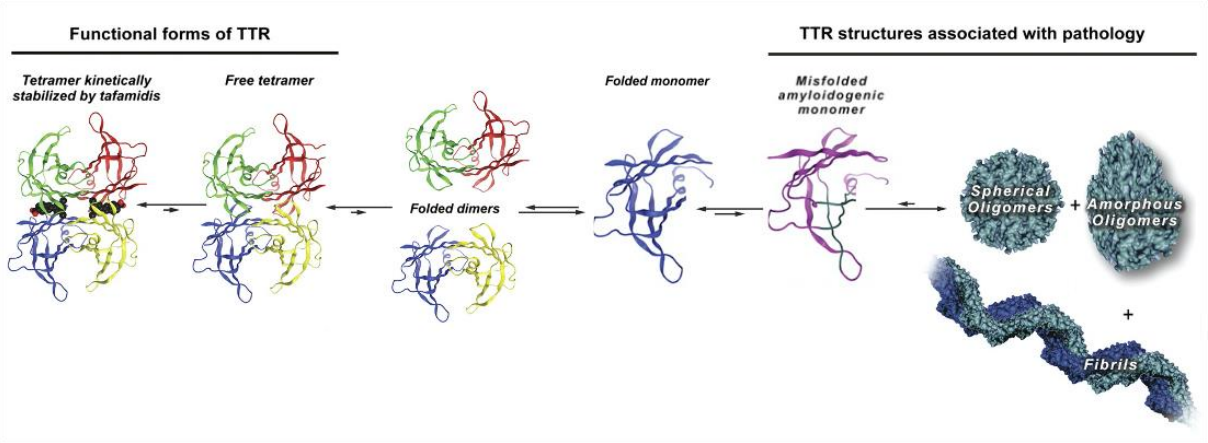

Figure 6. The TTR amyloid cascade. Amyloid formation by TTR requires rate-limiting tetramer dissociation to a pair of folded dimers, which then quickly dissociate into folded monomers. Partial unfolding of the monomers yields the aggregation-prone amyloidogenic intermediate. The amyloidogenic intermediate of TTR ("misfolded amyloidogenic monomer) retains much of its native structure (shown in purple), probably with some β-strand dissociation (shown in turquoise). The amyloidogenic intermediate can misassemble to form a variety of aggregate morphologies, including spherical oligomers, amorphous aggregates, and fibrils. Adapted from Bulawa et al., 2012.

FAMILIAL AMYLOID POLYNEUROPATHY

TTR is involved in Familial amyloid polyneuropathy (FAP), an autosomal dominant neurodegenerative disease, characterized as a "peculiar form of peripheral neuropathy", and firstly described in the north of Portugal, in 1952, by the Portuguese neurologist and researcher Corino de Andrade (Andrade, 1952). This neuropathy particularly present in fishermen kindreds in Póvoa de Varzim, was originally coined as "doença dos pézinhos" (foot disease) due to its distinctive symptomatology. In fact, this disorder is characterized by the initial loss of temperature and pain sensation, starting and predominating in the lower extremities, and histologically by generalized atypical amyloidosis, involving especially the peripheral nerves (Andrade, 1952). Besides Portugal, other major endemic areas include Sweden (R. Andersson, 1970) and Japan (Araki, Mawatari, Ohta, Nakajima, & Kuroiwa, 1968). The discovery of TTR as the main protein involved in the development of FAP (P. P. Costa, Figueira, & Bravo, 1978) represented the initial step to unveil the molecular pathogenesis of this disease.

PATHOPHYSIOLOGY OF FAP

FAP is a rare and fatal neurodegenerative disorder, transmitted as an autosomal dominant trait and characterized by the extracellular deposition of oligomers, aggregates

and amyloid fibres of mutant TTR, particularly in the PNS, which include the nerve trunks, plexuses, and sensory and autonomic ganglia (Coimbra & Andrade, 1971b). In Portugal, where the main TTR mutation is the Val30met, the onset of the disease occurs generally in the third decade of life leading to death within 10-20 years (Andrade, 1952; Barreiros, Galle, & Otto, 2013). However, the time of onset depends on the underlying mutation and geographic distribution. Differences exist in the presentation of early-onset cases in endemic areas and late-onset cases in non-endemic areas. Also, one reported case of late onset FAP (first clinical manifestations in the mid 50s) is the TTR Ala97Ser mutation-related FAP with a focus point in Taiwan.

The chemical nature of the amyloid protein involved in FAP was unknown until 1978, when Costa and colleagues described the amyloid fibril protein accumulated in tissues of Portuguese FAP patients as being an abnormal form of TTR (P. P. Costa et al., 1978). It was only later, in the 80s, that Saraiva and colleagues identified the molecular abnormality in TTR as a substitution of methionine for valine at position 30 of the TTR polypeptide chain (Saraiva et al., 1984). This variant has become a biochemical marker for FAP (Saraiva, Costa, & Goodman, 1985).

In the peripheral nerves, mutant TTR accumulates extracellularly in the form of amyloid fibres in the epineurium, perineurium and more predominantly in the endoneurium close to the Schwann cells' basal membrane, but without penetrating the cell membrane or cytoplasm, and causing axonal fibre degeneration mainly in the unmyelinated fibres (Fig. 7) (M. M. Sousa, Cardoso, et al., 2001). The extracellular deposition of amyloid fibrils throughout the connective tissue leads to general organ dysfunction (Coimbra & Andrade, 1971a, 1971b). Amyloid deposition also occurs in the DRG, more precisely in the stroma in close contact with satellite cells accompanied by a severe neuronal loss (preferential loss of smaller neurons), which correlates well with clinical loss of pain, temperature sensations and autonomic functions (Sobue et al., 1990).

One of the most striking features of FAP is the nerve length-dependent sensory-motor polyneuropathy which consists in the initial axonal loss, affecting first sensory unmyelinated fibres, followed by the loss of small-diameter myelinated fibres and, at later stages, of large myelinated fibres (Coimbra & Andrade, 1971b; Plante-Bordeneuve & Said, 2011). In addition to axonopathy, advanced neuronal degeneration is found in the autonomic and sensory nervous systems, which may be responsible for the characteristic progressive ascending neuropathy (dying-back type) of FAP (Luis, 1978). The dying-back axonopathy and the loss of sensory fibres are the pathological features responsible for the typical symptoms of FAP, which primarily include the loss of temperature and pain sensations, with nerves in the lower limbs being firstly affected, and progressing in a proximal direction (Plante-Bordeneuve & Said, 2011; Said, Ropert, & Faux, 1984). Motor

impairment also occurs later in the course of the disease, causing deterioration and weakness as well as a progressive loss of reflexes in the upper limbs occurring months to years after lower limb manifestations. CNS involvement was reported recently and is caused by accumulation of amyloidogenic TTR in the meninges and perivascular regions (Maia et al., 2015). Stroke-like, focal neurologic deficits, also named “amyloid spells” are the most common features in CNS TTR amyloidosis. Other rare presentations include dementia, hydrocephalus and myelopathy (Maia et al., 2015).

Autonomic dysfunction, involving cardiovascular disturbance, gastrointestinal dysfunction, bladder dysfunction and impotence, are also present in FAP patients (Ando & Suhr, 1998), and may begin in the very early stages of the neuropathy (Ando, Araki, Shimoda, & Kano, 1992). Cardiac involvement is the most common extra-neurological manifestation and observed in 80% of the patients and common clinical manifestations in cardiac amyloidosis include arrhythmias, syncope attacks due to conduction problems and heart failure caused by restrictive cardiomyopathy (Dubrey & Falk, 2010). There are additionally some reports of ocular manifestations associated to TTR amyloidosis that include vitreous deposits, glaucoma and pupillary abnormalities where TTR amyloid can be visualized as cotton wool inclusions in the vitreous body (Tripathy, Chawla, Selvan, & Venkatesh, 2018).

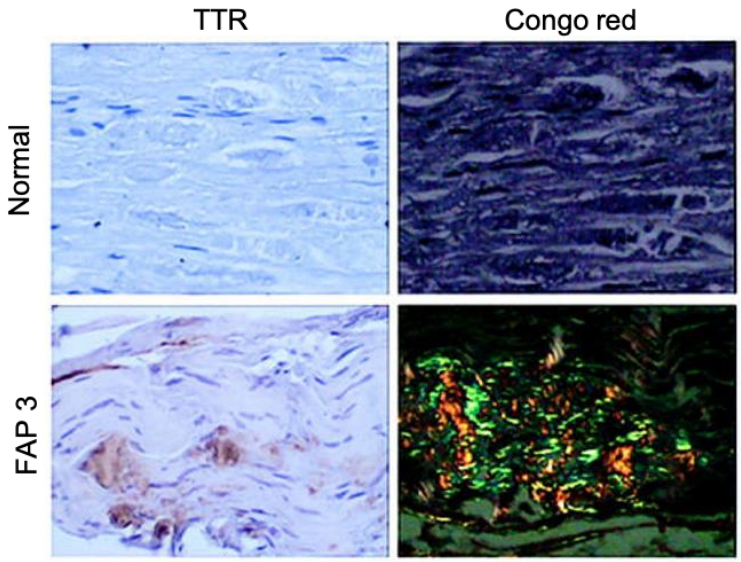

Figure 7. TTR deposition on the nerve of a FAP patient. TTR immunohistochemistry (left) and Congo Red staining (right) of a normal nerve (top) and a FAP 3 patient’s nerve (bottom). Adapted from M. M. Sousa, Cardoso, et al., 2001.

The causes of neurodegeneration in FAP remain to be fully disclosed. However, and taking into consideration the aforementioned pathological features, some hypotheses

have been proposed, namely lesions in DRG neurons, compression of nervous tissue by amyloid deposits and ischemia caused by amyloid deposits around blood vessels. However, none of these hypotheses can fully explain neurodegeneration in FAP and, in fact, neurodegeneration may occur involving the combination of more than one event.

THERAPEUTIC APPROACHES IN FAP

The best way to improve the overall scenario of an FAP patient is to start with an accurate diagnosis. Diagnosis in FAP includes different parameters which can be the presence of TTR V30M, considered a biomarker (Saraiva et al., 1985), proteomic tests by employing mass spectrometry analysis of serum to detect TTR variants (Ando & Ueda, 2012), histopathological sample analysis and the assessment of clinical manifestations typical of FAP.

One therapeutic option initiated immediately following diagnosis is symptomatic treatment. It is used combined with the more effective therapeutic approaches as an aiding option and includes neuropathic pain medication and treatment for orthostatic hypotension, incontinence and diarrhea. Moreover, cardiovascular events are the main cause of death in TTR amyloidosis, therefore treating cardiac failure is particularly important.

Treatment of TTR-FAP has significantly improved in recent years with new emerging therapeutic approaches. The first line of defence in any disease caused by malfunction of a given protein is to target its synthesis. Therefore, the major therapeutic approach for FAP is liver transplantation, since the liver is the major site of TTR synthesis. The rationale for this intervention is that it prevents the formation of additional amyloid deposits by removing the main source of mutant TTR. Liver transplantation can halt the progression by knocking down 95% of circulating TTR (Holmgren et al., 1991) and was first introduced for treatment of FAP in 1990, in Sweden, and, since then, it became a standard therapy worldwide. Transplanted patients present substantial and sustained reduction of mutant TTR in the serum (Holmgren et al., 1991) and a prolonged survival rate was observed in transplanted FAP V30M patients compared with the non-transplanted group (Okamoto et al., 2009; Yamashita et al., 2012). The outcomes of this therapeutic approach are considerably better when liver transplantation is performed early in the course of the disease (Adams et al., 2000). However, there are several major drawbacks associated with liver transplantation: i) it is not feasible in older patients or in those with advanced disease; ii) the outcomes are not as positive in patients with non-V30M mutations compared with those presenting V30M mutations (Wilczek, Larsson,

Ericzon, & Fapwtr, 2011); iii) in some patients, the disease progresses even after transplantation, and this can be due to the continued incorporation of WT TTR to the pre-existing amyloid deposits (Liepnieks & Benson, 2007) or continued deposition of mutant TTR synthesized by other sites, such as the choroid plexus (Said & Plante-Bordeneuve, 2009); iv) liver transplantation is a highly invasive surgical procedure (Hund, 2012). Given all the deleterious factors referred above, liver transplantation is losing its position as a standard treatment and other therapeutic approaches have been improved along the years.

Another way to supress the expression of mutant TTR is by gene therapy. This therapeutic approach is characterized by the silencing of the gene of interest through antisense oligonucleotides (ASO) and small interfering RNAs (siRNAs), which cause enzymatic degradation of mRNA and sequence-specific post-translational gene silencing, respectively (Ueda & Ando, 2014). Early studies have suggested a selective inhibition of mutant TTR production, using siRNAs (Kurosawa, Igarashi, Nishizawa, & Onodera, 2005), and the suppression of both WT and mutant TTR levels in the liver and brain, using ASO therapy (Benson et al., 2006), since the WT protein also contributes to amyloid formation in FAP. Patisiran, a siRNA encapsulated in lipid nanoparticle is a FDA approved gene therapy therapeutic approach for TTR-FAP treatment since August 2018. The first studies using Patisiran in healthy volunteers demonstrated a significant knock-down of circulating TTR by silencing the TTR gene (Coelho et al., 2013). Later studies in phase 2 showed that Patisiran presented promising results by halting disease progression (Suhr et al., 2015) and, in phase 3 trials, nearly half of the patients in Patisiran group had a decrease in modified neuropathy impairment score (mNIS+7) suggesting an improvement in clinical pathology.

Another promising drug, that recently completed the phase 3 trial, is Inotersen, an antisense oligonucleotide that selectively binds TTR mRNA and decreases its production. Early stage (1 and 2) patients without any prominent cardiac were used for this study. Patients showed significant decrease in mNIS+7 and Norfolk QOL (quality of life) scores and the study showed also a mean decrease in serum TTR of 74%. The drug is approved by European Medicine Agency for stage 1 and 2 of the disease and accepted for Priority Review by FDA (Benson, Dasgupta, & Monia, 2019; Benson et al., 2018).

Since TTR dissociation into monomers is the rate-limiting step in the pathway of fibrillogenesis, another form of therapy in FAP is the use of agents that stabilize TTR tetrameric structure, therefore preventing its dissociation and reducing amyloid production (Hammarstrom, Wiseman, Powers, & Kelly, 2003). These compounds are pharmacological chaperones of TTR that bind specifically to the tetramer, increasing its stability. Two drugs, diflunisal and tafamidis meglumine, were discovered as suitable TTR

stabilizers. Diflunisal, a nonsteroidal anti-inflammatory drug, is a TTR tetramer stabilizer (Sekijima, Dendle, & Kelly, 2006). In a study with 150 patients diflunisal administration was shown to promote better NIS+7 and QOL scores compared to placebo treated individuals. However, the safety profile was worrisome since side effects such as gastrointestinal and urinary complications appeared which caused high discontinuation rates, limiting the use of Diflunisal in FAP patients that already display cardiac and renal involvement (Berk et al., 2013). Tafamidis is a newly discovered small molecule that binds selectively and potently to the T4 binding site in the TTR tetramer (Connelly, Choi, Johnson, Kelly, & Wilson, 2010). Tafamidis was approved by the European Commission for the treatment of transthyretin amyloidosis. During a clinical trial, patients who received tafamidis showed reduction in neurologic deterioration, preservation of nerve fibre function, maintenance of QOL, and TTR stabilization compared to placebo patients (Coelho et al., 2013; Coelho et al., 2012). However, late onset treatment revealed to be less effective with a phase 2 study failing to demonstrate the efficiency of Tafamidis in TTR cardiomyopathy (Plante-Bordeneuve et al., 2017), showing, however that it may be effective in earlier stage cardiac disease (Maurer et al., 2015; Sultan, Gundapaneni, Schumacher, & Schwartz, 2017). A randomized study designed for the efficacy of Tafamidis in patients with TTR cardiomyopathy (Maurer et al., 2017) showed a significant reduction of mortality and cardiovascular events requiring hospitalization culminating in FDA granting Tafamidis the “Breakthrough Therapy” designation for the treatment of TTR cardiomyopathy in 2018.

There are some emerging therapies under development including the synergistic combination of Doxycycline, an antibiotic, and Tauroursodeoxycholic acid (TUDCA), a bile acid, showing promising results by reducing the amyloid deposition in tissues (Cardoso, Martins, Ribeiro, Merlini, & Saraiva, 2010). A phase 2 study showed consistent stabilization of cardiac disease in 75% of the patients receiving Doxycycline and TUDCA combination (Obici et al., 2012). Also, antibodies against serum amyloid P (SAP) and TTR, which clear amyloid from the tissues, are currently under development (Higaki et al., 2016; Richards et al., 2015). A phase 2 study aiming to evaluate the efficiency of the combination of Anti-SAP antibody (GSK2398852) and SAP inhibitor (GSK2315698) for the treatment of TTR amyloidosis, is currently ongoing.

In conclusion, even though there are some efficient available treatments targeting FAP pathogenesis, including liver transplantation as the most prevalent choice, these strategies are not able to treat symptomatic and late-onset patients. Although gene therapy is gaining some territory in the “race of treating late onset FAP pathology”, none of these therapeutic approaches is capable of targeting neurodegeneration, which remains irreversible.

FAP ANIMAL MODELS

Despite the vast knowledge gathered in the past decades regarding TTR-induced amyloidosis and FAP, many questions remain to be answered concerning the molecular mechanisms by which neurodegeneration events occur. Animal models that mimic the pathology of protein conformation associated diseases are of crucial importance since most protein misfolding diseases are experimentally difficult to study in detail owed by the availability of only autopsy and biopsy specimens.

Mice have long served as models of human biology and disease, due to their physiological similarity to humans and availability. They are, thus, helpful tools to answer the existing TTR-induced neuropathology questions and to search for possible pharmacological therapies targeting FAP.

FAP mouse models started being generated in the 80s using cDNA with the coding sequence for TTR V30M driven by the mouse metallothionein promoter and with zinc-derived induction (Sasaki et al., 1986). However, these animals presented low levels of plasma TTR V30M (2.5-12 µg/ml) and lacked amyloid deposition in the PNS and autonomic nervous system (ANS) (Sasaki, Nakazato, Saraiva, Matsuo, & Sakaki, 1989; Sasaki et al., 1986; Shimada et al., 1989). Later on, an improved transgenic mouse model was generated containing increased gene copies of TTR V30M (Yi et al., 1991). These animals developed some amyloid deposits in the kidneys, gastrointestinal tract and cardiovascular organs at 6 months of age. However, and despite the extensive amyloid deposition at 24 months of age, no amyloid deposition was observed in the PNS and ANS of these animals, the hallmark of the human disease (Yi et al., 1991). In subsequent years, several groups attempted to generate transgenic animals either from WT or mutant TTR gene constructs, but still all of them remained unsuccessful in producing congophilic deposits in peripheral nerves (Araki et al., 1994; Nagata et al., 1995; Takaoka et al., 1997).

Kohno and colleagues generated a new mouse model lacking the endogenous TTR but carrying the human TTR V30M and suggested that the presence of endogenous mouse TTR does not interfere with the human TTR deposition pattern since it was comparable to the previous models. This hypothesis was later contested where it was shown that mice expressing human TTR L55P in a TTR-null background presented TTR amyloid fibril deposition in the GIT at earlier stages when compared to animals expressing human TTR L55P carrying the endogenous murine TTR (M. M. Sousa et al., 2002). Therefore, the use of a mouse model expressing the mutant variant of human TTR in a TTR null background should be favoured. In fact, biophysical and biochemical studies revealed that endogenous murine TTR inhibits aggregation and deposition of highly

unstable human TTR via formation of a highly stable human-mouse heterotetramers (Reixach et al., 2008; Tagoe et al., 2007). Additionally, these transgenic animals carrying TTR L55P variants (M. M. Sousa et al., 2002; Teng et al., 2001), failed to present deposition phenotype in the nervous system even at late ages and after multiple generations.

Although these models provided already some good insights in disease onset modulation and were valuable tools for drug treatment research in amyloid deposition prevention, the lack of amyloid deposition in the PNS was still an important limitation to overcome.

The observations that inflammatory and oxidative stress pathways were activated by TTR deposits in patients (M. M. Sousa, Du Yan, et al., 2001; M. M. Sousa, Yan, Stern, & Saraiva, 2000) raised the hypothesis that disrupting the stress response pathway could accelerate TTR deposition. In this respect, in 2010, a new FAP mouse model was generated, carrying the human TTR V30M mutation in a heat shock transcription factor 1 (HSF1) null background. For the first time, a FAP model presented non-fibrillar TTR deposits in the autonomic and PNS, as well as an earlier and more extensive nonfibrillar deposition, when compared to the other available models. Fibrillar deposits were also detected in the DRG and the sciatic nerve at 24 months of age, accompanied by a decrease in the number of unmyelinated fibres (S. D. Santos, Fernandes, et al., 2010). This model has been used in a vast number of studies to assess pathogenesis of ATTR amyloidogenesis and the preclinical evaluation of pharmacotherapy and gene therapy studies (Butler et al., 2016; Cardoso et al., 2010; Ferreira et al., 2014). Nevertheless, one cannot set aside the fact that due to the absence of HSF1, which is directly involved in tissue damage protection, this model becomes less optimal for either fine analysis or experimental therapies because the absence of HSF1 could be responsible for making target cells more sensitive to toxicity of extracellular aggregates (Buxbaum, 2009).

Altogether, these observations stress out the inexistence of the ideal mouse model for FAP that mimics ATTR amyloidosis in patients, the observed symptoms and tissue-distribution of congophilic TTR amyloid deposits. In this regard, recently, two groups developed FAP mouse models with amyloid deposits in the PNS without additional genetic alterations such as HSF1 deficiency (Kan et al., 2018; Li et al., 2018). Li and colleagues generated humanized mouse strains at both *Ttr* and *Rbp* loci which allows human TTR (hTTR) to associate with human RBP4 (hRBP4). Although both, hTTR and hRBP4, were found at relatively low levels in the serum of these mouse lines, these mice present distinct amyloid deposition than previous conventional transgenic models. Both, normal and mutant mice presented amyloid deposition in the GIT at 12 and 18 months and in the heart at 24 months. Notably, at 24 months, Congo red positive amyloid deposits

were found in the perineurium of the sciatic nerve of the mutant line (Li et al., 2018). Kan and colleagues created a human knock-in mouse strain by replacing one allele of the mouse *Ttr* locus with human TTR A97S (hTTRA97S) (human TTR WT for control animals) and creating, thus, for the first time a mouse model that replicates the earliest signs of ATTR-FAP which is gradual degeneration of sensory nerve cells leading to abnormal sensations such as pain (Ando et al., 2013; Plante-Bordeneuve & Said, 2011). These mice presented congophilic amyloid deposits in the epineurium of the sural nerve and some early stage FAP symptoms were recapitulated including small fibre neuropathy, with degeneration of skin nerves and neuropathic pain, as well as large fibre sensory neuropathy. Despite lack of motor deficit in the hTTRA97S mouse, the presence of congophilic deposits plus display of early ATTR-FAP symptoms makes this mouse model a valuable tool to develop new therapies targeting the sensory polyneuropathy observed in early FAP (Kan et al., 2018).

In addition to the above-mentioned mouse models, other animals for FAP were generated, namely rats (Ueda et al., 2007). These animals, expressing human TTR V30M under the action of mouse albumin promoter, only presented non-fibrillar deposits in the large intestine and at late ages (24 months). Some cases of spontaneous ATTR amyloidosis were also reported in non-human primates (Chambers et al., 2010; Nakamura et al., 2008; Ueda et al., 2012). Vervet monkeys observed by Ueda and colleagues developed cardiac arrhythmia, bradycardia and reduced ejection fraction in the heart mimicking human ATTR WT amyloidosis.

Additionally, there are some successful reports of invertebrate FAP *C. elegans* (Madhivanan et al., 2018) and *D. melanogaster* models (Berg, Thor, & Hammarstrom, 2009; Pokrzywa, Dacklin, Hultmark, & Lundgren, 2007). The *C. elegans* model for ATTR amyloidosis were generated through expression of human WT and mutant (T119M, V30M, and D18G) TTR in the body wall muscle. *C. elegans* expressing TTR V30M presented nociception impairment analogous to human FAP patients and significant impairment on dendritic morphology of somatosensory neurons in ATTR V30M animals (Madhivanan et al., 2018). The transgenic flies were generated using WT and mutant forms of human TTR (TTR L66P, TTR V30M and TTR-A (highly destabilized mutant TTR with two aminoacid replacements V14N/V16E)). Although *Drosophila* flies don't express TTR, they express a TTR-like protein that shares some homology with TTR (CG30016). The first generation of a *Drosophila* FAP model used the glass multiple reporter (GMR) to promote the expression of TTR L55P and TTR-A, in the fly eye (Pokrzywa et al., 2007). This study showed the formation of aggregates in 30-day-old flies either expressing TTR L55P or TTR-A, adult retina degeneration and vacuolization of brain structures. Additionally, flies expressing TTR L55P and TTR-A presented dragged posture of the

wings, impaired climbing ability and decreased lifespan when compared to flies expressing TTR WT. A second fly model for FAP was generated by the expression of either TTR WT or TTR V30M with an HA-tag, in the nervous system (C155-Gal4 promoter) and in the eye (GMR-Gal4 promoter) (Berg et al., 2009). Expressing ATTR V30M in the nervous system resulted in progressive age-dependent reduced climbing activity and lifespan while TTR WT showed milder phenotype (Berg et al., 2009). Positive amyloid staining by Congo red was also seen in aged ATTR V30M flies as well as vacuolization of the brain and eye degeneration shown by scanning electron microscopy (SEM) (Berg et al., 2009).

MOLECULAR MECHANISMS UNDERLYING NEUROTOXICITY IN FAP

Over the last decades a remarkable progress has been made in uncovering the biology and structural basis of TTR amyloidogenesis in FAP. However, a lot is still to be deciphered regarding the molecular mechanisms underlying TTR amyloid neurotoxicity.

A mentioned above, mature fibrils are a less relevant structure in cellular damage whilst non-fibrillar aggregates are described as being the most toxic forms and responsible for the pathology of FAP. Cytotoxicity in FAP is suggested to start at early stages in the process of fibrillogenesis substantiated by the presence of non-fibrillar TTR aggregates in nerve biopsies from FAP patients at early stages of the disease (M. M. Sousa et al., 2002). Also, *in vitro* studies showed a high cellular toxicity from non-fibrillar TTR aggregates when compared to the more inert feature of TTR amyloid fibrils in promoting toxicity (M. M. Sousa, Cardoso, et al., 2001). Additionally, Reixach and colleagues demonstrated that TTR amyloid fibrils above 100kDa of size were not toxic as opposed to the monomeric or very low molecular mass TTR aggregates (Reixach et al., 2004) and there are even some suggestions that hypothesize that the presence of mature fibrils in tissues might represent a passive end stage that might even serve as a rescue mechanism (K. Andersson, Olofsson, Nielsen, Svehag, & Lundgren, 2002). However, the mechanism through which non-fibrillar aggregates cause neurotoxicity in FAP is still not fully understood.

A number of hypothesis, trying to clarify the mechanisms underlying the toxicity caused by TTR aggregates, have arisen in past years (Fig. 8): i) the activation of the Fas receptor, known to be involved in FAP pathology, and subsequent interaction and activation of caspase 8, followed by caspase 3, inducing apoptosis (Macedo, Batista, do Amaral, & Saraiva, 2007); ii) calcium dysregulation inside cells, either through disruption of the cellular membrane, which leads to calcium influx, or through the activation of

endoplasmic reticulum (ER) stress by TTR aggregates, which leads to calcium efflux from the ER, inducing caspase-mediated apoptosis (Saraiva et al., 2012; Teixeira, Cerca, Santos, & Saraiva, 2006); iii) oxidative stress, leading to the accumulation of reactive oxygen species (ROS), promoting dysregulation of normal functions in mitochondria (Saraiva et al., 2012); iv) TTR aggregates were shown to interact with the receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily, with a vast range of ligands, including amyloid-associated macromolecules (Bucciarelli et al., 2002), triggering inflammatory responses via extracellular signal-regulated kinase (ERK) and NF-kB (M. M. Sousa, Du Yan, et al., 2001; M. M. Sousa, Yan, et al., 2000). Moreover, the increased levels of oxidized molecules, promoted by oxidative stress, together with disruption of the extracellular matrix (ECM), via NF-kB signalling, could contribute to amyloid fibril formation (Saraiva et al., 2012).

As previously mentioned, FAP is characterized by a dying-back axonal degeneration, which might suggest a disturbance of the distal neuronal cytoskeleton as consequence of the TTR deposition. Interestingly, a study using a FAP Drosophila model expressing TTRV30M in the fly retina, reported a novel genetic interaction between TTRV30M and members of Rho GTPase-regulated pathways, well known regulators of actin cytoskeleton. The authors have shown that downregulation of Rac1/2, Cdc42, Pak and LIMK suppress TTRV30M-induced axonal defects on the retina of the flies (M, Lopes, & Liz, 2020). All these proteins are major players in actin dynamics and implicated in several other neurodegenerative disorders (DeGeer & Lamarche-Vane, 2013), which raised the possibility that Rho GTPase and the actin cytoskeleton are involved in FAP pathology. The actin cytoskeleton is closely associated with endocytosis, with actin dynamics being critical to remodel the cell surface and vesicular movement (Smythe & Ayscough, 2006). Noticeably, aggregated TTR was found to disrupt the endocytic transport within the cell, as the presence of aggregated TTR decreases transferrin endocytosis in human carcinoma cells and primary liver cells (Fong, Wong, & Vieira, 2017). This observation also adds to the idea of a possible link between TTR aggregation and disturbance in the actin cytoskeleton.

It is, however, still unknown whether TTR aggregates induce disturbances in neuronal actin regulators and in cytoskeleton dynamics in the distal axons but, as cytoskeleton disturbances would be an early event that precedes the degeneration of distal axons in peripheral nerves, this would be a new pathological pathway with great potential to be a therapeutic target for FAP.

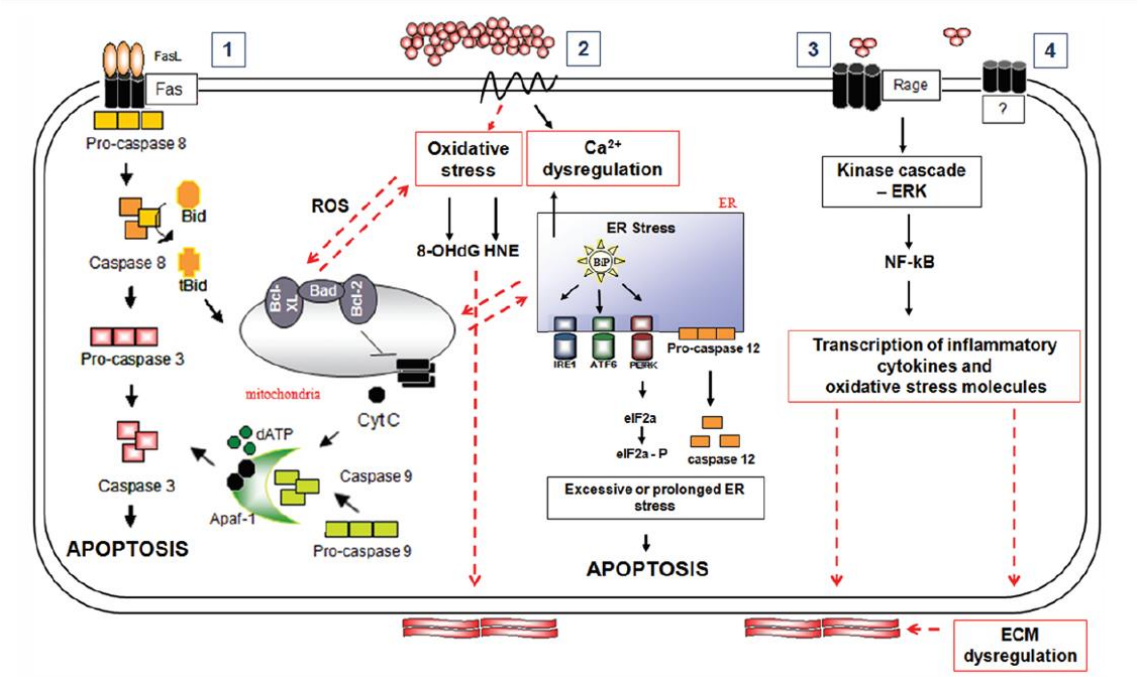

Figure 8. Schematic representation of the possible pathways described for TTR cytotoxicity in FAP. Extracellular small non-fibrillar TTR aggregates interact with the cytoplasmatic membrane by dependent and/or independent of receptors mediated processes leading to the activation of pathways that ultimately lead to neurodegeneration. 1) Small TTR aggregates activate the Fas receptor which leads to the activation of the caspase cascade and to cell death by apoptosis. 2) Disruption of the cellular membrane, mediated by the aggregates, leads to calcium influx from the extracellular medium. Small TTR aggregates were also shown to activate ER stress and to induce calcium efflux from the ER. Activation of ER stress activates apoptosis, through caspase-12 activation. Oxidative stress activated by TTR aggregates leads to oxidized products and reactive oxygen species (ROS) accumulation. Accumulation of ROS can induce changes in mitochondria function and homeostasis which can lead to apoptosis via caspases and to an increase in oxidative stress. 3) TTR aggregates interact with RAGE inducing a kinase cascade that activates NFkB, which in turn activates the transcription of inflammatory cytokines and oxidative stress molecules. 4) The existence of still unknown receptors for TTR aggregates is another hypothesis to explain the cellular toxicity induced by extracellular small non-fibrillar TTR aggregates. Black arrows: activation/expression; Red dashed arrows: possible actions. Adopted from Saraiva et al., 2012.

NEURONAL CYTOSKELETON

Neurons are amongst the most highly polarized cells containing two distinct structural and functional compartments, the somatodendritic and the axonal compartment that extend from the cell body (soma) to mediate the flow of information that passes through the nervous system. The somatodendritic compartment is composed of multiple branches of dendrites that are involved in protein synthesis and responsible to receive the neuronal input at highly specified regions – the dendritic spines – and transmit it to the cell body where it will be integrated. The axon is typically a single long projection that functions as a signal transmitter, which contacts with neighbouring cells conveying the electric impulse and neuronal information.

The neuronal cytoskeleton is crucial in maintaining this asymmetry in shape and polarity, since it provides not only the neuronal structural backbone, but also because it separates functionally the two different compartments. Axons can extend to remarkable distances (up to 1 meter in humans), which raises the need of a well-constructed machinery that shuttles vital cellular components from the cell body along the axonal projection. As such, an appropriately organized neuronal cytoskeleton is needed during nervous system development, maintenance and also during regenerative processes that follow injury. The maintenance of the neuronal structural integrity requires controlled internal organization of its three cytoskeletal components: the intermediate filaments, microtubules and actin microfilaments. These cytoskeleton components are shaped by proteins that can be assembled in a wide combination of ways, giving rise to different structures with different properties, and that can also be disassembled and reassembled into different shapes according to the specific spatio-temporal needs.

NEURONAL CYTOSKELETON COMPONENTS: THE BUILDING BLOCKS FOR POLARITY

The neuronal cytoskeleton is composed of three main components: neurofilaments, microtubules and actin microfilaments. As mentioned above, neurons present a distinctive polarization which is maintained by the elegant interplay of each purpose of each individual cytoskeleton component. In fact, all cytoskeleton components exhibit their own intrinsic characteristics but they share interdependent functions and, in the end, everything is connected.

Neurofilaments

Intermediate filaments (IFs) are rope-like structures with a diameter of approximately 8-10 nm and constitute the largest family of mammalian cytoskeleton proteins (Fuchs & Weber, 1994; McLachlan, 1978; Pauling & Corey, 1953). They provide mechanical strength and stabilize the cytoskeleton framework. IFs present a unique form of assembly comparing with other cytoskeleton components. In actin and microtubules (MTs), assembly occurs from either ends of the filament, but IFs assembly occurs along the whole filament, where exchange between the subunits of the monomeric and filamentous pools occurs (Godsel, Hobbs, & Green, 2008). Additionally, and in contrast to MTs and actin, IFs are a heterogeneous class of polymers. The assembly of the IFs is initiated by the formation of a dimer, in which the central rod domains of two polypeptides twist around each other, followed by the antiparallel association of two dimers, creating a tetramer. Tetramers join end-to-end, forming protofilaments, and laterally, forming filaments. IFs do not exhibit a dynamic behaviour, like MTs and actin filaments, and their major function is to provide structural support to the cell (Herrmann, Strelkov, Burkhard, & Aebi, 2009).

In neurons, intermediate filaments are denominated neurofilaments (NFs). NFs are heteropolymers that in the CNS are composed by 4 subunits of neurofilament light (NF-L), neurofilament medium (NF-M), neurofilament heavy (NF-H) (M. K. Lee, Xu, Wong, & Cleveland, 1993; Shaw & Weber, 1982) polypeptides as well as α-internexin (Fliegner, Kaplan, Wood, Pintar, & Liem, 1994; Yuan et al., 2006). In the PNS, neurofilaments are made up of NF-H, NF-M, NF-L and peripherin (Beaulieu, Robertson, & Julien, 1999; Troy, Brown, Greene, & Shelanski, 1990). Neurons may also express other intermediate filament proteins, including nestin, synemin, syncoilin and vimentin (Perrin et al., 2005).

One remarkable feature of NFs are the exceptional long lives they possess (Millecamps, Gowing, Corti, Mallet, & Julien, 2007; Nixon & Logvinenko, 1986; Yuan et al., 2009) and their elastic fibrous properties that allows the asymmetrical shape of neurons (Wagner et al., 2007). One of the most important functions of NFs is the contribution in intra-axonal volume, particularly in large myelinated axons. In fact, neurofilament levels in neurons ensure normal transmission of the nerve impulse by maintaining the normal axon calibre and proper neuronal function, since nerve conduction velocity is directly proportional to axonal diameter. Thus, axonal NFs loss leads to alterations in axonal calibre and consequently to slower conduction velocities culminating in delayed maturation during regeneration and abnormalities in animal behaviour (Kriz, Zhu, Julien, & Padjen, 2000; Sakaguchi, Okada, Kitamura, & Kawasaki, 1993; Zhu, Couillard-Despres, & Julien, 1997).

Microtubules

MTs are parallel bundles of 25nm in width composed by 13 protofilaments of α- and β-tubulin heterodimers. MTs play a significant role in cellular structure since they function as architectural backbones for neurons being crucial to maintain axonal integrity and form the tracks for long-range axonal transport (Prokop, 2013). Both α-tubulin and β-tubulin bind to GTP that regulates polymerization. Shortly after polymerization, GTP is hydrolysed and the affinity of tubulin for adjacent molecules weakens, favouring depolymerization and resulting in the dynamic state characteristic of MTs (Mitchison & Kirschner, 1984). Fast-growing ends mostly expose β subunits while the minus ends expose α subunits. While α-tubulin is always bound to GTP, in β-tubulin, GTP is converted to GDP after incorporation into the filament (Desai & Mitchison, 1997). MTs also undergo treadmilling, a dynamic process in which the plus end of the filament grows in length, while the other one shrinks, due to the removal of tubulin molecules bound to GTP from the minus end that travel to the plus end of the same MT (Walker et al., 1988). Fig. 9 depicts microtubule dynamic instability. In many cell types, MTs are nucleated at the centrosomal region in the microtubule-organizing centre (MTOC), which is the starting point of MT polymerization where α- and β-tubulin dimers associate with a previously formed γ-tubulin ring and start elongating from there (Joshi, Palacios, McNamara, & Cleveland, 1992; Sunkel, Gomes, Sampaio, Perdigao, & Gonzalez, 1995). Yet, neurons have the ability to be centrosomal independent regarding MT nucleation. Studies using *D. Melanogaster* neurons demonstrated that in the absence of functional centrosomes, neurons developed a normal MT network and a healthy axonal outgrowth (Basto et al., 2006; Nguyen, Stone, & Rolls, 2011). Moreover, another recent study using rat hippocampal neurons evidenced that centrosomes lose their MTOC function during neuronal development and that axon extension and regeneration is MTOC independent (Stiess et al., 2010).

Nevertheless, in neurons, MTs are still polarized filaments with the minus ends directed towards the cell body, that must be capped, and the plus ends directed outwards along the axon (Fig. 10). The capping of the minus ends of MTs, indispensable for preventing depolymerization, is assured by 3 homologous proteins, the calmodulin regulated spectrin-associated proteins 1–3 (CAMSAP1-3) (Baines et al., 2009). The MT cytoskeleton is, thus, a tightly organized structure with an effective crosstalk among the players that regulate its polarity and structure, which are crucial to determine the movement along MTs. MTs undergo extensive post-translational modifications (PTMs), which will be further explored in the next sections. Although PTMs don't influence directly de MT dynamic properties, they do correlate with the selective distribution of the MTs through the cell and even with MT stability which will impact in the binding of multiple

microtubule-associated proteins (MAPs) and, eventually, modulate MT function (Conde & Caceres, 2009).

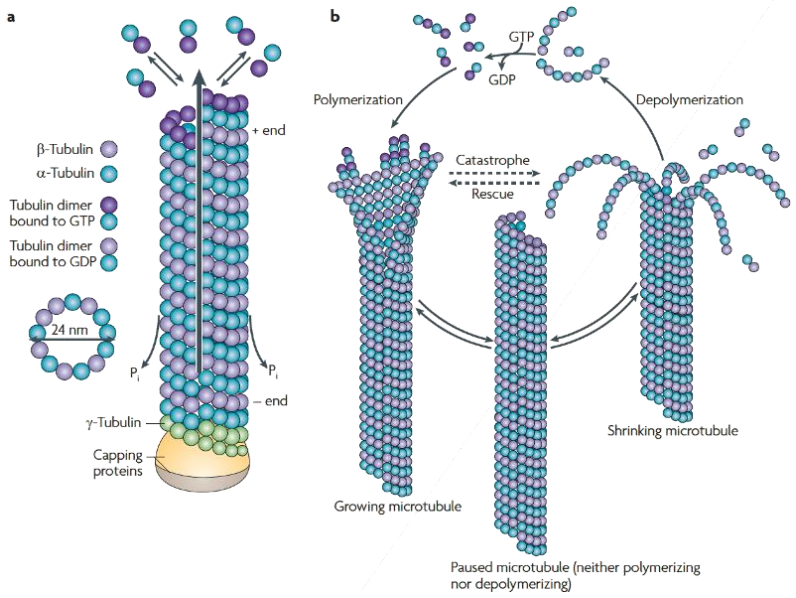

Figure 9. Microtubule polymerization and dynamics. (A) The head-to-tail association of the α/β heterodimers makes MTs polar structures, and they have different polymerization rates at the two ends. In each protofilament, the α/β heterodimers are oriented with their β-tubulin monomer pointing towards the faster-growing end (plus end) and their α-tubulin monomer exposed at the slower-growing end (minus end). The lateral interaction between subunits of adjacent protofilaments has been described as a B-type lattice with a seam (long arrow, a). A third tubulin isoform, γ-tubulin, functions as a template for the correct assembly of MTs. (B) Upon addition of a new dimer at the plus end, the catalytic domain of α-tubulin contacts the nucleotide exchangeable site of the previous β-subunit and becomes ready for hydrolysis; the plus end generally has a minimum GTP cap of one tubulin layer that stabilizes the MT structure. When this GTP cap is stochastically lost, the protofilaments splay apart and the MT rapidly depolymerizes. During or soon after polymerization, the tubulin subunits hydrolyse their bound GTP and become non-exchangeable. Thus, the MT lattice is predominantly composed of GDP–tubulin, with depolymerization being characterized by the rapid loss of GDP–tubulin subunits and oligomers from the MT plus end. Adopted from Conde & Caceres, 2009.

In neurons, MTs are responsible for the maintenance of neuronal polarization. MTs are more stable in axons than in dendrites, with the exception of those in the growth cone (Witte & Bradke, 2008). In fact, several proteins are responsible for this duality such as Stathmin/Op18 which is a MT destabilizing protein active specifically in dendrites (Watabe-Uchida, John, Janas, Newey, & Van Aelst, 2006). In vertebrate neurons, all axonal MTs display a plus-end-out orientation, whereas microtubules in dendrites have mixed orientations (Fig. 10) (Yau et al., 2016). These differences contribute to the observed polarised trafficking to axons and dendrites where, for example, in axons dynein mediated transport of cargos only occurs retrogradely, whereas in dendrites dynein can

perform vesicle transport in both directions. In *D. melanogaster* and *C. elegans*, however, all microtubules present a minus-end-out orientation in dendrites. The observations that before axonal specification MTs are mixed oriented raised the hypothesis that the uniform plus-end-out signature in vertebrate MTs is acquired during axonal specification (Yau et al., 2016).

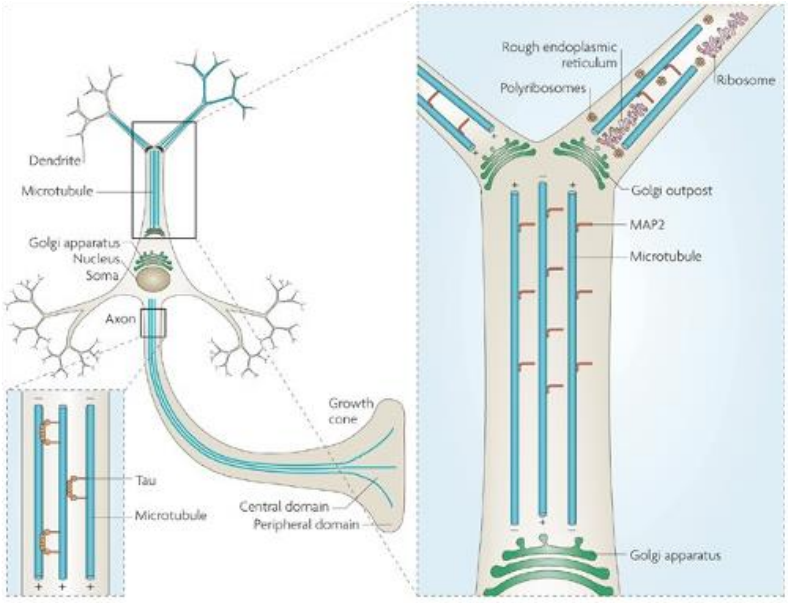

Figure 10. Microtubule organization and organelle distribution in axons and dendrites. Axons have tau bound microtubules of uniform orientation, whereas dendrites have microtubule-associated protein 2 (MAP2)-bound microtubules of mixed orientation. Dendrites also contain organelles that are not found in axons, such as rough endoplasmic reticulum, polyribosomes and Golgi outposts. Adopted from Conde & Caceres, 2009.

Actin

The actin cytoskeleton is composed of globular actin monomers (G-actin) that have tight binding sites that enable head-to-tail interactions with two other actin monomers, such that they polymerize to form thin, flexible actin filaments (F-actin) of approximately 7 nm in diameter (Spudich, Huxley, & Finch, 1972). Mammals express three actin isoforms, α, β and γ, of which α and mostly β are expressed in neurons (Letourneau, 2009). In neurons, actin is highly abundant comprising about 4–5% of total protein and, interestingly, neurons are able to synthesise actin. Both, actin protein and actin mRNA are transported in axons and studies suggested a crucial role for actin mRNA in growing axons since local translation of actin in actively extending axonal regions provides the necessary building blocks for cytoskeleton rearrangements (A. C. Lin & Holt, 2007).

Actin filaments are organized into higher-order structures, forming bundles or three-dimensional networks. The process of actin polymerization proceeds through three

sequential steps: nucleation, elongation and depolymerization. Nucleation consists in the formation of a small aggregate, composed of three actin monomers. As soon as trimers are formed, they are able to elongate by adding G-actin monomers to both ends of the filament. Similar to MTs, F-actin displays intrinsic polarity, due to the presence of two distinguishable ends with different polymerization rates: a fast-growing plus (or barbed) end, which is more dynamic and elongates five to ten times faster than the slow-growing minus (or pointed) end. The reversible addition of monomers happens in both ends. (Pollard & Mooseker, 1981). Actin assembles spontaneously through non-covalent interaction, thus the binding between actin monomers and ATP is not required for polymerization. However, energy contributes to a faster polymerization. Following filament assembly, ATP bound to actin is hydrolysed to ADP (Hayashi & Rosenbluth, 1962; Offer, Baker, & Baker, 1972). Since actin polymerization is a reversible process, ADP-actin monomers or F-actin fragments are removed from the pointed end. The cycle is completed when ADP bound to dissociated monomers is exchanged for ATP, and they can be recruited back to the growing filament at the barbed end.

The different rates of polymerization in the two ends contribute to the steady-state dynamics of polymerization/depolymerization, known as actin filament treadmilling, which explains the dynamic behaviour of actin filaments. The dynamics of F-actin assembly/disassembly are strongly regulated by actin-binding proteins (ABPs): i) proteins that promote nucleation and subsequent polymerization of filaments and branched networks, such as the Arp2/3 complex and formins; ii) proteins that are bound to monomeric G-actin and promote its assembly into the barbed end of the actin filament, such as profilin; iii) proteins that bind to actin filaments, promoting their stabilization (tropomyosin) or cross-linking (α-actinin, filamin and fimbrin); iv) proteins responsible for the capping of both barbed and pointed ends, such as CapZ and tropomodulin; and v) proteins that sever actin filaments, such as the actin depolymerizing factor (ADF)/cofilin family (Coles & Bradke, 2015). Besides kinetic modulation, ABPs also serve to spatially organise actin. Figure 11 depicts the schematic of actin dynamics modulation.

In neurons, actin is seen as the engine behind the generation of the force necessary to regulate the neuronal shape and cellular internal and external movements (Luo, 2002). Actin also plays an important role during developmental growth since this process requires rapid restructuring of actin to form complex and dynamic structures as lamellipodia, filopodia, stress fibres, focal adhesions and actin waves. Some of these structures such as lamellipodia and filopodia will be further dissected in the next sections.

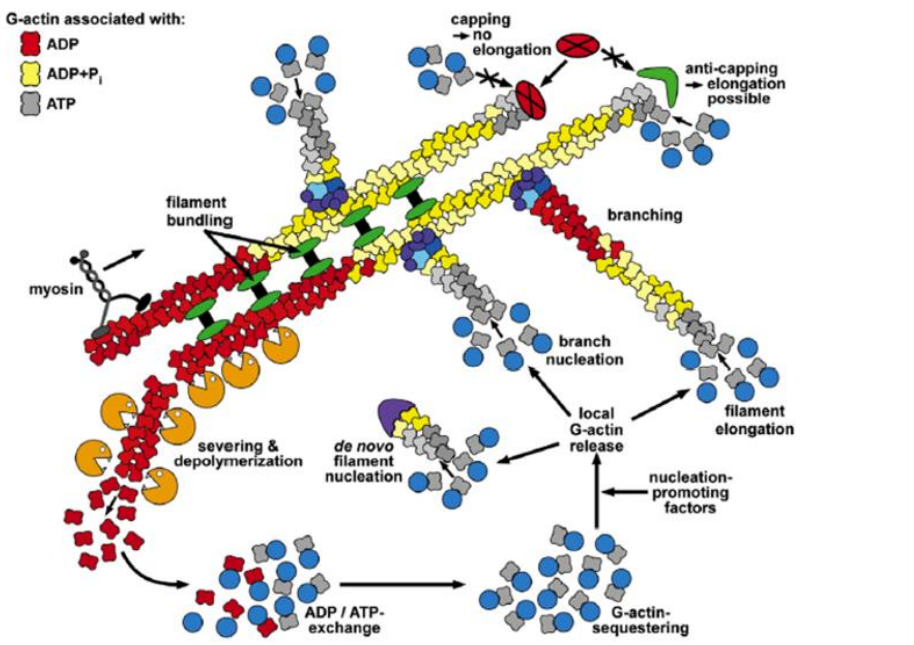

Figure 11. The regulation of actin dynamics. ATP-bound globular actin (G-actin) polymerizes to form helical actin filaments (F-actin), the basis of the actin cytoskeleton. In F-actin, hydrolysis of the bound ATP and subsequent dissociation of inorganic phosphate occurs. During axon formation, actin dynamics are modulated by various means. For example, actin filaments branch and can be bundled, for example, by fascin. Actin dynamics are increased by filament severing and depolymerization, for example, by cofilin. Released G-actin is set for repolymerization by profilin-mediated ADP/ATP exchange. To avoid its uncontrolled polymerization, G-actin is sequestered, for example, by profilin or thymosin b4. Elongation of existing or newly nucleated filaments occurs upon local release of G-actin from profilin. The release is triggered by nucleation-promoting factors, such as WASP-family proteins, for example, upon external signals. Capping proteins such as CapZ prevent filament elongation while anti-capping proteins, for example, of the Ena/VASP family, prevent the binding of capping proteins and thereby allow filament elongation. Myosin, an actin-based motor protein mediates transport and actin contractility. Adopted from Witte & Bradke, 2008.

NEURONAL CYTOSKELETON ORGANIZATION

During axonal growth either in a context of neuronal development or in the context of regeneration in the PNS, there is the formation of a growth cone. First described by Ramón y Cajal, this distinctive actin-rich structure, present in the tip of the growing axon is an accurate sensor that interprets extracellular stimuli of different origins and transduces signals to the cell interior through cytoskeleton rearrangements. It is currently accepted that cytoskeleton dynamics at the growth cone generates mechanical forces driving axonal elongation. The role of the growth cone in neurons is therefore to control both the rate and the direction of growth.

The growth cone is organized into three different domains. The peripheral (P) domain is predominantly enriched with actin, shaped as both long F-actin bundles, named

filopodia, and sheet-like F-actin meshwork, named lamellipodia (Fig. 12). Both types of actin filaments have their barbed ends facing the periphery of the cell. Additionally, dynamic MTs are able to explore this domain. The central (C) domain is composed by bundles of stable MTs, entering the growth cone from the axon shaft, as well as vesicles, organelles and proteins transported into this domain. The transition (T) zone is located between the P and C domains and is composed by actin arcs (actomyosin contractile structures) creating a hemi-circumferential ring (Fig. 12). F-actin treadmilling and actin retrograde flow are the engines responsible for the movement of the growth cone in response to directional cues (Lowery & Van Vactor, 2009). Actin treadmilling is the combination of both polymerisation and depolymerisation activities that ensure the constant length of the actin polymer promoting, at the same time, polymer spatial advance. However, under non-stimulatory conditions, i.e., when filopodia/lamellipodia are not engaged to the substrate, actin polymerisation occurs against the plasma membrane which in turn generates a mechanical force counteracting polymer advance. This leads to backward movement of actin microfilaments from the leading edge to the T-domain, where actin filaments are rearranged by ABPs. This process is called the actin retrograde flow which is not only a physical consequence resulting from the plasma membrane tension against F-actin polymerisation, but is also actively controlled by other molecules, belonging to the myosin family (C. H. Lin, Espreafico, Mooseker, & Forscher, 1996).

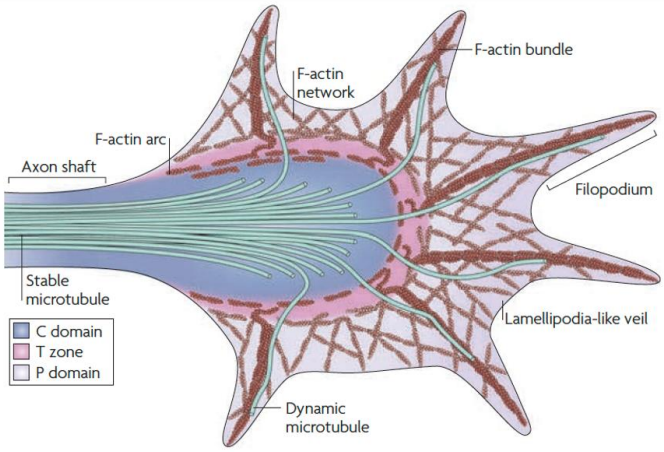

Figure 12. Structure of the growth cone. The leading edge consists of dynamic, finger-like filopodia that explore the road ahead, separated by sheets of membrane between the filopodia called lamellipodia-like veils. The growth cone can be separated into three domains based on cytoskeletal distribution. The peripheral (P) domain contains filopodia, lamellipodia and individual dynamic 'pioneer' MTs exploring the region, usually along F-actin bundles. The central (C) domain encloses stable, bundled MTs that enter the growth cone from the axon shaft, the transition (T) zone sits at the interface between the P and C domains, where actomyosin contractile structures (termed actin arcs) lie perpendicular to F-actin bundles and form a hemi-circumferential ring. The dynamics of these cytoskeletal components determine growth cone shape and movement during development. Adapted from Lowery & Van Vactor, 2009.

Under growth promoting conditions, filopodia extend and create points of attachment – the engorgement phase – which is characterised by the disassembly of F-actin and reorientation of F-actin arcs in the T-domain towards the adhesive sites. Reoriented F-actin generates a corridor which is rapidly invaded by C-domain MTs carrying vesicles and organelles determining the new axonal growth direction (Schaefer et al., 2008). In addition to MT-based transport, filopodia extension is also facilitated by actin-based motor proteins such as myosin-X or myosin V, that use an ATP-dependent 'walking' mechanism to deliver key material to support outgrowth. Consolidation phase occurs as the proximal part of the growth cone assumes a cylindrical shape and the transport of organelles becomes bidirectional, thus forming a new segment of the axon shaft (Dent & Gertler, 2003). In summary, growth cone migration and the consequent neurite elongation involve 3 fundamental cytoskeleton phenomena: actin-based protrusion of the leading edge, invasion of microtubule to the distal regions of the cone and microtubule and actin-based transport of vesicles/organelles from the C-domain to the P-domain (Heidemann, Lamoureux, & Buxbaum, 1995).

Growth cone filopodia/lamellipodia progression is a highly orchestrated process. Several factors have a fundamental role in the overall migration of the growth cone. In fact, the reactiveness of the growth cone to external cues depends on the differential expression and accumulation of specific proteins throughout the P-domain (Dent & Gertler, 2003). For example, in the presence of a chemical gradient either of attractant nature, such as nerve growth factor (NGF) and netrin, or repulsive nature, such as semaphorin-3A, slit3 and ephrin-A2, growth cones accumulate different proteins at different locations, modulating their own turning (Bashaw & Klein, 2010; Dent & Gertler, 2003). Specifically, on the side of a chemoattractant cue several processes occur: progressive expression of more membrane receptors, increased local vesicle transport and increased rate of exocytic events. On the contrary, as the growth cone turns towards the chemoattractant gradient, its rear side collapses, mostly because the cytoskeleton is disassembled, membrane receptors are endocytosed leading to destruction of adhesive contacts and macropinosomes are formed (Vitriol & Zheng, 2012). Therefore, differential expression of molecules during growth cone advance creates the curvature necessary for directional growth.

The axon has a distinctive cytoskeletal organization, containing bundles of long stable MTs which provide support and represent the roads for the transport of proteins. The unipolar orientation that MTs display along the axon is a crucial feature for the correct transport in neurons (Kapitein & Hoogenraad, 2011). The transport of organelles and vesicles occurs bidirectionally: from the soma to the periphery (anterograde transport) and from the periphery to the soma (retrograde transport). Kinesin superfamily proteins (KIFs)

are responsible for the anterograde transport, moving towards the plus end, and, conversely, dynein is responsible for the retrograde transport of cargoes, moving towards the minus end (Fig. 13D) (Hirokawa, Niwa, & Tanaka, 2010). In the last decade, it was found that the axon shaft contains unique actin-rich structures, such as actin rings, where actin is arranged in periodic isolated rings, in association with adducin, and separated by spectrin tetramers (Fig. 13B) (Xu, Zhong, & Zhuang, 2013); and actin trails, which are dynamic "hotspots" that undergo constant assembly/disassembly (Fig. 13C) (Ganguly et al., 2015). It is believed that actin rings provide additional structural support to the axon and that actin trails confer a much-needed dynamic flexibility. Moreover, patches of actin filaments have been described in the axon initial segment (AIS), where these structures mediate the capture of cargoes, limiting their transport into the axon (Watanabe et al., 2012).

MTs are also enriched in dendrites, in which, contrary to what is described in the axon, exhibit a mixed polarity, with both plus and minus ends oriented towards the periphery (Kapitein & Hoogenraad, 2011). MTs are predominantly restricted to the dendritic shaft but some of them are able to protrude into the dendritic spine (Eira, Silva, Sousa, & Liz, 2016). Moreover, a network of actin filaments (actin patch) is present in dendritic spines, with the barbed ends pointing towards the cellular membrane (Fig. 13A), contributing to the formation and organization of dendritic spines (Matus, Ackermann, Pehling, Byers, & Fujiwara, 1982).

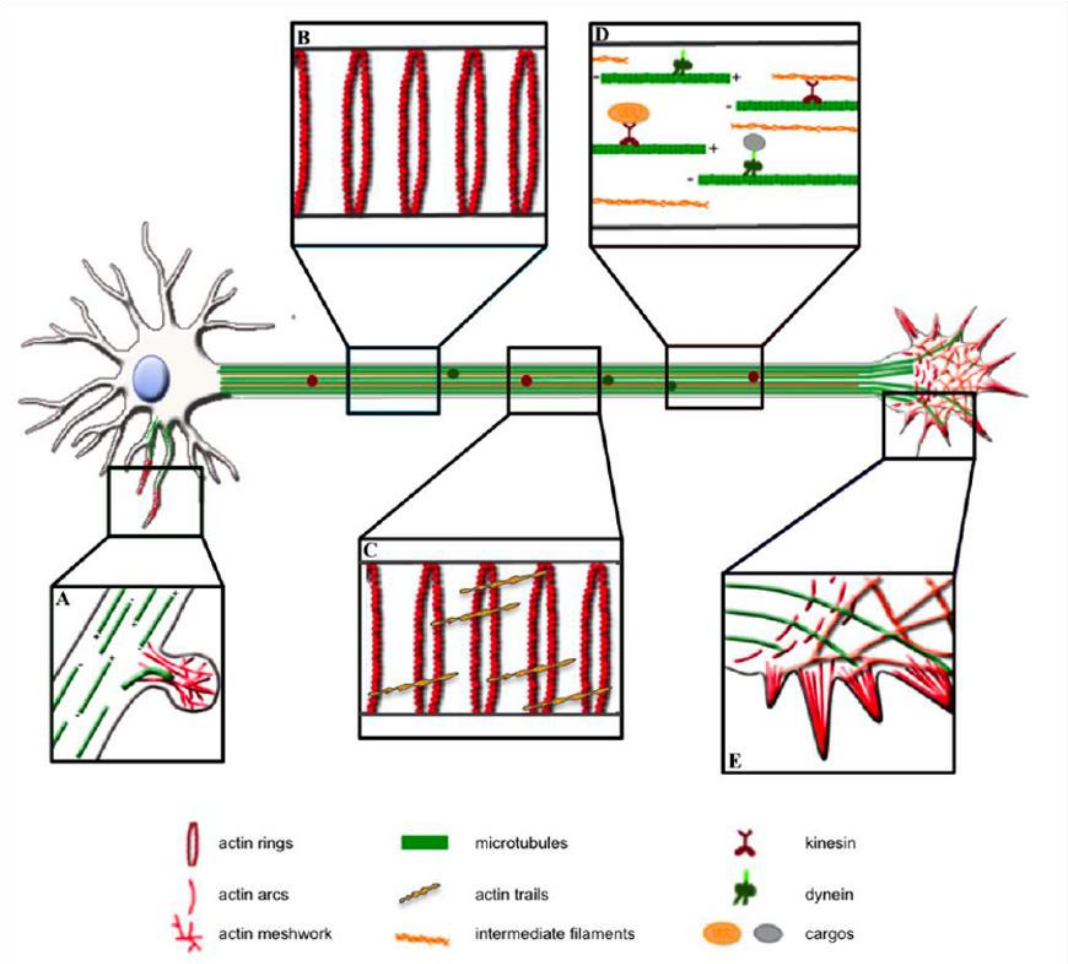

Figure 13. Cytoskeletal architecture of a developing/growing neuron. (A) In dendrites, MTs present a mixed polarity, mostly located in the dendritic shaft, with some of them protruding to the dendritic spine. Actin patches are also enriched in dendritic spine. (B) Actin is enriched in the axon, in the form of isolated rings, in association with adducin and spectrin. (C) Dynamic hotspots of actin filaments, known as actin trails, are located along the axon. (D) Throughout the axon, MTs are oriented with their plus end facing the axon tip and represent the roads for cargo transport, mediated by kinesin and dynein. (E) The growth cone is a unique structure composed of dynamic MTs and actin filaments arranged as filopodia (bundles) and lamellipodia (sheet like network). Adopted from Eira et al., 2016.

REGULATION OF THE NEURONAL CYTOSKELETON: MOLECULAR PLAYERS

The regulation of the neuronal cytoskeleton is crucial for the proper development and maintenance of the neuron, and especially crucial during axon formation, outgrowth and guidance. Despite the high level of structural conservation, MTs present different identities and properties that are greatly defined by the incorporation of distinct tubulin isoforms (Wilson & Borisy, 1997) and by tubulin PTMs. PTMs are crucial regulators of MT properties and functions and they occur through the action of several tubulin-modifying enzymes (Janke & Bulinski, 2011). The most common and abundant PTMs include

detyrosination, Δ2-tubulin generation, acetylation, polyglutamylation and polyglycylation (Fig. 14). Most α-tubulins are expressed in a tyrosinated state; hence they originally encode a carboxy(C)-terminal tyrosine amino acid (Song & Brady, 2015), which is associated with a highly dynamic state of MTs (Ponstingl, Krauhs, Little, & Kempf, 1981). The cycle of tyrosination/detyrosination initiates with the proteolytic removal of the terminal tyrosine residue (detyrosination) in the polymerized MT, by a recently discovered tubulin carboxypeptidase, vasohibins/SVBP (Aillaud et al., 2017), whereas re-addition of tyrosine (tyrosination) is catalysed by an enzyme called tubulin tyrosine ligase (TTL), working exclusively on soluble dimers of tubulin (Janke & Bulinski, 2011; Raybin & Flavin, 1977). Tyrosination of tubulin has a major impact on neuronal function; TTL knockout mice die at the perinatal stage due to neuronal abnormalities (Erck et al., 2005). Detyrosinated tubulin has been associated with long-lived MTs, although a direct causal relationship between MT stability and tubulin detyrosination has not been established. Following detyrosination, tubulin exposes a C-terminal glutamate, that can be further, and irreversibly, removed by deglutamylase enzymes of the cytosolic carboxypeptidase (CCP) family, generating Δ2-tubulin (Janke & Bulinski, 2011). Acetylation is the only PTM that occurs in the lumen of MTs, in the lysine 40 (Lys40) residue of α-tubulin (L'Hernault & Rosenbaum, 1985), although additional α-tubulin and β-tubulin acetylation sites were already identified (Janke & Kneussel, 2010). Tubulin acetylation is predominantly catalysed by α-tubulin N-acetyltransferase 1 (α-TAT1/ATAT1) (Kalebic et al., 2013) and deacetylation is promoted by deacetylating enzymes such as histone deacetylase 6 (HDAC6) (Hubbert et al., 2002) and Sirtuin-2 (SIRT2) (North, Marshall, Borra, Denu, & Verdin, 2003). Instead of a direct effect on MT stability, both detyrosination (Konishi & Setou, 2009; Kreitzer, Liao, & Gundersen, 1999) and acetylation (Reed et al., 2006) were suggested to impact on axonal transport by regulation of kinesin motors. Another tubulin modification with a high impact on MT integrity is polyglutamylation. Polyglutamylation consists in the reversible addition of side chains of glutamates in gene-encoded glutamate residues of both α- and β-tubulins, which is catalysed by members of the tubulin tyrosine ligase-like (TTLL) family (Janke et al., 2005; van Dijk et al., 2008) and reverted by enzymes of the CCP family (Rogowski et al., 2010). This tubulin PTM is highly abundant in neurons (Song & Brady, 2015).

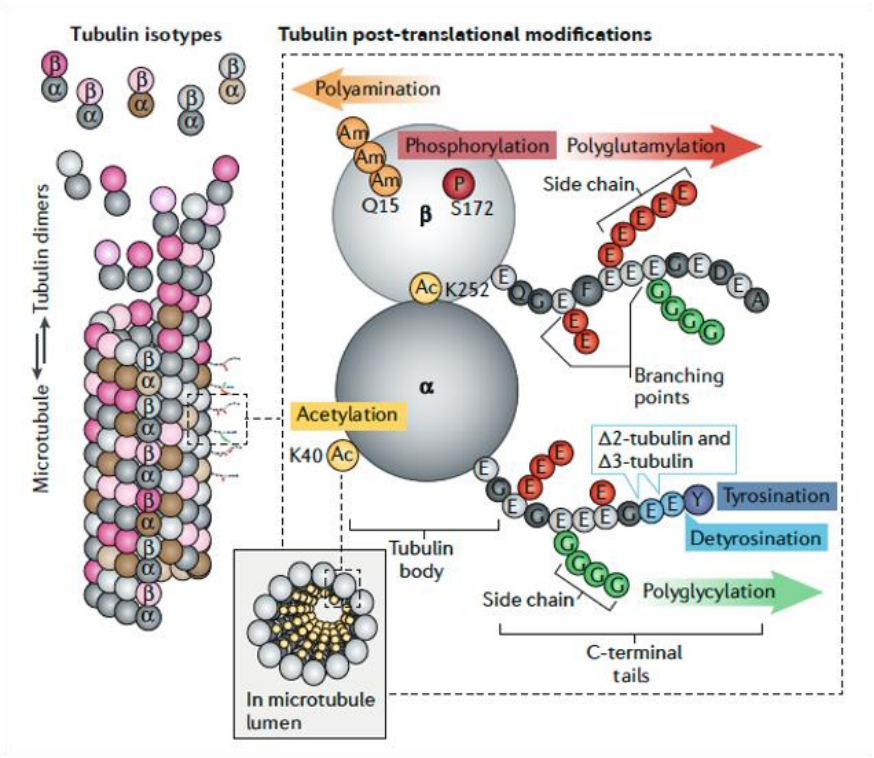

Figure 14. Tubulin post-translational modifications. Schematic representation of the α-tubulin/β-tubulin dimer and its associated modifications. The tubulin code refers to the concept that different tubulin gene products, together with a variety of post-translational modifications (PTMs), modulate the composition of individual microtubules. Tubulin isotypes (α-tubulins, dark grey and brown; β-tubulins, light grey and pink) are encoded by different tubulin genes and can mix during microtubule assembly. Tubulin PTMs are catalysed by a range of enzymes and are located either at the globular, highly structured tubulin bodies (acetylation, phosphorylation, polyamination), or at the unstructured C-terminal tails of tubulin (glutamylation, glycylation, tyrosination, detyrosination and removal of glutamate residues to produce Δ2- ubulin and Δ3-tubulin). Tubulin PTMs can generate binary switches (on–off signals) by adding or removing single functional residues (acetylation, phosphorylation or detyrosination) or can gradually modulate the strength of their signals by adding different numbers of residues (polyamination, polyglutamylation and polyglycylation). Polymodifications are initiated by the generation of a branching point, from which side chains are further elongated Adopted from Janke & Magiera, 2020.

Tyrosinated tubulin has been associated with dynamic MTs, thereby being enriched in the growth cone, while detyrosinated and acetylated tubulin are associated with more long-lived and stable MTs, present in the axon shaft (Ahmad, Pienkowski, & Baas, 1993; Janke & Kneussel, 2010; Schulze, Asai, Bulinski, & Kirschner, 1987). However, the PTM is not considered the cause, but the consequence of MT stability or dynamicity. In fact, MTs are not stabilized by detyrosination per se, but this happens due to the fact that detyrosinated MTs are less susceptible to depolymerization by kinesin 13 family motor proteins, such as mitotic centromere-associated kinesin (MCAK) and the neuronal kinesin family member 2A (KIF2A) (Peris et al., 2009). Additionally (in the same reasoning),

tubulin acetylation does not contribute to MT stabilization. Instead, this PTM occurs in already stable MTs, due to its slow activity rate (Kull & Sloboda, 2014). PTMs have also been shown to alter the association of MTs to motor proteins. Kinesin-1 has been shown to bind preferentially to acetylated and detyrosinated, thus more stable, MTs (Dunn et al., 2008; Reed et al., 2006), while tubulin tyrosination regulates the initiation of dynein-driven retrograde transport in neurons, with the involvement of +TIPS, such as CLIP-170 and EBs (Nirschl, Magiera, Lazarus, Janke, & Holzbaur, 2016).

Additionally, MTs and actin dynamics must be under precise control, which is regulated by several signalling pathways. Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase, with two isoforms, GSK3α and GSK3β, and is a known regulator of the neuronal cytoskeleton, being involved in processes such as neurogenesis, neuronal migration, neuronal polarization and axon growth and guidance. GSK3β, in particular, displays a wide range of predicted substrates and is able to regulate the activity of several MAPs, through phosphorylation, including the MT-depolymerizing factor stathmin, APC, CLASP, collapsing response mediator protein 2 (CRMP2), MAP1B and Tau, thereby controlling local MT dynamics, mainly in the growth cone (Hur & Zhou, 2010; Liu, Hur, & Zhou, 2012). GSK3β activity is regulated by several signalling pathways, including the one involving the triggering of the receptor tyrosine kinase (RTK) and consequent activation of phosphatidylinositol 3-kinase (PI3K), followed by activation of Akt, which mediates the phosphorylation of the serine 9 (Ser9) residue of GSK3β, inhibiting its activity (Cross, Alessi, Cohen, Andjelkovich, & Hemmings, 1995; Hur & Zhou, 2010). At the growth cone, during axon outgrowth, GSK3β activity towards MAP1B is preserved, promoting MT dynamics, while the activity of this kinase towards CRMP2 and APC is blocked, increasing MT polymerization and stability (Hur & Zhou, 2010). In fact, recently it was found that, following spinal cord injury, the inactivation of GSK3β increases MT dynamics (increased MT growth speed), promoting axon regeneration, via decrease in the phosphorylation of CRMP2, and independently of MAP1B and CLASP2 (Liz et al., 2014).

The Rho family of GTPases play central roles in regulating the organization of the actin cytoskeleton, being involved in the control of cytoskeletal dynamics in neuronal growth cones (B. J. Dickson, 2001) as well as in dendrites and dendritic spines (Nakayama, Harms, & Luo, 2000). The most extensively studied members of this family of proteins are the Ras homologous member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42). The activation of GTPases is controlled by cycling between an active GTP-bound state and an inactive GDP-bound state, a process regulated by guanine nucleotide exchange factors (GEFs), that promote the activation of the protein, which activate the protein by catalysing the exchange of GDP for GTP, GTPase-activating proteins (GAPs), that switch GTPases “off”,

by stimulating their intrinsic GTPase activity, and guanine nucleotide dissociation inhibitors (GDIs), which maintain the proteins in an inactive state (Jaffe & Hall, 2005). It has been shown that RhoA is responsible for the formation of contractile actin and myosin filaments (stress fibers) and focal adhesions, while Rac1 and Cdc42 are able to promote the establishment of lamellipodia and filopodia, respectively (Hall, 1998).

Rho GTPases regulate a high number of cellular processes and represent upstream regulators of several ABPs. The Arp2/3 complex is crucial for F-actin nucleation in lamellipodia and filopodia and both Rac1 and Cdc42 activate Arp2/3 indirectly through members of the Wiskott-Aldrich syndrome protein (WASP). WASP and neural(N)-WASP exist in an autoinhibited conformation, due to the interaction between the C-terminus and N-terminus. The autoinhibited conformation is released when Cdc42 binds to WASP and promotes its binding to Arp2/3 and monomers of G-actin, initiating the polymerization of a branched network (Jaffe & Hall, 2005; Pollitt & Insall, 2009). Additionally, activation of Arp2/3 by Rac1 is mediated by WASP-family verprolin-homologous protein (SCAR/WAVE) (Eden, Rohatgi, Podtelejnikov, Mann, & Kirschner, 2002). RhoA kinase (ROCK) is one of the major downstream targets of RhoA, being responsible for the increased activity of myosin II and consequent increase in actin retrograde flow, resulting in growth cone collapse and retraction, in response to repulsive guidance cues (Gomez & Letourneau, 2014). Furthermore, ROCK phosphorylates LIM kinase (LIMK), which, in turn, phosphorylates ADF/cofilin in the serine 3 residue, inhibiting the cofilin-driven severing of F-actin resulting in a decrease in actin turnover and growth cone collapse; this process is reversed by the action of slingshot phosphatase-1 (SSH1) (Aizawa et al., 2001). LIMK-dependent phosphorylation of cofilin can also be induced by Rac1, acting through one of its downstream targets, p21-activated kinase (PAK) (Endo et al., 2003).

Besides the crucial role in actin dynamics regulation, Rho GTPases also display an involvement in the regulation of MTs, being one of the most striking examples the MT stabilization via RhoA-mDia where RhoA signalling pathway was shown to induce the formation of a stable detyrosinated MT subset involved in cell migration where the radially oriented array of MTs reorganized and polarized in response to an external cue by the spatio-temporal transformation of a group of dynamic MTs into a stable one that contained detyrosinated or acetylated α-tubulin (Gundersen & Bulinski, 1988; Gundersen et al., 2005). Additionally, activation of mDia was shown to stimulate the formation of stable MTs and to be sufficient to generate and orient stable MTs by regulating the dynamics of MT plus ends (Palazzo, Cook, Alberts, & Gundersen, 2001). A more recent study has provided evidence suggesting that actin capping protein induces release of mDia from actin filaments near the leading-edge allowing binding to MTs and subsequent stabilization (Bartolini, Ramalingam, & Gundersen, 2012).

CYTOSKELETON REMODELLING DURING PERIPHERAL NERVE REGENERATION

The first descriptions of axonal regeneration were made in 1928, when Ramon y Cajal described the PNS (Fig. 15) as a supportive environment that could orient the regrowth of injured axons. While axons in the CNS were described as incapable to regrow forming swellings at the tip in the lesion site, the retraction bulbs, PNS axons are able to regenerate due to their ability to form a growth cone at their transected tip.

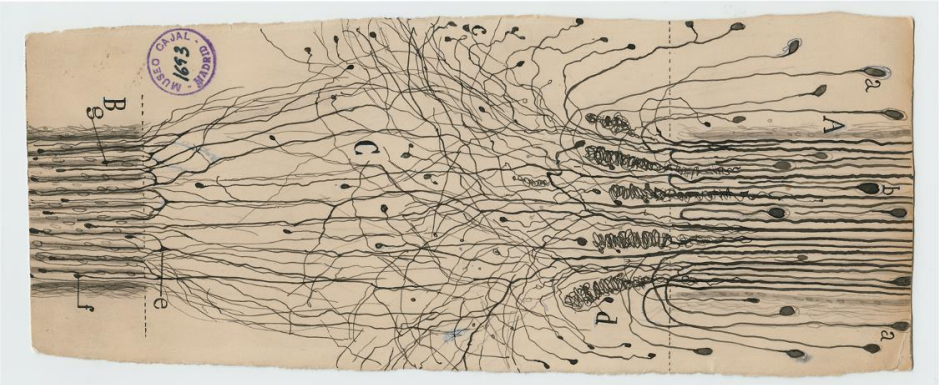

Figure 15. First schematic drawing by Ramon y Cajal showing the regeneration of a cut peripheral nerve. Adopted from Ramon y Cajal, 1905.

Following injury to the PNS, both motor and sensory axons are able to spontaneously regenerate and regrow to a significant extent. The process which allows axonal regeneration in the PNS is Wallerian Degeneration, a response from non-neuronal cells to injury. The key event in this process is the degeneration of the disconnected distal portion of the axons, which triggers a cascade of responses by Schwann cells and macrophages that eventually lead to the clearing of the inhibitory debris, as well to a production of a growth-permissive environment (Gaudet, Popovich, & Ramer, 2011). This regenerative process can be viewed as an attempt to recapitulate the developmental process. During development, neural progenitor cells divide asymmetrically, giving rise to a daughter progenitor cell and to a neuron. That unpolarized neuron starts migrating to its target and along that process becomes polarized, originating an axon and dendrites (Barnes & Polleux, 2009). Both the axon formation and its elongation are strongly dependent on cytoskeleton dynamics, since actin destabilization and MT stabilization are essential for these processes.

The other fundamental moment in axon regeneration is the formation of the growth cone. As previously mentioned, these structures are responsible for integrating extracellular signals and are able to guide growing axons to reach their correct targets. MT dynamics modulation is a key event in peripheral axon regeneration not only in

promoting the correct assembly of a growth cone but also in promoting regeneration by modifying axonal transport (Fig. 16).

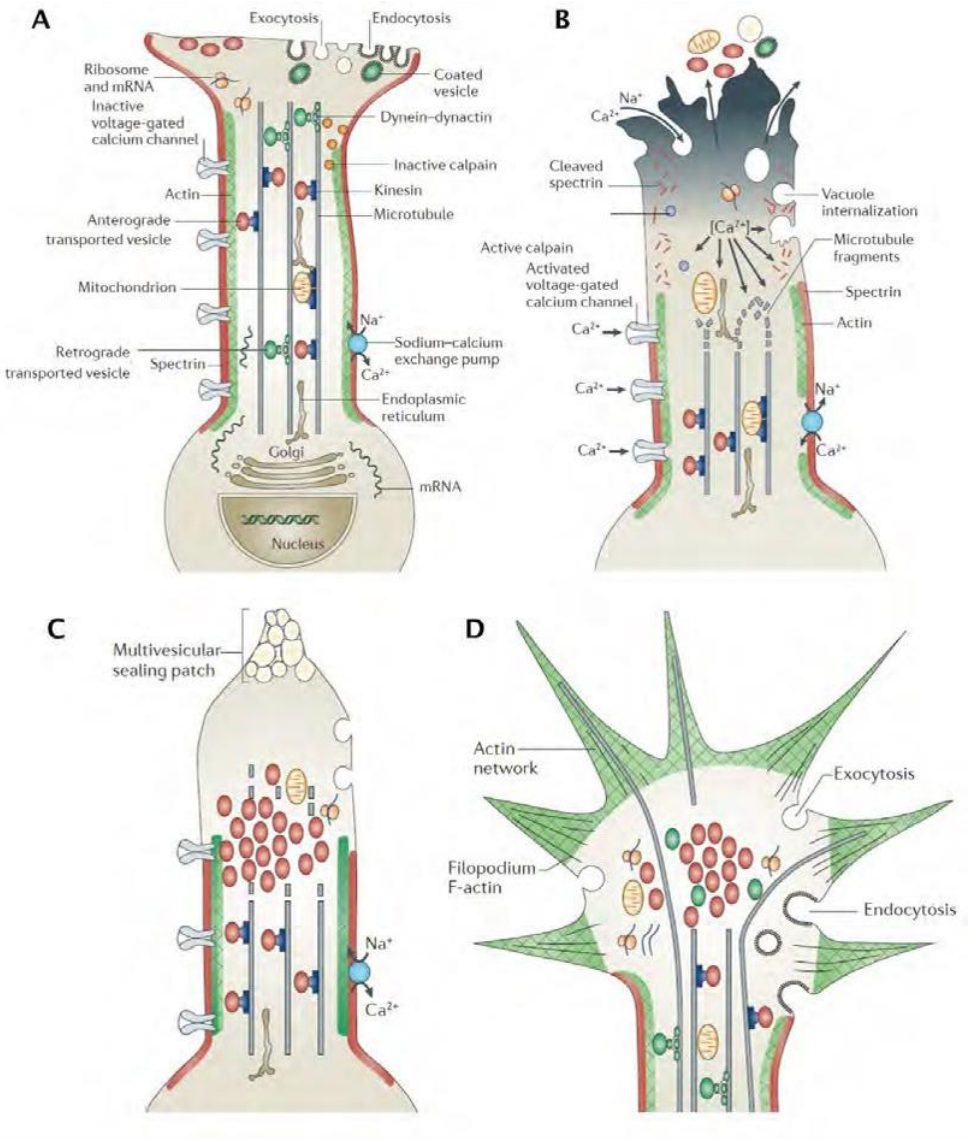

Figure 16. Formation of a new growth cone after axotomy. Upon lesion, several steps are required to mount a competent growth cone. After being cut, a mature axon (A) generates distal and proximal severed segments that are permeable to the extracellular environment (B). This will allow calcium entry and the consequent activation of axonal proteases, cytoskeleton degradation and stimulation of vacuole internalisation. (C) The transient calcium influx induces membrane retrieval by forming a sealing patch composed of vesicles. Thereafter, membrane sealing also allows the decrease of intracellular calcium levels. Calcium influx initiates several pro-regenerative processes inside severed axons but it must be transient to prevent cell death. (D) After reorganising actin and microtubule cytoskeletons, a restored growth cone requires the local expression of proteins as well as axonal transport (via stabilised microtubules) of vesicles and mitochondria to sustain the regenerative response. Once reconstructed, the competent growth cone uses lamellipodia and filopodia to generate forces to support outgrowth. Adopted from Bradke, Fawcett, & Spira, 2012.

After axotomy, local calcium influx is necessary for the assembly of a new competent growth cone and afterwards for axon regrowth (Bradke et al., 2012). Moreover, axotomy also triggers membrane depolarisation which further promotes local calcium influx by activating voltage-gated calcium channels and calcium release from internal stores. In mouse peripheral sensory neurons, calcium influx elicits a back-propagating calcium wave that reaches the cell body and causes dramatic epigenetic changes switching axons from a non-elongating to actively elongating state (Cho, Sloutsky, Naegle, & Cavalli, 2013). Importantly, epigenetic changes result in the expression of a large repertoire of regeneration-associated genes (RAGs) promoting axon regeneration (Finelli, Wong, & Zou, 2013). The elevation of free intracellular calcium triggers intracellular signalling required for specific initial events, including membrane retrieval by sealing and accumulation of Golgi-derived anterogradely transported vesicles, local cytoskeleton restructuring characterised by depolymerisation of microtubules and actin, activation of calcium-dependent calpains which proteolytically cleave the submembrane spectrin cortex, activation of local protein translation and retrograde molecular signalling (Bradke et al., 2012). Moreover, the calcium transient activates calcium-dependent adenylate cyclase leading to increased cAMP which is described to potentiate axonal regrowth, reconnection of axonal fragments and formation of branching to postsynaptic targets (Ghosh-Roy, Wu, Goncharov, Jin, & Chisholm, 2010). In addition, increased levels of cAMP signal to dual leucine zipper kinase (DLK-1), an injury- induced axonal protein that governs the balance between degeneration and regeneration. Thus, active DLK-1 ultimately promotes cytoskeleton remodelling needed for growth cone assembly.

Recently, it has also been emphasized the possibility of promoting regeneration by targeting the MT cytoskeleton. It has been shown that stabilizing the microtubule network reduces fibroblasts migration towards the lesion site (A. C. Lin & Holt, 2007) and prevents the release of inhibitory proteins by astrocytes and fibroblasts (A. C. Lin & Holt, 2007; Pollard, 2016). PTMs that occur on MTs have been increasingly recognized as crucial controllers of MT properties and functions (Hammond, Cai, & Verhey, 2008) and targeting MT dynamics is a known therapeutic approach to promote regeneration. Acetylated tubulin is enriched in older, stable microtubules along the axon shaft and, therefore, it is the PTM more frequently correlated with MT stability (Janke & Bulinski, 2011). Since moderate MT stability has already been reported as required to promote axon growth and regeneration (Erturk, Hellal, Enes, & Bradke, 2007; Hellal et al., 2011), drugs that target MT-acetylation appear as a useful strategy to enhance axon re-growth after injury. Nevertheless, the impact of modulating acetylating or deacetylating enzymes on MT stability is not fully clear, since determining the roles of the different modifications *in vivo* is

challenging because PTMs rarely occur in isolation and the level of each modification is singularly regulated by the cell (Janke & Bulinski, 2011).

In summary, modifying cytoskeleton-dynamics is a multifaceted strategy which can restore growth cones, enhance axon growth and reduce the environmental inhibition to axon regeneration. Hence, this dynamics represents an appealing target and may hold promise as a basis for the development of a regenerative therapy in peripheral injury contexts.

CYTOSKELETON DYSFUNCTION AND NEURODEGENERATION: A NEW EMERGING CONNECTION

There is a strong link, emerged mainly in the last decade, that connects neuronal cytoskeletal impairments with neurodegeneration. The vast majority of the cytoskeletal dysfunctions involved in the neurodegenerative framework include alterations in MT stability, which may have direct implications in the normal functioning of axonal transport, and also give rise to alterations in actin dynamics. Mutations in NFs have also been related to neurodegenerative disorders, as described above.

Alterations in microtubule stability as a causative effect in neurodegeneration

For a proper functioning of the nervous system, neurons need a controlled stability of the MTs and thereby impairments in this cytoskeleton component are related to several neurodegenerative disorders. In fact, MT fragmentation is the first detectable event during Wallerian degeneration (Zhai et al., 2003), and destabilization of the MT network accompanies the formation of retraction bulbs and axonal retraction (Erturk et al., 2007). Adding to the fact that alterations in MT stability might precede axonal transport impairment (Cartelli et al., 2010), these findings suggest a causative link that connects MT disturbances with the initial steps of the neurodegeneration processes.

Studies indicate that the mass and length of MTs in cells is affected by MT polyglutamylation, as the modification increases MT-severing catalysed by spastin, a MT interacting protein (Lacroix et al., 2010).

MT alterations related to variations in levels of tubulin modifications have been reported in several neurodegenerative diseases. Some examples include: i) a mouse model for AD, where decreased levels of α-acetylated tubulin were observed in the hippocampus and coincided with increased levels of HDAC6 (L. Zhang et al., 2014); ii) Aβ oligomers that *in vitro* induce MT loss via tau recruitment of TTLL6, promoting tubulin

polyglutamylation and recruitment of the MT-severing enzyme spastin (Zempel et al., 2013); iii) a model of induced Parkinson's disease (PD) using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimide (MPTP), where early alterations in α-tubulin modifications were observed in dopaminergic neurons, leading to impaired axonal transport and disease progression (Cartelli et al., 2013); iv) brains of Huntington's disease (HD) patients where a dramatic decrease in the level of α-acetylated tubulin was observed and related with a defective axonal transport (Dompierre et al., 2007); and v) a Charcot-Marie-Tooth (CMT) mouse model, induced by mutation of the small heat-shock protein 1 gene (HSPB1), where decreased levels of α-acetylated tubulin and severe axonal transport deficits were observed in peripheral nerves (d'Ydewalle et al., 2011). These reports point mainly to α-acetylation of tubulin, with a concomitant impairment in axonal transport, as a common pathological hallmark in several neurodegenerative disorders. In fact, these findings supported the use of drugs targeting MT acetylation as a useful strategy for treating neurodegenerative diseases. Nevertheless, the impact of modulating acetylating/deacetylating enzymes on MT stability is not clear. Strikingly, αTAT promotes MT destabilization and accelerates MT dynamics in a mechanism independent of its function as a α-tubulin acetylase (Kalebic et al., 2013). In line with the same observation, catalytically inactive HDAC6 mutants also modulate MT dynamics without altering acetylation (Zilberman et al., 2009).

Besides the regulation of MT functions by tubulin modifications, MAPs also play a key role in MT stabilization and in the regulation of kinesin- and dynein-mediated transport (Mandelkow & Mandelkow, 1995). Tau is the most studied MAP due to its involvement in AD. However, besides AD, tau dysfunction is at the centre of several neurodegenerative disorders (V. M. Lee, Goedert, & Trojanowski, 2001; Poorkaj et al., 1998; Spillantini et al., 1998). Hyperphosphorylation of tau leads to detachment of MAP from MTs, and thereby to MT disassembly, loss of neural shape and impairment of axonal transport (A. C. Alonso, Grundke-Iqbal, & Iqbal, 1996; A. D. Alonso, Grundke-Iqbal, Barra, & Iqbal, 1997). Moreover, dissociation of tau from MTs provides access for MT-severing proteins such as katanin, which break down MTs and lead to axon degeneration (Qiang, Yu, Andreadis, Luo, & Baas, 2006). Although tau is traditionally perceived as an axonal protein, it is also localized to the dendritic compartment including spines, albeit at lower levels than those found in the axon (Papasozomenos & Binder, 1987). In fact, tau has been implicated in synaptic function as part of the molecular pathway leading to Aβ-driven synaptotoxicity (Frandemiche et al., 2014).

Another MAP which is emerging as a crucial target in neuronal dysfunction is CRMP-2 which is regulated by phosphorylation, which on one hand disrupts its

association with tubulin heterodimers blocking MT polymerization (Fukata et al., 2002) and on the other hand, blocks CRMP-2 association with kinesin, blocking the anterograde vesicular transport of molecules important for synaptic integrity at the distal end of axons (Kawano et al., 2005). Regarding neurodegeneration, CRMP-2 has been implicated in AD (Williamson et al., 2011), in Multiple Sclerosis and PD (Fang et al., 2015; Petratos et al., 2012). In fact, upstream regulators of MT dynamics include signalling kinases that modulate the functions of MAPs, such as GSK3β which is intimately related with AD which is mainly derived from its role on tau phosphorylation (Flaherty, Soria, Tomasiewicz, & Wood, 2000), and as such affects MT dynamics and axonal transport in this disorder.

Numerous studies link MT alterations with a wide range of events that occur in neurodegenerative disorders. This relation of dysregulation of the MT cytoskeleton with disease onset received a major attention in the design of novel therapeutic approaches aimed at preventing/reverting neurodegeneration through cytoskeleton modulation.

Axonal transport defects as a direct cause of neurodegeneration or a downstream effect of altered MT stability

Most proteins essential for the axon and the synaptic terminals are synthesized in the cell body and need then to be transported down the axon. Therefore, intracellular transport is crucial for neuronal morphogenesis, function and survival. Transport through the axon is divided in the slow axonal transport of cytosolic and cytoskeletal proteins and the fast axonal transport of membranous organelles and vesicles. Over the years, many studies have been directed to a common premise linking axonal transport impairment with the first steps of neurodegeneration. However, in some neurodegenerative disorders it is still unknown whether one is a cause or a consequence of the other. One should though consider that, given the thin calibre of axons and the complex cytoskeletal and proteinaceous milieu inside them, the accumulation of motor proteins associated with their cargos that might occur as a consequence of impaired axonal transport, can create a toxic environment to neurons thereby leading to neurodegeneration.

Besides alterations in MT-stability, already referred to above, alterations in axonal transport are caused by mutations in motors as well as in non-motor proteins involved in neurodegenerative diseases. In the sporadic forms of amyotrophic lateral sclerosis (ALS), KIF1Bβ and KIF3Aβ the two major KIFs isoforms enriched in the motor cortex, present decreased gene expression in the brain (Pantelidou et al., 2007). Moreover, mutations in dynactin (DCTN1) and in the cytoplasmic dynein heavy chain (DNCHC1) genes were found in ALS patients causing a defective retrograde transport (Munch et al., 2004).

Additionally, mutations in superoxide dismutase 1 (SOD1), implicated in ALS, could directly or indirectly affect axonal transport by: i) interfering with cargo binding; ii) generating a physical barrier composed by the SOD1 aggregates; iii) disrupting MT stability; and iv) disrupting motor activity (Strom et al., 2008).

In fact, transport defects might also be caused by protein aggregates, the pathological hallmark of several neurodegenerative disorders (Goldstein, 2012). Two putative mechanisms for axonal transport poisoning by protein aggregates have been proposed although not fully analysed yet: i) nonspecific physical blockage of the transport along axons and, ii) selective blockage of certain cargos by aggregate-dependent sequestration of motors away from cargoes (Coleman, 2011). These mechanisms could be the cause of axonal transport defects in diseases such as AD, PD and HD.

As suggested in this section, several causes can lead to disruption of axonal transport in neurodegenerative disorders. Although further disclosure of the mechanisms leading to such defects in specific neurodegenerative disorders should be performed, the similar features of the reported axonal transport alterations in several distinct disorders support common therapeutic interventions.

Disclosing key actin cytoskeleton components involved in neurologic disorders

In mature neurons, actin is enriched in dendritic spines and disruption of the actin cytoskeleton leads to altered dendritic spines, decreased dendritic spine density and ultimately to synaptic loss (W. Zhang & Benson, 2001). As such, dysregulation of actin dynamics in addition to impaired MT stability also plays a key role in neurodegenerative disorders. Actin cytoskeleton dynamics is regulated by ABPs. The impaired function of several ABPs has been related to the development of neurodegeneration as is discussed below.

The ABP cofilin1 has a concentration dependent function, acting as an actin stabilizer at very low concentrations, as an actin severing protein at medium concentrations and as an actin nucleation protein at high concentrations (Andrianantoandro & Pollard, 2006). Cofilin1 is associated to a neurodegeneration-related structure present in stressed and degenerating neurons: the cofilin-actin rods, which are emerging key players in neurodegeneration mainly in the context of AD. Cofilin1 hyperactivation by dephosphorylation leads to its saturated binding to actin filaments causing them to form rod structures - the cofilin-actin rods that were identified in neurites of cultured neurons treated with Aβ oligomers (Maloney, Minamide, Kinley, Boyle, & Bamburg, 2005) and in human AD brains mainly in the hippocampus and cerebral cortex

(Minamide, Striegl, Boyle, Meberg, & Bamburg, 2000). Cofilin-actin rods act by promoting axonal transport alterations and leading to synapse disruption (Cichon et al., 2012), indicating their possible involvement in other neurodegenerative diseases unrelated to AD such as HD (Munsie & Truant, 2012) and PD (Lim et al., 2007). In fact, it was shown that α-synuclein induces cofilin1 inactivation and actin stabilization in primary neurons and in fibroblasts derived from PD patients (Bellani et al., 2014). These studies pinpoint the actin cytoskeleton as a putative therapeutic avenue since cofilin-actin rod formation can be reverted by modulating the activity of LIMK, which phosphorylates and deactivates cofilin 1, leading to cofilin-actin rod clearance (Davis et al., 2011).

Hirano bodies are highly ordered rod- or disk-shaped sheets (Izumiyama, Ohtsubo, Tachikawa, & Nakamura, 1991) deposited in the hippocampus of AD patients (Schmidt, Lee, & Trojanowski, 1989), enriched in actin and actin binding proteins, with cofilin1 as one of the major constituents of the inclusions (Galloway, Perry, & Gambetti, 1987; Maciver & Harrington, 1995). Different studies suggested that Hirano bodies are precursors of cofilin-actin rods (Maselli, Furukawa, Thomson, Davis, & Fechheimer, 2003) or vice-versa (Minamide et al., 2000). Hirano bodies were also observed in models of tauopathy, implicating interactions between tau and the actin cytoskeleton (Fulga et al., 2007). In this respect, tau was shown to promote bundling of actin filaments leading to their stabilization (Fulga et al., 2007). Profilin is another ABP with an important role in actin polymerization where it enhances the rate of polymerization by delivering ATP-G-actin to the barbed end of the filament (Selden, Kinosian, Estes, & Gershman, 1999). Profilin has been recently associated with neurodegeneration in different ALS cases where four mutations were identified in the profilin 1 gene (PFN1) localizing these mutations close to actin binding sites (Wu et al., 2012). Primary motor neurons expressing mutant PFN1 form insoluble PFN1 aggregates *in vitro*, which inhibit axonal outgrowth and growth cone size, and test positive for ALS markers, supporting the role of profilin in familial ALS pathology (Wu et al., 2012). Although the mechanism underlying ALS pathology caused by PFN1 mutations is still not clear, recent studies suggest that there is a mutation-derived destabilization of the native PFN1 structure that leads to its aggregation and, either due to a loss of function or gain of toxic function, will cause ALS (Boopathy et al., 2015).

The actin cytoskeleton is strongly regulated by signalling pathways, namely by the Rho GTPase family (Nobes & Hall, 1995; Ridley & Hall, 1992). In fact, dysregulation of Rho GTPase signalling occurs in several neurodegenerative diseases: in PD, its inhibition leads to increased neurite extension in a mouse model of the disease (Zhou et al., 2011); in CMT mutations in frabin/FDG4, a GEF of the Rho GTPase Cdc42 (Delague et al., 2007; Stendel et al., 2007), lead to peripheral nerve demyelination; in HD, huntingtin interacts

with several players of the Rho GTPase signalling pathways (Tourette et al., 2014); in AD several independent evidence show the involvement of Rho GTPases in disease including increased levels of RhoA in neurons close to amyloid plaques in the cerebral cortex of APP(Swe) Tg2576 mice and; increased activity of RhoA accompanied by decreased neurite length induced *in vitro* by Aβ (Petratos et al., 2008) and, alterations in the levels of Cdc42/Rac with concomitant actin cytoskeletal alterations in hippocampal neurons treated with Aβ (Mendoza-Naranjo, Gonzalez-Billault, & Maccioni, 2007).

As illustrated above, the actin cytoskeleton, although less explored than the MT component, is being implicated in several neurodegenerative disorders. On one hand, dysregulation of ABPs induces the formation of structures enriched in actin that perturb neuronal integrity, and on the other hand, regulatory proteins of actin dynamics are altered in neurodegenerative disorders.

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REVIEW ARTICLES

In this thesis I was the first author of a review article which contributed to disseminate knowledge on the importance of neuronal cytoskeleton organization on neurodegenerative disorders:

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Review article

The cytoskeleton as a novel therapeutic target for old neurodegenerative disorders

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ABSTRACT

Cytoskeleton defects, including alterations in microtubule stability, in axonal transport as well as in actin dynamics, have been characterized in several unrelated neurodegenerative conditions. These observations suggest that defects of cytoskeleton organization may be a common feature contributing to neurodegeneration. In line with this hypothesis, drugs targeting the cytoskeleton are currently being tested in animal models and in human clinical trials, showing promising effects. Drugs that modulate microtubule stability, inhibitors of posttranslational modifications of cytoskeletal components, specifically compounds affecting the levels of tubulin acetylation, and compounds targeting signaling molecules which regulate cytoskeleton dynamics, constitute the mostly addressed therapeutic interventions aiming at preventing cytoskeleton damage in neurodegenerative disorders. In this review, we will discuss in a critical perspective the current knowledge on cytoskeleton damage pathways as well as therapeutic strategies designed to revert cytoskeleton-related defects mainly focusing on the following neurodegenerative disorders: Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, Amyotrophic Lateral Sclerosis and Charcot-Marie-Tooth Disease.

Contents

Abbreviations: 6-OHDA, 6-hydroxydopamine; ABP, actin binding protein; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; Aβ, amyloid-β; Cdk5, cyclin-dependent kinase 5; CMT, Charcot-Marie-Tooth disease; CMT2, Charcot-Marie-Tooth disease type 2; CRMP-2, collapsin response mediator protein 2; Epo, Depothilone D; GSK3β, glycogen synthase kinase 3β; HD, Huntington disease; HDAC6, histone deacetylase 6; HSP, hereditary spastic paraplegia; KIF, kinesin superfamily; LKE, lanthionine ketamine ester; MAP, microtubule associated protein; MAP1B, microtubule associated protein 1B; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimide; MT, microtubule; NFT, sneurofibrillary tangles; PD, Parkinson's disease; SIRT-2, sirtuin-2; SOD1, superoxide dismutase 1.

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1. The neuronal cytoskeleton

Neurons are amongst the most highly polarized cells containing two distinct functional compartments, the axonal and the somatodendritic compartment (Craig and Banker, 1994). The axon is a unique projection that functions as a signal transmitter, which contacts with neighboring cells conveying the electric impulse and neuronal information. The somatodendritic compartment is composed by the neuronal cell body and multiple branched dendrites which receive signals. The neuronal cytoskeleton is crucial in maintaining this asymmetric shape and polarity, since it provides not only the neuronal structural backbone, but also because it separates functionally the two different compartments (Witte and Bradke, 2008). Axons can extend to remarkable distances (up to 1 m in humans), which raises the need of a well-constructed machinery that shuttles vital cellular components from the cell body along the axonal projection (Kapitein and Hoogenraad, 2011). As such, an appropriately organized neuronal cytoskeleton is needed during nervous system development, maintenance and also during regenerative processes that follow injury. The maintenance of the neuronal structural integrity requires controlled internal organization of its three cytoskeletal components: the intermediate filaments, actin microfilaments and microtubules (Kapitein and Hoogenraad, 2015; Luo, 2002; Yuan et al., 2012). Of note, these cytoskeleton components are shaped by proteins that can be assembled in a wide combination of ways, giving rise to different structures with different properties, and that can also be disassembled and reassembled into different shapes according to the specific spatio temporal needs (Fletcher and Mullins, 2010).

1.1. Neuronal cytoskeleton components: the building blocks of polarity

The neuronal cytoskeleton is composed by intermediate filaments (IFs), actin filaments, and microtubules (MTs). IFs constitute the largest family of mammalian cytoskeleton proteins and they provide mechanical strength and stabilize the cytoskeleton framework. In adult neurons, 5 types of IFs are expressed: the type IV neurofilament (NF) triplet proteins (NF-light (NF-L), NF-medium (NF-M) and NF-heavy (NF-H)) (Shaw and Weber, 1982), α-internexin (Fliegner et al., 1994) and the type III peripherin (Troy et al., 1990). Each IF subunit is composed by a globular N-terminal head, a α-helical rod domain, and variable C-terminal tail domains that differ in length and amino acid composition. Neuronal IF assembly starts with the formation of parallel, side-to-side, coiled-coil dimers of IF subunits, mediated by the association of their rod domains. Subsequently, two dimers line up in an anti-parallel manner, forming a tetramer. Aggregates formed by approximately eight tetramers constitute a unit-length-filament (ULF) of approximately 55 nm in length (Herrmann et al., 1999; Hisanaga and Hirokawa, 1988). Elongation occurs through the axial aggregation of ULFs forming immature filaments of about 16 nm in diameter and many microns in length. Finally, radial compaction takes place resulting in packing of the filaments to constitute the final 10 nm filament. Filaments are lengthened by dynamically joining ends of shorter filaments. The core of the filaments is composed by the rod domains while the C-terminal tails form flexible extensions that link the filaments to each other and to other elements in the cytoplasm (Uchida et al., 2013). NF assembly is regulated by post-translational modifications namely phosphorylation of the N-terminal head domain (Sihag and Nixon, 1989, 1990) which in the case of NF-L inhibits its assembly suggesting that this modification has a role in maintaining NFs in a disassembled state (Hisanaga et al., 1990). The NF head phosphorylation occurs in the cell body and is reverted for its assembly and transport along the axon where NFs exert their function (Nixon and Lewis, 1986 Sihag and Nixon, 1991). The C-terminal tail of NFs, namely of NF-M and NF-H, is also phosphorylated in a spatially regulated manner. In the cell body NFs have predominantly non-phosphorylated tails which become extensively phosphorylated in mature axons (Nixon and Logvinenko, 1986; Sihag and Nixon, 1990). Phosphorylation of the tail of NFs is important for the interaction between NF domains themselves and also for their interaction with MTs (Hisanaga and Hirokawa, 1989; Hisanaga et al., 1991). Moreover, tail phosphorylation confers NFs resistance to proteolysis (Pant, 1988).

The actin cytoskeleton is composed of actin monomers (G-actin) that have tight binding sites that enable head-to-tail interactions with two other actin monomers, such that they polymerize to form thin, flexible actin filaments (F-actin) of approximately 7 nm in diameter (Spudich et al., 1972). These filaments are organized into higher-order structures, forming bundles or three-dimensional networks. Since the actin monomers are oriented in the same direction, actin filaments display polarity with distinguishable plus and minus ends. The reversible addition of monomers happens in both ends, but the plus end elongates five to ten times faster than the minus end (Pollard and Mooseker, 1981). Actin monomers bind ATP, which leads to a faster polymerization and ATP is then hydrolyzed to ADP after assembly (Hayashi and Rosenbluth, 1962; Offer et al., 1972). In neurons, actin is seen as the engine behind the generation of the force necessary to regulate the neuronal shape and cellular internal and external movements (Luo, 2002).

MTs are parallel bundles of protofilaments of α- and β-tubulin which play a significant role in cellular structure since they function as architectural backbones for neurons being crucial to maintain axonal integrity and form the tracks for axonal transport (Prokop, 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 depolymerization and resulting in the dynamic state characteristic of MTs (Mitchison and Kirschner, 1984). MTs also undergo treadmilling, a dynamic process in which the plus end of the filament grows in length while the other one shrinks, due to the removal of tubulin molecules bound to GTP from the minus end that travel to the plus end of the same MT (Walker et al., 1988). In many cell types, MTs are nucleated at the centrosomal region in the microtubule-organizing center (MTOC) which is the starting point of MT polymerization where α- and β-tubulin dimers associate with a previously formed γ-tubulin ring and start elongating from there (Joshi et al., 1992; Sunkel et al., 1995). Yet, neurons have the ability to be centrosomal independent regarding MT nucleation. Studies using *Drosophila* neurons demonstrated that in the absence of functional centrosomes, neurons developed a normal MT network and a healthy axonal outgrowth (Basto et al., 2006; Nguyen et al., 2011). Moreover, another recent study using rat hippocampal neurons evidenced that centrosomes lose their MTOC function during neuronal development and that axon

extension and regeneration is MTOC independent (Stiess et al., 2010). Nevertheless, in neurons, MTs are still polarized filaments with the minus ends directed towards the cell body, that must be capped, and the plus ends directed outwards along the axon. The capping of the minus ends of MTs, indispensable for preventing depolymerization, is assured by 3 homologous proteins, the calmodulin-regulated spectrin-associated proteins 1–3 (CAMSAP1-3) (Baines et al., 2009). MTs operate together with a large number of proteins, the MT associated proteins (MAPs) that either influence MTs themselves or transmit signals from the MT cytoskeleton to other parts of the cell. Classical MAPs, which bind to the MT lattice are involved in MT stabilization and include tau, MAP1a, MAP1b, and MAP2 (Takemura et al., 1992). A particular group of MAPs include the MT plus-end-tracking proteins which are distinguished by their ability to concentrate at growing microtubule ends to regulate MT dynamic instability, being some examples the end-binding proteins 1, 2 and 3 (EB-1/2/3), the cytoplasmic linker protein of 170 kDa (CLIP170), the cytoplasmic linker associated protein 2 (CLASP2) and the adenomatous polyposis coli (APC) (Akhmanova and Steinmetz, 2008). The MT cytoskeleton is, thus, a tightly organized structure with an effective crosstalk among the players that regulate its polarity and structure, which are crucial to determine the movement along MTs, as is discussed below.

1.2. Organization of the cytoskeleton in different neuronal compartments

During neuronal development and regeneration, a growth cone is formed at the distal tip of the growing axon. The growth cone has received significant attention, and much has been learnt about cytoskeleton organization and dynamics in this specialized cellular domain. The growth cone can be separated into three domains based on the distribution of cytoskeletal components. The central domain (C-domain) is composed by bundles of MTs that enter from the axon shaft into the growth cone, by central actin bundles, and by vesicles, organelles and proteins that are being transported into this domain (Letourneau, 1983; Schaefer et al., 2002). The actin filaments, existing as both filopodia (long and packed actin bundles) and lamellipodia (sheet-like F-actin

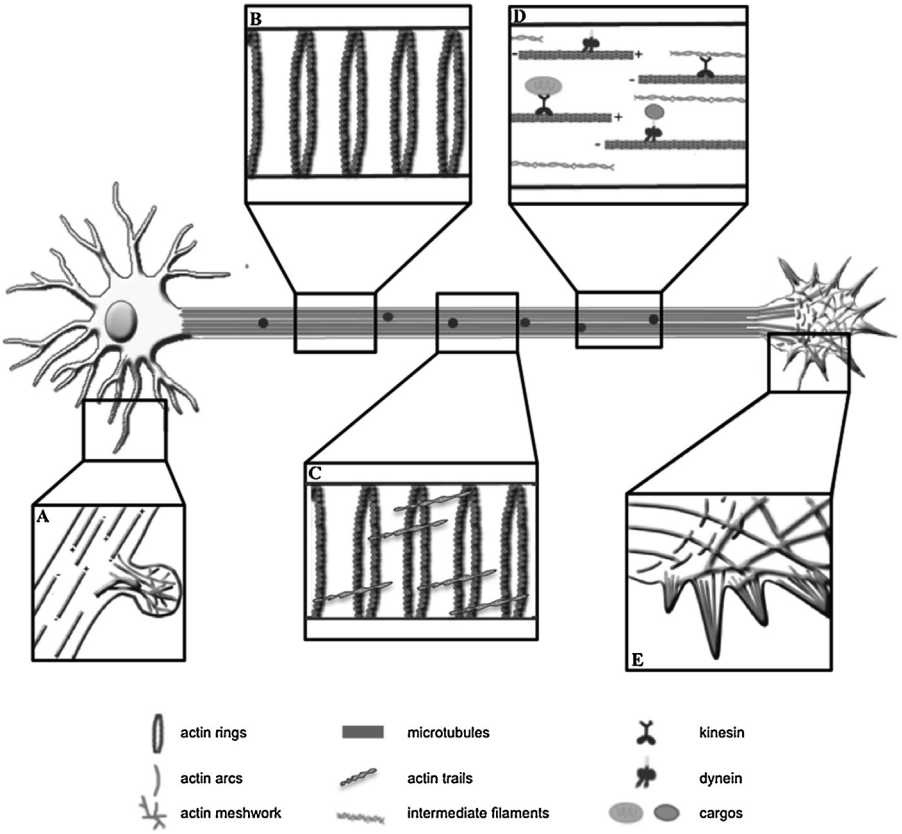

Fig. 1. Representation of the neuronal structure and key components of the neuronal cytoskeleton. Neurons have a tightly regulated cytoskeleton, which encloses three types of filaments (IFs, actin microfilaments and MTs) that organize themselves in varying ways, depending on the neuronal region. (A)—At the somatodendritic compartment, the dendrites are composed by MTs, which are mixed oriented and mostly restricted to the dendritic shaft, with some of the MTs protruding into de dendritic spine. Actin filaments form the so-called actin patches and are localized inside the dendritic spine. (B)—Actin rings are disposed as isolated rings associated with adducin and spectrin and localize in the axon and dendrites. (C)—Actin trails are dynamic short actin filaments along the axon that undergo constant assembly/disassembly cycles and believed to be involved in the delivery of actin to the pre-synaptic terminals. (D)—Throughout the axon, MTs are displayed in a polarized fashion with the minus end directed towards the cell body and the plus end towards the tip of the axon. MTs are the highways in which the molecular motors move in different directions: kinesin towards the plus end of MTs and dynein towards the minus end. Here, IFs form a structural meshwork that encloses MTs. (E) – At the growing tip of the axon, the growth cone, the cytoskeleton intermediates organize themselves in order to favor growing conditions. Actin forms sheet-like structures (lamellipodia) and rod-like structures (filopodia) and at the distal part of the axon shaft, MTs protrude into the growth cone running in parallel to F-actin in filopodia.

meshwork), constitute the peripheral domain (P-domain) and shape the growth cone and direct its propagation. Axonal MTs present different dynamic properties according to their localization. While in the axon shaft acetylated and detyrosinated forms of α- tubulin are present, which are post-translational modifications occurring mainly in stable MTs, in the growth cone dynamic MTs are located, composed by tyrosinated α- tubulin, running along filopodia in the P-domain (Robson and Burgoyne, 1989). These distal dynamic MTs play a role in growth cone guidance being involved in turning in the presence of an inhibitory cue (Challacombe et al., 1997). Moreover, neurons presenting decreased dynamic MTs in the distal part of the axon present aberrant growth cones and decreased axon outgrowth (Gonzalez-Billault et al., 2001). Between the P- and C-domains is formed a transition zone (T-zone), where actomyosin contractile structures termed actin arcs lie perpendicular to F-actin bundles, forming a hemi-circumferential ring (Schaefer et al., 2002). In Fig. 1 the cytoskeleton architecture of a developing neuron is depicted.

In mature neurons, actin is highly enriched in dendritic spines, contributing to their formation and organization (Cohen et al., 1985). Dendrites present a branched actin network in which the barbed ends are pointed towards the cellular membrane (Matus et al., 1982). Both in axons and dendrites, patches of actin filaments organized in the form of meshwork are present, similar to those observed in the lamellipodia of growth cones (Korobova and Svitkina, 2010; Spillane et al., 2011; Watanabe et al., 2012). Actin patches have been reported in the axon initial segment, suggested to mediate the capture of axonal transport cargoes and limit their advance into the axon (Watanabe et al., 2012), and along the more distal axon shaft, where they serve as precursors to the formation of axonal filopodia (Spillane et al., 2011). More recent studies which contributed to a significant breakthrough in our understanding of the neuronal cytoskeleton described, using super resolution microscopy, a new actin structural organization present both in axons and dendrites, the actin rings, where actin is disposed in isolated rings associated with adducin spaced approximately 200 nm apart, and separated by spectrin tetramers (D'Este et al., 2015; Xu et al., 2013; Zhong et al., 2014). Moreover, another new actin structure was disclosed recently using fluorescent probes for F-actin and 3D high resolution microscopy that revealed deep axonal actin filaments in the axon shaft, the actin trails (Ganguly et al., 2015). These actin trails are dynamic F-actin "hotspots" that undergo constant assembly/disassembly processes and are related functionally to actin enrichment in synapses. These recently described structures are believed to confer to the axon structural support provided by actin rings, and a dynamic cytoskeletal meshwork provided by the actin trails.

In the case of MTs, these aligned parallel bundles support axon elongation and shape. Besides cellular support, MTs are highly important in axonal transport being the rails along which vesicles, organelles, proteins and mRNA complexes are transported. Axonal transport along MTs is carried out by two essential molecular motor super-families: kinesin and dynein. Kinesin superfamily proteins (KIFs) are heterotetramers with two heavy chains and two light chains. The kinesin heavy chains can be divided into three domains: the head, which contains MT and ATP binding sites and works as a motor domain, and stalk and tail domains which recognize and bind to the cargo(s) (Hirokawa et al., 2010). Dynein consists of a huge protein complex containing multiple polypeptide subunits: two heavy chains with ATPase activity, two intermediate chains, four intermediate light chains and several light chains. Both kinesin and dynein use ATP to move along MTs in different directions: kinesins move towards the plus end (anterograde transport) and dyneins move towards the minus end (retrograde transport) (Hirokawa et al., 2010; Karki and Holzbaur, 1999). Besides the MT-based molecular motors, there are also molecular motors that drive movement along actin filaments: myosins. These motors form a dimer and consist of a motor domain, a neck region and a tail region. They have a prominent function in neuronal elongation at the leading edge of axons and in synapses where they interact with actin filaments in the synaptic regions and use the energy of ATP hydrolysis to generate force and movement (Lin et al., 1996).

In growth cones MTs and actin filaments are known to cooperate (Dent and Kalil, 2001; Schaefer et al., 2002). In dendritic spines, initially described as enriched in actin filaments, the presence of MTs was more recently confirmed and this cytoskeleton component was suggested to impact on spine development, structure maintenance and function (Gu et al., 2008; Hu et al., 2008; Jaworski et al., 2009). In hippocampal neurons, dynamic MTs bound to the plus tip protein EB3 invade dendritic spines in response to neuronal activity (Hu et al., 2008). PAR1B, a cell polarity-regulating serine/threonine protein kinase, is responsible for the regulation of microtubule dynamics in dendritic spines mediating the maintenance of dendritic spine morphology (Hayashi et al., 2011). Similarly to what happens in growth cones, a bidirectional interplay between MTs and actin was reported in dendritic spines. EB3 was shown to interact with p140Cap regulating actin dynamics in dendritic spines through association of p140Cap with the F-actin binding protein cortactin (Jaworski et al., 2009). Besides the impact of invading MTs on actin dynamics, an increase in actin polymerization in the spine neck (the basal region of the dendritic spine in close contact with the dendritic shaft) in response to calcium influx induced by synaptic activity promotes the recruitment of drebrin, an actin binding protein that binds EB3 in MTs. The increase in drebrin leads to the recruitment of EB3 present on shaft microtubules in the spine neck region which are then guided into the spine head by F-actin (Merriam et al., 2013).

Finally, NFs are mainly present in axons regulating axonal diameter, neuronal differentiation, axon outgrowth and regeneration (Zhu et al., 1997). Mutations in NFs have been reported in numerous neurodegenerative disorders (Perrot et al., 2008), including: i) amyotrophic lateral sclerosis (ALS), where overexpression of NF forms occurs, namely NF-heavy, which leads to NF aggregation leading to motor neuron disease, that can be successfully rescued by NF-light overexpression in suitable stoichiometric proportions (Meier et al., 1999); ii) Alzheimer's Disease (AD), with NFs suggested to be present in the neurofibrillary tangles (NFTs), although this was a subject of controversy (Ishii et al., 1979; Nukina et al., 1987); iii) Parkinson's Disease (PD), where a mutation in NF-medium was found in a PD patient (Lavedan et al., 2002), although a consistent link between the NF-medium mutation and PD pathology was never established; and iv) Charcot-Marie-Tooth Disease (CMT), where mutations in all the functional domains of NF-light have been reported. Furthermore, NF accumulation was shown in CMT type II (CMT2) (Vogel et al., 1985) and, *in vitro*, the expression of mutated forms of NF-light lead to axonal transport disruption, mitochondria accumulation and defective NF assembly (Brownlees et al., 2002; Perez-Olle et al., 2005). Additionally, dysregulation of NF phosphorylation, namely aberrant phosphorylation, leads to an accumulation of NFs in the cell body and has been observed in several neurodegenerative disorders, namely AD, ALS, PD and CMT (Rudrabhatla, 2014).

In order to support their physiological functions, neurons need their cytoskeleton to maintain the appropriate balance between dynamics/stability and interaction amongst its different components. Disturbances to this complex interplay and to axonal transport are emerging as cellular damage pathways in several

unrelated neurodegenerative disorders. In the following sections of this review cytoskeleton alterations in the context of neurodegenerative diseases and therapeutic strategies aimed at reverting them, will be covered and discussed in a critical perspective.

2. Cytoskeleton damage and neurodegeneration: a new emerging connection

There is a strong link, emerged mainly in the last decade, that connects neuronal cytoskeletal impairments with neurodegeneration. The vast majority of the cytoskeletal dysfunctions involved in the neurodegenerative framework include alterations in MT stability, which may have direct implications in the normal functioning of axonal transport, and also alterations in actin dynamics. Mutations in NFs have also been related to neurodegenerative disorders, as described above. In this section we will review these alterations mainly in the context of the neurodegenerative disorders for which a larger body of data has been accumulated: i) AD, that is associated with extracellular deposits of amyloid-β peptides (Aβ) in senile plaques and accumulation of hyperphosphorylated tau protein in NFTs leading to progressive cognitive decline (Blennow et al., 2006), and represents the leading disease where cytoskeleton damage has been addressed; ii) PD, 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 (Dauer and Przedborski, 2003); iii) Huntington's Disease (HD), characterized by the formation of neuronal intranuclear inclusions constituted by mutated huntingtin, that affect muscle coordination and lead to mental decline and behavioral symptoms (Walker, 2007); iv) ALS, typified by progressive degeneration of upper and lower motor neurons (Rowland and Shneider, 2001); and v) CMT, the most common inherited disorder of the peripheral nervous system (Pareyson and Marchesi, 2009). In Table 1 are summarized the major cytoskeleton dysfunctions observed in the above-mentioned disorders.

2.1. Alterations in microtubule stability as a causative effect in neurodegeneration

For a proper functioning of the nervous system, neurons need a controlled stability of the MTs and thereby impairments in this cytoskeleton component are related to several neurodegenerative disorders. In fact, MT fragmentation is the first detectable event during Wallerian degeneration (Zhai et al., 2003), and destabilization of the MT network accompanies the formation of retraction bulbs and axonal retraction (Erturk et al., 2007). Adding to the fact that alterations in MT stability might precede axonal transport impairment (Cartelli et al., 2010), these findings suggest a causative link that connects MT disturbances with the initial steps of the neurodegeneration processes. Besides, alterations on MT organization in spines might impact on their function and consequently on neurodegeneration. In fact, a decrease or shortening of dendritic spines has been observed in neurodegenerative conditions, namely in AD. In this respect, treatment of primary neurons with Aβ oligomers was shown to promote the misslocalization of tau to the somatodendritic compartment which correlated with the loss of MTs and consequently loss of mature spines and decreased synaptic activity (Zempel and Mandelkow, 2015).

Table 1
Main cytoskeletal defects observed in neurodegenerative disorders.

Cytoskeleton dysfunction	Molecular dysregulation	Neurodegenerative disorder
Altered microtubule dynamics and axonal transport defects	Decreased α-tubulin acetylation	AD (Zhang et al., 2014) HD (Dompierre et al., 2007) CMT (d'Ydewalle et al., 2011)
	Tau hyperphosphorylation	AD (Alonso et al., 1996; Alonso et al., 1997)
	Increased βIII tubulin levels	PD (Cartelli et al., 2013)
	GSK3β hyperactivation	AD (Flaherty et al., 2000)
	Mutations in SOD1	ALS (Strom et al., 2008)
MT severing	Increased tubulin polyglutamylation/recruitment of MT severing enzymes	AD (Zempel et al., 2013)
Axonal transport defects (microtubule independent)	Mutations in molecular motors	ALS (Munch et al., 2004) CMT (Zhao et al., 2001)
	Increased kinesin phosphorylation by GSK3β	AD (Morfini et al., 2002; Pigino et al., 2003)
	Mutated Huntingtin	HD (Trushina et al., 2004)
	Protein aggregation	AD (Coleman, 2011; Goldstein, 2012) PD (Coleman, 2011; Goldstein, 2012) HD (Coleman, 2011; Goldstein, 2012)
Actin cytoskeleton dysregulation	Cofilin-actin rods (cofilin hyperdephosphorylation)	AD (Minamide et al., 2000) HD (Munsie and Truant, 2012)
	Hirano bodies	AD (Schmidt et al., 1989)
	Cofilin inactivation	PD (Bellani et al., 2014)
	Profilin1 mutations	ALS (Wu et al., 2012)
	Dysregulation of Rho GTPases	PD (Zhou et al., 2011) CMT (Delague et al., 2007; Stendel et al., 2007) HD (Tourette et al., 2014) AD (Mendoza-Naranjo et al., 2007; Petratos et al., 2008)

MT stability is regulated by several tubulin post-translational modifications. The α-tubulin isoform is expressed in a tyrosinated state (with a tyrosine aminoacid in the C-terminus), which is associated with a highly dynamic state of MTs (Ponstingl et al., 1981). Tyrosinated tubulin is modulated by detyrosination, a process catalyzed by a yet undescribed carboxypeptidase (Kalinina et al., 2007), but reverted by tubulin tyrosine ligase (Raybin and Flavin, 1977). Tyrosination of tubulin has a major impact on neuronal function; tubulin tyrosine ligase knockout mice die at the perinatal stage due to neuronal abnormalities (Erck et al., 2005). Detyrosinated tubulin has been associated with long-lived MTs, although a direct causal relationship between MT stability and tubulin detyrosination has not been established. Another relevant modification conceptually associated with stable MTs is tubulin acetylation. This modification is performed by a specific acetylase (α-tubulin N-acetyltransferase (αTAT)) and the most-studied acetylation residue is Lysine 40 of α-tubulin which in contrast to the other tubulin post-translational modifications occurs in the lumen of the microtubule (L'Hernault and Rosenbaum, 1985; Soppina et al., 2012). Tubulin acetylation is a reversible modification, being deacetylation catalyzed by histone deacetylase 6 (HDAC6) (Hubbert et al., 2002) and Sirtuin-2 (SIRT2) (North et al., 2003). Instead of a direct effect on MT stability, both detyrosination (Konishi and Setou, 2009; Kreitzer et al., 1999) and acetylation (Reed et al., 2006) were suggested to impact on axonal transport by regulation of kinesin motors. The impact of tubulin acetylation on kinesin regulation is questionable as this modification occurs within a region away from the MT interaction sites with molecular motors. In fact, in *in vitro* studies using purified tubulin, acetylation was shown not to affect kinesin binding to the MT surface (Soppina et al., 2012) nor to impact on MT structure (Howes et al., 2014). These results suggest that the influence of tubulin acetylation on motor-dependent transport is not a direct effect and might occur due to additional events taking place after tubulin acetylation, which are not recapitulated in *in vitro* studies.

Another tubulin modification with a high impact on MT integrity is polyglutamylation. Polyglutamylation consists in the reversible addition of side chains of glutamates in gene-encoded glutamate residues of both α- and β-tubulins, which is catalyzed by members of the tubulin tyrosine ligase-like family (Janke et al., 2005) and reverted by enzymes of the cytosolic carboxypeptidase family (Rogowski et al., 2010). Studies indicate that the mass and length of MTs in cells is affected by MT polyglutamylation, as the modification increases MT-severing catalyzed by spastin, a MT interacting protein (Lacroix et al., 2010).

MT alterations related to variations in levels of tubulin modifications have been reported in several neurodegenerative diseases. Some examples include: i) a mouse model for AD, where decreased levels of α-acetylated tubulin were observed in the hippocampus and coincided with increased levels of HDAC6 (Zhang et al., 2014); ii) Aβ oligomers that *in vitro* were shown to induce MT loss *via* tau recruitment of tubulin tyrosine ligase-like 6, promoting tubulin polyglutamylation and recruitment of the MT-severing enzyme spastin (Zempel et al., 2013); iii) a model of induced PD using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimide (MPTP), where early alterations in α-tubulin modifications were observed in dopaminergic neurons, leading to impaired axonal transport and disease progression (Cartelli et al., 2013); iv) brains of HD patients where a dramatic decrease in the level of α-acetylated tubulin was observed and related with a defective axonal transport (Dompierre et al., 2007); and v) a CMT mouse model, induced by mutation of the small heat-shock protein gene, where decreased levels of α-acetylated tubulin and severe axonal transport deficits were observed in peripheral nerves (d'Ydewalle et al., 2011). These reports point mainly to α-acetylation of tubulin, with a concomitant impairment in axonal transport, as a common pathological hallmark in several neurodegenerative disorders. In fact, these findings supported the use of drugs targeting MT acetylation as a useful strategy for treating neurodegenerative diseases. Nevertheless, the impact of modulating acetylating/deacetylating enzymes on MT stability is not clear. Strikingly, αTAT promotes MT destabilization and accelerates MT dynamics in a mechanism independent of its function as a α-tubulin acetylase (Kalebic et al., 2013). In line with the same observation, catalytically inactive HDAC6 mutants also modulate MT dynamics without altering acetylation (Zilberman et al., 2009).

Besides the regulation of MT functions by tubulin modifications, MAPs also play a key role in MT dynamics. Tau, a MT stabilizer, is the most studied MAP due to its involvement in AD. However, besides AD, tau dysfunction is at the center of several neurodegenerative disorders (Lee et al., 2001; Poorkaj et al., 1998; Spillantini et al., 1998). Hyperphosphorylation of tau leads to detachment of MAP from MTs, and thereby to MT disassembly, loss of neural shape and impairment of axonal transport (Alonso et al., 1996; Alonso et al., 1997). Moreover, dissociation of tau from MTs provides access for MT-severing proteins such as katanin, which break down MTs and lead to axon degeneration (Qiang et al., 2006). The above observations suggest a disruption of tau normal function promoted by hyperphosphorylation leading to neurodegeneration. Supporting this hypothesis studies with tau knockout mice demonstrated that the absence of this MAP is associated with decreased neurite outgrowth, decreased MT stability and causes degeneration in neurons from AD mice (Dawson et al., 2001, 2010; Harada et al., 1994). Nevertheless, tau knockout mice present normal axonal transport rates (Yuan et al., 2008) suggesting that the impairment in axonal transport in AD is a secondary effect resultant from the induction of neurodegeneration by abnormal tau.

Besides the loss of function induced by tau hyperphosphorylation, it is important to refer the gain of toxic function by NFTs, which on one hand sequester normal tau affecting MT dynamics (Alonso et al., 1996) and on the other hand become physical obstacles for axonal transport (Velasco et al., 1998). Although tau is traditionally perceived as an axonal protein, it is also localized to the dendritic compartment including spines, albeit at lower levels than those found in the axon (Papasozomenos and Binder, 1987). In fact, recently, tau has been implicated in synaptic function as part of the molecular pathway leading to Aβ-driven synaptotoxicity (Frandemiche et al., 2014). Besides tau, the MT associated protein 1B (MAP1B), also an axonal MT stabilizer, was also shown to be present in NTFs in brains of AD patients (Ulloa et al., 1994). In *Drosophila*, mutants of the MAP1B fly homologue Futsch led to a destabilization of the MT cytoskeleton resulting in axonal transport defects and neurodegeneration (Bettencourt da Cruz et al., 2005). However, although alterations in MT organization were also observed in neurons from MAP1B knockout mouse models (Gonzalez-Billault et al., 2001; Teng et al., 2001), no impact on vesicle transport was observed (Teng et al., 2001). MAP1B has also been related to cytoskeleton alterations in ALS. In a fly model of ALS, where human TDP-43, a RNA binding protein linked to the disease, was overexpressed in motor neurons, an altered localization of Futsch was observed when compared to controls, being increased on the cell bodies of motor neurons and reduced at the neuromuscular junction. Moreover, overexpression of Futsch in the fly model of ALS rescued the reduced life span, locomotor dysfunction, and the decreased acetylated tubulin levels at the neuromuscular junction (Coyne et al., 2014). In spinal cords of ALS patients a similar misslocalization of MAP1B was observed, with a significant accumulation of MAP1B in motor neuron cell bodies (Coyne et al., 2014). Besides being a MT-associated protein MAP1B was shown to interact with actin (Cueille et al., 2007; Togel et al., 1998). In this respect, MAP1B was demonstrated to play a role on dendritic

spine morphology and synaptic function by regulation of the actin cytoskeleton through modulation of Rho GTPase activity (Tortosa et al., 2011). These results suggest that MAP1B dysregulation might impact on synaptic dysfunction in neurodegenerative diseases.

The family of collapsin response mediator proteins (CRMPs) consists of five cytosolic proteins (CRMP1–CRMP5) highly expressed in the nervous system and playing critical roles in mediating growth cone guidance, neuronal polarity, and axonal elongation. CRMPs are very similar in terms of protein sequence (with the exception of CRMP5), are negatively regulated by phosphorylation and their ultimate target is the cytoskeleton. Although CRMPs 2, 4 and 5 play a key role in the regulation of the neuronal cytoskeleton (Brot et al., 2010; Fukata et al., 2002; Inagaki et al., 2001; Rosslenbroich et al., 2005), hyperphosphorylated CRMP2 has been particularly associated with neurodegenerative disorders, specifically AD (Williamson et al., 2011). CRMP2 phosphorylation on one hand disrupts its association with tubulin heterodimers blocking MT polymerization (Fukata et al., 2002) and on the other hand, blocks CRMP2 association with kinesin, blocking the anterograde vesicular transport of molecules important for synaptic integrity at the distal end of axons (Kawano et al., 2005). CRMP2 is highly implicated in axonal regeneration: following spinal cord injury decreased phosphorylation of CRMP2 mediated by inactivation of glycogen synthase kinase 3β (GSK3β), promotes axonal regeneration by increasing MT dynamics (Liz et al., 2014). These results support modulation of CRMP2 activity as a therapeutic target to induce axonal regeneration. Concerning neurodegenerative disorders, CRMP2 has been mainly implicated in AD (Soutar et al., 2009; Uchida et al., 2005; Yoshida et al., 1998), where CRMP2 phosphorylation was shown to be induced by Aβ oligomers mediating the disease characteristic cognitive deficits (Isono et al., 2013). More recently CRMP2 hyperphosphorylation was also implicated in multiple sclerosis and PD (Fang et al., 2015; Petratos et al., 2012). Concerning multiple sclerosis, increased levels of phospho-CRMP2 were observed in degenerating spinal cord neurons of experimental autoimmune encephalomyelitis (EAE) animal model and active lesions from patients with multiple sclerosis. Moreover, transduction of retinal ganglion cells from EAE-induced mice with a phospho resistant mutant of CRMP2 decreased axonal degeneration (Petratos et al., 2012). In PD, the involvement of CRMP2 was demonstrated in an in vitro model of the disease, where cultures of dopaminergic neurons treated with MPP+ presented axonal degeneration mediated by an increase in CRMP2 phosphorylation (Fang et al., 2015). As demonstrated by the works referred, CRMP2 phosphorylation is receiving increasingly relevance in several neurodegenerative disorders, which together with the research on drugs modulating the molecule, namely the studies from Hensley et al. with lanthionine ketimine ester (LKE) (Hensley et al., 2013), promote CRMP2 as promising therapeutic target on neurodegeneration, as discussed in section 4.

Besides the impact of CRMP2 hyperphosphorylation in neurodegeneration, CRMP4 was suggested to be implicated in ALS: i) increased levels of CRMP4 were detected in lumbar spinal cord motor neurons in mutant SOD1 mice; ii) *in vivo* overexpression of CRMP4 in wild-type animals induced motor neuron death and neuromuscular denervation; and iii) a mutation in CRMP4 was detected in ALS patients and expression of the variant in motor neuron cultures resulted in decreased axonal outgrowth and cell survival (Blasco et al., 2013; Duplan et al., 2010). While CRMP2 has been mainly implicated in the regulation of the microtubule cytoskeleton, CRMP4 was shown to play a role both on microtubules and actin dynamics, what underlies its neuritogenic effect in hippocampal neurons (Khazaei et al., 2014). Nevertheless, in motor neurons the impact of CRMP4 on the neuronal cytoskeleton was not addressed.

Upstream regulators of MT dynamics include signaling kinases that modulate the functions of MAPs, such as GSK3β. GSK3β is a multifunctional serine/threonine kinase that phosphorylates several MT-interacting proteins, namely CRMP2, MAP1B and tau, MT plus end-tracking proteins (APC and CLASP2) and the MT-depolymerizing factor stathmin (Liu et al., 2012). GSK3β is intimately related with AD what is mainly derived from its role on tau phosphorylation (Flaherty et al., 2000), and as such affects MT dynamics and axonal transport in this disorder. Another tau kinase which has been implicated in the pathogenesis of AD, is cyclin-dependent kinase-5 (Cdk5) (Tseng et al., 2002). In fact, Cdk5 acts as a "priming kinase" for the subsequent GSK3β phosphorylation of tau and CRMP2 (Cole et al., 2006; Li et al., 2006), impacting on the regulation of MT dynamics. Additionally, Cdk5 is also responsible for the hyperphosphorylation of NFs (Sun et al., 1996). Consequently, the aberrant activity of Cdk5 observed in AD results in hyperphosphorylation of both NFs and tau, two major hallmarks of neurodegeneration.

Numerous studies link MT alterations with a wide range of events that occur in neurodegenerative disorders. This relation of dysregulation of the MT cytoskeleton with disease onset received a major attention in the design of novel therapeutic approaches aimed at preventing/reverting neurodegeneration through cytoskeleton modulation.

2.2. Axonal transport defects as a direct cause of neurodegeneration or a downstream effect of altered MT stability

Most proteins essential for the axon and the synaptic terminals are synthesized in the cell body and need then to be transported down the axon. Therefore, intracellular transport is crucial for neuronal morphogenesis, function and survival. Transport through the axon is divided in the slow axonal transport of cytosolic and cytoskeletal proteins and the fast axonal transport of membranous organelles and vesicles. Over the years, many studies have been directed to a common premise linking axonal transport impairment with the first steps of neurodegeneration. However, in some neurodegenerative disorders it is still unknown whether one is a cause or a consequence of the other. One should though consider that, given the thin caliber of axons and the complex cytoskeletal and proteinaceous milieu inside them, the accumulation of motor proteins associated with their cargos that might occur as a consequence of impaired axonal transport, can create a toxic environment to neurons thereby leading to neurodegeneration.

Besides alterations in MT-stability, already referred to above, alterations in axonal transport are caused by mutations in motors as well as in non-motor proteins involved in neurodegenerative diseases. In the sporadic forms of ALS, KIF1Bβ and KIF3Aβ the two major KIFs isoforms enriched in the motor cortex, presented decreased gene expression in the brain (Pantelidou et al., 2007). Moreover, mutations in dynactin and in the cytoplasmic dynein heavy chain genes were found in ALS patients causing a defective retrograde transport (Munch et al., 2004). Additionally, mutations in superoxide dismutase 1 (SOD1), implicated in ALS, could directly or indirectly affect axonal transport by: i) interfering with cargo binding; ii) generating a physical barrier composed by the SOD1 aggregates; iii) disrupting MT stability; and iv) disrupting motor activity (Strom et al., 2008). CMT2 that involves peripheral axonopathy, was shown to be caused by mutations in KIF1Bβ that were related to decreased anterograde transport (Zhao et al., 2001). In AD, besides the effect of hyperphosphorylated tau on axonal transport, the impaired binding of amyloid precursor protein (APP) to kinesin-I was demonstrated as a potential mechanism for reduced axonal transport (Gunawardena and Goldstein, 2001). Moreover, in familial AD, mutations in presenilin were described to increase the activity of GSK3β leading to

increased phosphorylation of kinesin light chains, and promoting kinesin detachment from its cargo and impairment of axonal transport (Morfini et al., 2002; Pigino et al., 2003). Concerning HD, expression of mutant huntingtin was shown to cause defective axonal trafficking in multiple model organisms, including squid, *Drosophila,* and mice (Gunawardena et al., 2003; Szebenyi et al., 2003; Trushina et al., 2004). Axonal transport defects were suggested to occur due to the sequestering of motor proteins by the aggregates of mutant huntingtin (Trushina et al., 2004). Moreover, wild-type huntingtin was show to interact with motor proteins having a physiological role in the intracellular transport of vesicles and organelles (Engelender et al., 1997; McGuire et al., 2006). Thus, disruption of axonal transport in HD was suggested to result from the loss of the physiological huntingtin function in combination with the gain of toxic function of the mutant protein (Caviston and Holzbaur, 2009).

As already briefly mentioned above for SOD1, transport defects might also be caused by protein aggregates, the pathological hallmark of several neurodegenerative disorders (Goldstein, 2012). Two putative mechanisms for poisoning of axonal transport by protein aggregates have been proposed although not fully analyzed yet: i) nonspecific physical blockage of the transport along axons and, ii) selective blockage of certain cargos by aggregate-dependent sequestration of motors away from cargoes (Coleman, 2011). These mechanisms could be the cause of axonal transport defects in diseases such as AD, PD and HD.

As suggested in this section, several causes can lead to disruption of axonal transport in neurodegenerative disorders. Although further disclosure of the mechanisms leading to such defects in specific neurodegenerative disorders should be performed, the similar features of the reported axonal transport alterations in several distinct disorders support common therapeutic interventions.

2.3. Disclosing key actin cytoskeleton components involved in neurologic disorders

In mature neurons, actin is enriched in dendritic spines and disruption of the actin cytoskeleton leads to altered dendritic spines, decreased dendritic spine density and ultimately to synaptic loss (Zhang and Benson, 2001). As such, dysregulation of actin dynamics in addition to impaired MT stability also plays a key role in neurodegenerative disorders.

The actin cytoskeleton is strongly regulated by signaling pathways, namely by the Rho GTPase family (Nobes and Hall, 1995; Ridley and Hall, 1992). The three canonical Rho GTPases include RhoA, Rac1 and Cdc42. Activation of Rho GTPases is modulated by cycling between an inactive GDP-bound state and an active GTP-bound state, a process regulated by guanine exchange factors (GEFs), which activate the protein by promoting the exchange of GDP for GTP, GTPase-activating proteins (GAPs), which increase the intrinsic GTPase activity, inactivating the protein, and guanosine nucleotide dissociation inhibitors (GDIs) that maintain GTPases in an inactive state (Jaffe and Hall, 2005). Dysregulation of Rho GTPase signaling occurs in several neurodegenerative diseases: in PD, its inhibition leads to increased neurite extension in a mouse model of the disease (Zhou et al., 2011); in CMT mutations in frabin/FDG4, a GEF specific to the Rho GTPase Cdc42 (Delague et al., 2007; Stendel et al., 2007), lead to peripheral nerve demyelination; in HD, huntingtin interacts with several players of the Rho GTPase signaling pathways (Tourette et al., 2014); in AD several independent evidence show the involvement of Rho GTPases in disease including increased levels of RhoA in neurons close to amyloid plaques in the cerebral cortex of APP(Swe) Tg2576 mice, increased activity of RhoA accompanied by decreased neurite length induced *in vitro* by Aβ (Petratos et al., 2008), and alterations in the levels of Cdc42/Rac with concomitant actin cytoskeletal alterations in hippocampal neurons treated with Aβ (Mendoza-Naranjo et al., 2007).

The Rho GTPase signaling is upstream the regulation of actin binding proteins (ABPs). The impaired function of several ABPs has been related to the development of neurodegeneration as is discussed below.

The ABP cofilin has a concentration dependent function, acting as an actin stabilizer at very low concentrations, as an actin severing protein at medium concentrations and as an actin nucleation protein at high concentrations (Andrianantoandro and Pollard, 2006). Cofilin is associated to a neurodegeneration-related structure present in stressed and degenerating neurons: the cofilin-actin rods, which are emerging as key players in neurodegeneration mainly in the context of AD. Cofilin hyper-activation by dephosphorylation leads to its saturated binding to actin filaments causing them to form rod structures – the cofilin-actin rods. Cofilin phosphorylation leading to its inactivation is mediated by Lim kinase (LIMK), a process reverted by the action of Slingshot phosphatase-1 (SSH1). LIMK is regulated by the action of either p21-activated kinase (PAK), a downstream effector of Rac and Cdc42 (Edwards et al., 1999), or ROCK downstream of RhoA activation (Sumi et al., 1999). Both ROCK and PAK1 phosphorylate and activate LIMK. Cofilin-actin rods were identified in neurites of cultured neurons treated with Aβ oligomers (Maloney et al., 2005) and in human AD brains mainly in the hippocampus and cerebral cortex (Minamide et al., 2000). The function of cofilin-actin rods is still controversial, but they might promote axonal transport alterations and lead to synapse disruption (Cichon et al., 2012), indicating their possible involvement in other neurodegenerative diseases unrelated to AD. In fact, the presence of cofilin-actin rods has also been suggested in HD, although localized in the nucleus (Munsie and Truant, 2012). In PD, dysregulation of cofilin activity was also observed prompting the actin cytoskeleton as putative therapeutic target in this disorder. Parkin a protein mutated in several cases of familial PD was shown to interact with LIMK leading to reduction of cofilin phosphorylation (Lim et al., 2007). A more recent study demonstrated that α-synuclein induces cofilin inactivation and actin stabilization in primary neurons and in fibroblasts derived from PD patients (Bellani et al., 2014). As a possible therapeutic avenue, cofilin-actin rod formation was shown to be reverted by modulating the activity of LIMK (Davis et al., 2011).

Hirano bodies are also actin related structures, deposited in the hippocampus of AD patients (Schmidt et al., 1989). Whereas cofilin-actin rods are needle-like structures composed exclusively by cofilin and actin and that can assemble either in the nucleus (Fukui, 1978) or in the cytoplasm (Minamide et al., 2000), Hirano bodies are highly ordered rod or disk shaped sheets (Izumiyama et al., 1991) enriched in actin and ABPs, with cofilin as one of the major constituents of the inclusions (Galloway et al., 1987; Maciver and Harrington, 1995). Different studies suggested that Hirano bodies are precursors of cofilin-actin rods (Maselli et al., 2003) or vice-versa (Minamide et al., 2000). Hirano bodies were also observed in models of tauopathy, implicating interactions between tau and the actin cytoskeleton (Fulga et al., 2007). In this respect, tau was shown to promote bundling of actin filaments leading to their stabilization (Fulga et al., 2007).

Drebrin, a F-actin interacting protein which, as referred above, binds to the MT-binding protein EB3 promotes a dynamic interaction between F-actin and MTs that is crucial for growth cone formation, neuritogenesis and dendritic spine morphology (Geraldo et al., 2008; Merriam et al., 2013). Besides linking actin and MTs, drebrin interaction with the actin cytoskeleton is crucial for the maintenance of dendritic spine size, number and

morphology (Harigaya et al., 1996). Drebrin levels are decreased in AD patients (Harigaya et al., 1996), which is suggested to be linked to disease progression (Julien et al., 2008). Dendritic spine regulation by drebrin is related with an interplay with cofilin, that when impaired may result in synaptic dysfunction in AD (Kojima and Shirao, 2007). In AD, the mechanism of synaptic dysfunction and cognitive decline is suggested to be caused by reduction of PAK activity by Aβ accumulation which leads on one hand to decreased drebrin levels in spines and on the other hand to the activation of cofilin and the consequent disruption of actin dynamics (Kojima and Shirao, 2007). While cofilin has an actin severing activity, drebrin stabilizes the actin filament inhibiting actin depolymerization. Drebrin and cofilin competitively bind to actin filaments, with drebrin inhibiting the cofilin-mediated severing activity (Grintsevich and Reisler, 2014). Thus, the balance between drebrin and cofilin activity plays a key role in maintaining actin dynamics in dendritic spines.

Additionally, tau-induced neurodegeneration is associated with accumulation of F-actin and the formation of cofilin-actin-rich rods in *Drosophila* and mouse models of tauopathy (Fulga et al., 2007). Profilin is another ABP with an important role in actin polymerization where it enhances the rate of polymerization by delivering ATP–G-actin to the barbed end of the filament (Selden et al., 1999). Profilin has been recently associated with neurodegeneration. Exome sequencing of different ALS families allowed the identification of four mutations in the profilin1 gene localizing these mutations close to actin binding sites (Wu et al., 2012). Primary motor neurons expressing mutant profilin1 form insoluble profilin1 aggregates *in vitro,* which inhibit axonal outgrowth and growth cone size, and test positive for ALS markers, supporting the role of profilin in familial ALS pathology (Wu et al., 2012). Although the mechanism underlying ALS pathology caused by profilin1 mutations is still not clear, recent studies suggest that there is a mutation-derived destabilization of the native profilin1 structure that leads to its aggregation and, either due to a loss of function or gain of toxic function, will cause ALS (Boopathy et al., 2015).

As illustrated above, the actin cytoskeleton, although less explored than the MT component, is being implicated in several neurodegenerative disorders. On one hand, dysregulation of ABPs induces the formation of structures enriched in actin that perturb neuronal integrity, and on the other hand, regulatory proteins of actin dynamics are altered in neurodegenerative disorders.

3. Drugs targeting cytoskeleton functions: impact on neurodegenerative disorders

As discussed in the previous section, several cytoskeleton alterations exist in unrelated neurodegenerative disorders, which raised the interest to develop therapeutic strategies

Table 2
MT stabilizers/destabilizers and HDAC6 inhibitors applied in neurodegenerative disorders.

Compound	Pathology	Function	Outcome	Clinical trial
Taxol (paclitaxel)	Tauopathy	MT stabilizers	Increased MT number and stabilization; restored axonal transport; ameliorated motor impairments	No
Epothilone D	Tauopathy		Corrected MT density, axonal transport defects, axonal dystrophy, cognitive impairment, neuronal loss and tau pathology	Yes (AD)
	PD		Prevented neurodegeneration; rescued MT defects	No
NAP (Davunetide)	AD, ALS, PD		Preserved axonal transport; decreased tau hyperphosphorylation; delayed symptomatology	Yes (AD)
Noscapine	ALS		Decreased MT hyperdynamicity; restored axonal transport restoration; delayed disease onset; improved motor performance	No
	PD		Corrected axonal transport alterations	No
Vinblastine	iPSC-derived neurons from HSP patients	MT destabilizer	Rescued axonal swelling phenotype	No
Tricostatin A and Tubastatin A	CMT2	HDAC6 inhibitors	Increased α-tubulin acetylation; reestablished axonal transport; increased motor performance	No
Tubastatin A and ACY-1215	AD		Increased α-tubulin acetylation; reduced Aβ and hyperphosphorylated tau levels; rescued cognitive impairment	No
Tricostastin A	HD		Improved transport deficits	No
AK-7	HD	Sirt2 inhibitor	Improved motor function; extended survival; reduced brain atrophy	No
	PD		Decreased α-synuclein toxicity; prevented dopaminergic neuronal loss	

Table 3
ROCK and GSK3β inhibitors applied in neurodegenerative disorders.

Compound	Pathology*	Function	Outcome	Clinical trial
Fasudil	AD	ROCK inhibitor	Ameliorated cognitive impairment; attenuated neuronal loss	No
	PD (MPTP mouse model)		Decreased loss of dopaminergic neurons; increased motor performance	No
	PD (6-OHDA mouse model)		No improvement of survival	No
	ALS		Improved motor function	Yes
Y-27632	HD		Inhibition of huntingtin aggregation; prevented neuronal death; improved motor performance	No
	ALS		Increased motor performance (less successful than fasudil)	No
MMBO	AD	GSK3β inhibitor	Decreased tau phosphorylation; improved memory and cognition	No
Tideglusib	AD		Decreased tau phosphorylation; improved memory and cognition; decreased Aβ pathology and neuronal death	Yes
Pyridotriazol	AD		Decreased tau phosphorylation; improved memory and cognition; decreased Aβ pathology and neuronal death	No
Morin	AD		Ameliorated tau pathology	No
Lithium	AD		Decreased tau phosphorylation; variable results in Aβ levels and behavior	Yes
	PD (MPTP mouse model)		Decreased loss of dopaminergic neurons	No
	PD (6-OHDA mouse model)		No effect on dopaminergic neuron degeneration	No
	HD (Drosophila model)		Decreased neurodegeneration	No
	HD (R2-6 mouse model)		Improved motor performance (not in all animals tested)	No
	ALS		Slow disease progression	Yes
Lithium with Valproate	ALS, HD, PD		Improved motor performance and survival; reduced neurological defects	Yes (HD and AD)
GSK3β inhibitor VII	ALS		Improved motor performance and survival; delayed disease onset; loss of drug effect on symptomatic stage	No
JGK-263	ALS		Improved motor function; delayed disease onset	No
indirubin-3′-oxime and AR-A014418	PD		Prevented loss of dopaminergic neurons	No

*Studies performed in mouse models, except when mentioned.

targeting the neuronal cytoskeleton. The majority of drugs being tested in the context of neurodegenerative disorders were selected based on promising effects in axonal regeneration after CNS injury. These include: i) MT stabilizers, given that taxol and epothilone B have a positive effect on axonal growth after spinal cord injury (Hellal et al., 2011; Ruschel et al., 2015); ii) drugs targeting tubulin post-translational modifications, more specifically α-tubulin acetylation, which has been associated with increased axonal growth (Rivieccio et al., 2009); and iii) compounds targeting regulatory proteins of cytoskeleton dynamics, namely RhoA, a regulator of actin dynamics, and GSK3β a major regulator of MT stability. Following spinal cord injury, inhibition of each of these signaling molecules showed positive effects on axonal regeneration (Fehlings et al., 2011; Liz

et al., 2014). In this section we address the commonly used therapeutic strategies targeting cytoskeleton dysfunction in neurodegenerative disorders. In Tables 2 and 3 the therapeutic strategies discussed in this section are summarized.

3.1. Microtubule stabilizers: can cancer drugs show promising effects on neurodegenerative disorders?

Over the past years several MT-stabilizing drugs were characterized as cancer therapeutics due to the crucial role of MTs in cell division. The concept of utilizing MT-stabilizing drugs for the treatment of neurodegenerative diseases was initially considered for tauopathies and AD, with the rationale that these drugs could compensate for the loss of tau stabilizing function (Zhang et al., 2005). *In vivo*, the first studies addressing the therapeutic potential of MT stabilizers were performed with taxol (paclitaxel), a natural product which initially emerged as an anti-cancer drug (Schiff et al., 1979). Taxol was tested *in vivo* in a mouse model of tauopathy, which exhibits tau pathology in spinal motor neurons that project outside the blood brain barrier, showing beneficial effects: i) increased MT numbers and stabilization; ii) restored fast axonal transport; and iii) ameliorated motor impairments (Zhang et al., 2005). However, taxol has limited brain bioavailability being an unsuitable therapeutic candidate for diseases affecting the brain. Another natural compound with anti-cancer activity and functionally similar to taxol in promoting MT stabilization is epothilone D (EpoD), which has the advantage of being brain penetrant and was shown to correct MT density and axonal transport defects, axonal dystrophy, cognitive impairment, neuronal loss, and tau pathology in different tau transgenic mouse models, both in preventive and interventional studies (Barten et al., 2012; Brunden et al., 2010; Zhang et al., 2012). A relevant characteristic of EpoD is that it is highly effective at low doses, meaning that low doses of MT stabilizers are sufficient to restore MT dynamics and axonal transport defects, avoiding an over-stabilization of MTs that could lead to side effects such as peripheral neuropathy (Lee and Swain, 2006). Based on the promising results obtained with EpoD, a phase I clinical trial was initiated in AD patients in 2012, but ended in October 2013 without published results (ClinicalTrials.gov identifier NCT01492374). Besides AD and related tauopathies, the MT stabilizer EpoD showed promising effects in a model of experimental parkinsonism induced by MPTP. As previously referred to, in this model MPTM affects MT stability leading to axonal transport impairment and dopaminergic neuron degeneration. In this model treatment with EpoD prevented neurodegeneration and rescued MT defects (Cartelli et al., 2013). These results support MT stabilization as a possible therapeutic strategy for other neurodegenerative diseases apart from tauopathies. Nevertheless, the major disadvantage of EpoD is the preferential intravenous administration.

NAP peptide (davunetide), a short peptide fragment (NAPVSIPQ) derived from the activity-dependent neuroprotective protein (ADNP), is also a naturally occurring compound described has having MT-stabilizing properties. NAP beneficial effects, including preservation of axonal transport, decrease of tau hyperphosphorylation, and delay of symptomatology, were demonstrated in many different animal models namely AD, ALS and PD (Jouroukhin et al., 2013; Magen et al., 2014; Matsuoka et al., 2007; Matsuoka et al., 2008). The great advantage of NAP over other MT-stabilizing agents lies in its clean toxicology profile, and ability to penetrate the blood brain barrier by systemic administration as well as the non-invasive intranasal application. Based on a safety profile in phase I studies, phase II studies were performed addressing intranasal administration of NAP in a patient population suffering from amnestic mild cognitive impairment (aMCI), a precursor of AD. NAP was generally safe and well tolerated with an equal percentage of adverse events reported by placebo subjects and NAP-treated subjects. More importantly, NAP showed successful effects in cognitive tests (Gozes et al., 2009). Ongoing phase II clinical trials are being performed with NAP in patients with tauopathy (ClinicalTrials.gov identifier NCT01056965). Phase II/III clinical trials with NAP were also performed in patients with progressive supranuclear palsy (ClinicalTrials.gov identifier NCT01110720), a neurodegenerative cause of atypical Parkinsonism, but no positive effects were observed (Boxer et al., 2014). The MT interacting properties of NAP derive from the SIP domain of the peptide which binds to the MT end binding proteins EB1 and EB3 (Oz et al., 2014). In a study aiming to identify NAP-like peptides with neuroprotective and reduction of tau pathology properties, the peptides NATLSIHQ (NAT) and STPTAIPQ derived from tubulin, and TAPVPMPD (TAP) derived from tau were selected. These peptides displayed neuroprotection against Aβ induced neurotoxicity with NAT and TAP also preventing tau-like aggregation. Moreover, NAT treatment promoted beneficial effects *in vivo* in a mouse model of frontotemporal dementia (Gozes et al., 2014). These results suggest NAP-derivatives as clinically relevant in neurodegenerative diseases, namely the ones involving cognitive dysfunction.

MT stabilizing effects of several drugs are related with their effect on promoting MT polymerization. Other MT modulating agents have a distinct stabilizing activity, acting on MT dynamicity by decreasing the MT growing and shrinkage rates and increasing the pausing time of MTs, rather than changing MT polymer mass or altering the structural features of MTs. An example of a modulator of MT dynamics is noscapine, which was tested in a mutant SOD1 transgenic mouse model of ALS. SOD1 mice have hyperdynamic MTs in axons from the spinal cord and sciatic nerve, associated with impairments in axonal transport. Noscaspine decreased MT hyperdynamicity, restored axonal transport and delayed disease onset with an improvement of motor performance (Fanara et al., 2007). Correction of axonal transport alterations induced by injection of MPTP in mice (a model of experimental parkinsonism) was also observed with noscapine treatment (Fanara et al., 2012).

Non-naturally occurring small molecule MT-stabilizing compounds were also subject of investigation leading to the identification of selected triazolopyrimidines and phenylpirimidines that are orally bioavailable and brain penetrant. Despite showing therapeutic potential by enhancement of MT stabilization in brains of wild-type mice, studies with mouse models for neurodegenerative diseases are still lacking (Lou et al., 2014). Although treatment with MT stabilizers is being highly addressed in the context of neurodegenerative disorders, it is crucial to refer that pathology might also derive from hyperstabilization of MTs, which causes a too rigid neuronal cytoskeletal structure, as described for hereditary spastic paraplegia (HSP) (Denton et al., 2014). HSP is mainly characterized by degeneration of corticospinal tract axons, being spastic paraplegia 4 (SPG4) the most common form of the disease, which is caused by mutations in the gene encoding for the MT severing enzyme spastin. Induced pluripotent stem cells (iPSC)-derived neurons from a SPG4 patient display a significant increase in axonal swellings, decreased axonal transport and increased levels of acetylated tubulin. Treatment with the MT destabilizing drug vinblastine rescued the axonal swelling phenotype of SPG4 neurons (Denton et al., 2014). This study provides evidence that stabilization of MTs may also underlie neurodegeneration, and raises the possibility that therapy with MT destabilizing drugs may be efficacious in spastin-associated HSP and other disorders related to MT dysfunction.

In conclusion, there is substantial evidence for alterations in MT stabilization and/or dynamicity, with consequent axonal transport

deficiencies, being a critical feature of a number of neurodegenerative diseases. Accordingly, brain-penetrant MT-directed agents that can stabilize MTs and/or normalize MT dynamics, administrated in low doses to avoid polyneuropathy, hold considerable promise as therapeutics for neurodegenerative disorders (Table 2). As referred to above, given that pathology might derive either from hyperstabilization of MTs, as described for HSP, or in contrast, as in AD, from accentuated MT destabilization, it is crucial to analyze the dynamic state of MTs among different neurodegenerative disorders, since the correct balance of MT dynamics is crucial to maintain neuronal integrity.

3.2. Targeting tubulin deacetylases: impact on microtubules, axonal transport and autophagy

Histone deacetylases (HDACs) are enzymes that deacetylate lysine residues from histones as well as from several other non-histone proteins. From the several HDACs, HDAC6 and SIRT2 were shown to take part in the MT network by acting as specific α-tubulin deacetylases (Hubbert et al., 2002; North et al., 2003). HDAC6 emerged as a promising target in neurodegenerative disorders in which axonal transport is disrupted since HDAC6 inhibition strongly enhanced mitochondrial transport in hippocampal neurons, due to the enhanced binding of acetylated tubulin to the motor protein kinesin-1 (Chen et al., 2010). However, it is important to refer that HDAC6 is able to regulate MT stability independently of tubulin acetylation which raises the concern that its impact on axonal transport might not be directly related with levels of acetylated tubulin (Zilberman et al., 2009). Moreover, accumulating evidence supports the notion that HDAC6 participates in the transport and autophagic clearance of misfolded proteins (Pandey et al., 2007), impacting thus on neurodegenerative disorders resulting from protein aggregation.

In a mouse model of CMT2, presenting severe axonal transport defects in peripheral nerves as a consequence of decreased levels of acetylated α-tubulin, inhibition of HDAC6 showed positive effects. Treatment with tricostatin A (a pan-HDAC inhibitor) and tubastatin A (selective HDAC6 inhibitor) not only promoted an increase in acetylated α-tubulin and the reestablishment of axonal transport but also showed beneficial effects in the CMT2 phenotype by increasing motor performance (d'Ydewalle et al., 2011).

In models of tauopathy and AD, HDAC6 inhibitors also showed positive effects. In a transgenic mouse model of tau deposition, increased α-tubulin acetylation in the brain promoted by treatment with tubastatin A correlated with reversion of behavioral defects including memory impairment and hyperactivity (Selenica et al., 2014). Nevertheless, the mechanism by which HDAC6 inhibition was protective in tau transgenic mice was also related with a reduction in total tau levels, evidencing that HDAC6 inhibitors might act by multiple modes of action in neurodegenerative disorders. In fact, it cannot be excluded that HDAC6 inhibitors act on additional substrates, namely cortactin and HSP90 (Kovacs et al., 2005; Zhang et al., 2007). Cortactin deacetylation by HDAC6 creates an actin network that stimulates autophagosome-lysosome fusion contributing to protein clearance (Lee et al., 2010a). Concerning the molecular chaperone HSP90, its deacetylation mediated by HDAC6 favors the formation of a "refolding" complex leading to tau accumulation (Cook et al., 2012). Additionally, tau was suggested to be a HDAC6 substrate and tau acetylation was proposed to impair its interaction with MTs promoting an increase in the pool of cytosolic tau prone to aggregation (Cohen et al., 2011). These evidences question the use of HDAC6 inhibitors in tauopathies. In accordance with multiple modes of action for HDAC6 inhibitors, in a model of AD, tubastatin A and ACY-1215 (another selective HDAC6 inhibitor) not only increased tubulin acetylation, but also reduced the production and facilitated the clearance of Aβ and hyperphosphorylated tau, leading to a rescue of cognitive deficits (Zhang et al., 2014). Further confirming HDAC6 as a therapeutic target in AD, a severe mouse model of AD crossed with HDAC6 knockout mice (APPPS1-21-*HDAC6*$^{-/-}$) presented improvement of the AD cognitive pathology. APPPS1-21-*HDAC6*$^{-/-}$ had increased tubulin acetylation and improved mitochondrial trafficking, although no differences in Aβ plaque load when compared to APPPS1-21 mice (Govindarajan et al., 2013).

In ALS, genetic ablation of HDAC6 in the SOD1G93A mice (by crossing with *HDAC6*$^{-/-}$ mice) promoted increased tubulin acetylation in the spinal cord, improved motor function and extended survival (Taes et al., 2013). Although a dramatic decrease in acetylated α-tubulin was observed in HD brains (Dompierre et al., 2007), a potential therapeutic application of HDAC6 inhibitors in HD should be carefully addressed. In one hand, HDAC6 inhibition compensates for the transport deficit in striatal cells that contain a pathological polyQ expansion in the huntingtin protein (Dompierre et al., 2007) but on the other hand autophagic clearance of aggregated huntingtin in a HD neuronal model requires the neuroprotective presence of HDAC6 (Iwata et al., 2005). In the case of PD, protein aggregates and impaired mitochondria are processed by a common pathway involving HDAC6, suggesting it might also have a neuroprotective effect in this disorder (Lee et al., 2010b). However, no specific HDAC6 activators or agonists have been studied in animal or clinical trials for either PD or HD.

Due to HDAC6 participation in MT-dependent transport and in the autophagy clearance of protein aggregates, HDAC6 modulation particularly in neurodegenerative disorders related to aggregation, requires careful assessment. Nevertheless, inhibition of tubulin deacetylases showed beneficial effects in several neurodegenerative disorders (Table 2), suggesting that correction of axonal transport may counteract the importance of enhanced autophagy.

SIRT2 is another α-tubulin deacetylase. SIRT2 belongs to the sirtuin family which includes 7 homologues in mammals, from which SIRT2 and also sirtuin-1 (SIRT1) modulation have been mainly addressed in neurodegenerative diseases. Concerning the impact on the neuronal cytoskeleton, tau is among the variety of SIRT1 substrates, and deacetylation of this MAP was shown to contribute to its degradation (Min et al., 2010). In brains from AD patients, SIRT1 levels were reduced correlating with tau accumulation and aggregation (Julien et al., 2009). These results proposed SIRT1 activation as a therapeutic option for tauopathies. In fact, resveratrol, an activator of SIRT1, demonstrated positive effects not only in models of tauopathy but in mouse models of several neurodegenerative disorders namely: AD (Capiralla et al., 2012; Wang et al., 2006; Wang et al., 2008), PD (Lofrumento et al., 2014), ALS (Mancuso et al., 2014; Song et al., 2014), and HD (Parker et al., 2005). These studies categorized resveratrol as a very promising compound in neurodegenerative diseases what prompted the initiation of several clinical trials namely on HD (ClinicalTrials.gov identifier NCT02336633) and AD (ClinicalTrials.gov identifiers NCT01504854; NCT00678431; NCT02502253; NCT01716637). While the trial in HD is still recruiting, some of the studies with AD were completed (Kennedy et al., 2010; Mecocci and Polidori, 2012). However, a complete large-scale clinical trial providing a conclusion is missing. While the compound seems to be well tolerated without evident toxicity, resveratrol presents several drawbacks namely poor bioavailability, low solubility and chemical instability (Walle et al., 2004; Walle, 2011).

In contrast to the neuroprotective effect of SIRT1 activation, in the case of SIRT2, its inhibition was suggested to have a positive role in neurodegenerative diseases. AK7, an inhibitor of SIRT2, has a

neuroprotective effect in models of PD as it ameliorates α-synuclein toxicity *in vitro* and prevents MPTP-induced dopamine depletion and dopaminergic neuron loss *in vivo* (Chen et al., 2015). Similarly, in HD, treatment with AK7 improved motor function, extended survival, and reduced brain atrophy in two genetic mouse models (Chopra et al., 2012). In ALS, the effect of SIRT2 was investigated by crossing the SOD1G93A mice with SIRT2 knockout mice; surprisingly no alterations in tubulin acetylation or beneficial effects were observed. These results suggested that at least in ALS, in contrast to HDAC6 inhibition, inhibition of SIRT2 is not a promising therapeutic target (Taes et al., 2013). The opposing effect of manipulating SIRT1 or SIRT2 indicates the need to develop highly selective molecules to target these enzymes. Focusing on the major topic of the present review, although both SIRT1 and SIRT2 regulate the microtubule cytoskeleton by regulating tau and alpha-tubulin acetylation, respectively, it has not been demonstrated whether the beneficial outcomes of modulating these deacetylases derives from an effect on microtubule stability, or in alternative from an effect on another unrelated processes in which these enzymes might be involved. In this context, similarly to HDAC6, SIRT1 and SIRT2 also play a role on autophagy (Ng and Tang, 2013).

3.3. Benefits and drawbacks of inhibiting upstream regulatory kinases

Several signaling molecules have been linked with neuronal cytoskeleton disassembly and are emerging as therapeutic targets for various neurological disorders. It is however important to note that the signaling pathways regulating cytoskeleton dynamics involve multifunctional kinases and thus the outcomes of the *in vivo* studies using kinase inhibitors cannot be directly related with the neuronal cytoskeleton. Nevertheless, very promising results are being obtained with kinase inhibitors in several neurodegenerative conditions (Table 3).

The RhoA/ROCK pathway is the major regulator of the actin cytoskeleton. Multiple extrinsic factors mediate RhoA/ROCK activation, which regulates cytoskeleton reorganization such as growth cone collapse and neurite outgrowth inhibition (Fujita and Yamashita, 2014). RhoA/ROCK inhibitors are being mainly used in the context of injury to the nervous system. In fact, a phase I/II clinical trial using Cethrin, a recombinant fusion protein composed of C3, a Rho inhibitor, was conducted in patients with spinal cord injury improving functional recovery (Fehlings et al., 2011). RhoA/ROCK signaling has also been implicated in several neurodegenerative disorders namely AD, PD, HD and ALS. Concerning *in vivo* studies in AD, administration of fasudil, a ROCK inhibitor, significantly ameliorated cognitive impairment, attenuated neuronal loss, and neuronal injury induced by the intracerebroventricular injection of Aβ1-42 to rats (Song et al., 2013). In the case of PD, fasudil was tested in the MPTP mouse model and oral gavage administration of the drug not only resulted in decreased loss of dopaminergic neurons but also improved motor performance (Tonges et al., 2012). In a more severe model of PD, the 6-hydroxydopamine (6-OHDA) mouse model, fasudil treatment increased the striatal levels of the dopamine metabolite DOPAC suggesting a regenerative response; however, fasudil failed to significantly improve survival of tyrosine hydrolase (TH)-positive neurons in this model (Tatenhorst et al., 2014). Nevertheless, AAV. shRNA-mediated downregulation of ROCK2 (isoform strongly expressed in the CNS), by injection in the substantia nigra of the 6-OHDA mouse model, attenuated the loss of TH-positive neurons and improved motor behavior (Saal et al., 2015). The distinctive results obtained with the different strategies blocking ROCK in this PD model probably derive from the fact that fasudil inhibits other kinases rather than ROCK or that specific inhibition of the ROCK2 isoform is required.

The ROCK inhibitor Y-27632, a more specific ROCK inhibitor in comparison with fasudil, inhibits huntingtin aggregation and prevents striatal neuronal death induced by polyQ-huntingtin (Deyts et al., 2009) *in vitro*. Moreover, the oral administration of the drug showed beneficial effects *in vivo* both in a *Drosophila* model for HD and in the R6/2 mouse model of HD. In the *Drosophila* model Y-27632 attenuated the progressive neurodegeneration of photoreceptor neurons (Pollitt et al., 2003). In R6/2 mice, although Y-27632 did not impact on life span and striatal medium spiny neuron loss, treated animals presented improved performance on rotarod function and reduced levels of soluble huntingtin (Li et al., 2009).

In ALS both fasudil and Y-27632 were tested in the SOD1G93A mouse model, leading to improved motor function although results were more successful with fasudil (Gunther et al., 2014; Tonges et al., 2014). This is probably related with the decreased selectivity of fasudil that might be acting on other kinases. In fact, this study reflects one of the drawbacks of using kinase inhibitors, which is the fact that most of them are not specific for a single kinase. Nevertheless, a phase II clinical trial with fasudil was initiated in ALS patients but results were not reported (ClinicalTrials.gov identifier NCT01935518).

GSK3β is a multifunctional kinase and its dysregulation is implicated in the pathology of several neurodegenerative disorders (Lei et al., 2011). GSK3β is ubiquitously expressed, constitutively active and it is essential for several physiological functions. In pathological conditions such as AD, GSK3β activity is exacerbated (Hooper et al., 2008). The common requirements for a GSK3β inhibitor are not primarily related to potency, as a mild inhibition is necessary to restore the aberrant levels of kinase activity to physiological levels, but with selectivity. Lithium is the most common GSK3β inhibitor being the standard pharmacological therapy for bipolar disorder treatment since 1950, although it is largely non-specific. A variety of GSK3β inhibitors with diverse chemical structures, biological potency, selectivity and mode of enzyme binding, have shown efficacy in different animal models (Martinez et al., 2013).

Regarding a disruption of the neuronal cytoskeleton, GSK3β has been mainly implicated in AD. On one hand, GSK3β phosphorylates tau, affecting its function namely on MT stability (Lovestone et al., 1996), self-assembly of MTs (Utton et al., 1997), and regulation of axonal transport (Tatebayashi et al., 2004), and on the other hand Aβ oligomers induce axonal transport defects *via* activation of GSK3β (Ramser et al., 2013). In this respect, lithium and several small molecules targeting GSK3β were tested in AD mouse models, and although all compounds decreased tau phosphorylation, variable results were observed in terms of Aβ load and memory and cognitive functions. MMBO, a synthetic optimized molecule with high selectivity for GSK3β and brain penetrant when administrated orally, decreased tau phosphorylation and improved memory and cognition without affecting Aβ pathology (Onishi et al., 2011). Other two synthetic optimized molecules, namely Tideglusib and Pyrido-triazol, presented similar results as MMBO in terms of tau phosphorylation and behavioral functions, but also decreased Aβ pathology and neuronal death (Noh et al., 2013; Sereno et al., 2009). Morin, an isolated natural product, ameliorated tau pathology but no other disease characteristics were tested with this compound (Gong et al., 2011). Concerning lithium distinct studies were performed: i) in human APP transgenic mice, lithium treatment ameliorated the behavior deficits, reduced both Aβ levels and plaques and tau phosphorylation (Rockenstein et al., 2007); ii) in an aged mouse model of AD, presenting both tangles and plaques, lithium decreased tau phosphorylation although without affecting Aβ load and memory (Caccamo et al., 2007); iii) in a different mouse model, also presenting both tau and Aβ pathology, lithium promoted similar

results decreasing the levels of phosphorylated tau, without affecting behavior, neuronal loss, or Aβ levels, but it increased amyloid deposition (Sudduth et al., 2012). All together the above reports raise questions about the benefit of GSK3β inhibitors in AD, definitely showing an effect in tau pathology but having variable results in terms of Aβ pathology. Nevertheless, clinical trials were initiated with GSK3β inhibitors. A phase II trial was performed with Tideglusib in AD patients showing safety of the drug although without clinical benefit; however the report of the trial warranted further dose finding studies in early disease stages and for longer duration (Lovestone et al., 2015). Additionally, several clinical trials with lithium were performed in AD (Forlenza et al., 2011; Hampel et al., 2009; Macdonald et al., 2008; Nunes et al., 2013) and the outcome from the various studies suggested that long-term treatment starting at the initial stages of the disease might have a protective effect on the progression of cognitive impairment to dementia (Forlenza et al., 2011).

Besides the regulation of MT dynamics, GSK3β impact on neurodegenerative disorders is also associated with other cellular damage pathways such as inflammation, endoplasmic reticulum-stress, mitochondrial dysfunction and apoptosis. In this respect, GSK3β inhibition has also been investigated in PD, HD and ALS; although results were not directly related with an impact on the neuronal cytoskeleton, it is important to discuss that data as it shows the variability in the outcome obtained with GSK3β inhibition. In experiments with the MPTP induced mouse model of PD, treatment with lithium (Youdim and Arraf, 2004) and with the inhibitors indirubin-3′-oxime and AR-A014418 (Wang et al., 2007) prevented the loss of dopaminergic neurons. However, contradictory results were also reported since in the 6-OHDA animal model of PD, lithium was unable to protect against dopaminergic neurodegeneration (Yong et al., 2011). Moreover, two elderly human patients, without neurodegenerative disorders, receiving chronic treatments with lithium revealed signs of PD namely neuronal apoptosis in basal ganglia structures and impaired motor coordination (Basile et al., 2014; Lecamwasam et al., 1994). The neurotoxicity of lithium was related with alterations in drug pharmacokinetics with aging, what suggests that dose adjustment and regular monitoring of serum lithium concentrations in older people is recommended (Trifiro and Spina, 2011). A pilot trial with lithium was performed in patients with progressive supranuclear palsy or corticobasal degeneration, diseases that share some of the symptoms of PD. Only one participant completed the study, as the others stopped drug early due to intolerability (ClinicalTrials.gov identifier NCT00703677), and as such one of the goals, which was to analyze the impact on levels of phosphorylated tau, was not achieved.

In HD, lithium showed a protective effect against neurodegeneration in a Drosophila model expressing mutant huntingtin (Sarkar et al., 2008). In the R2/6 mouse model for HD lithium showed variable results. While no effects were observed when treatment started pre-symptomatically, for post-symptomatic treatment, two types of results were obtained; one group of mice lost weight faster, died earlier and showed rotarod performance similarly to the vehicle-treated controls, and another group lost weight at a normal rate, died at a similar age, but showed greatly improved motor performance compared to controls (Wood and Morton, 2003). Though the variability of results, the improvement in motor function was an important finding.

In SOD1 transgenic mouse model of ALS, GSK3β inhibition using the GSK3β inhibitor VII (a cell-permeable pyrrolopyrimidine compound that acts as a potent GSK-3β inhibitor) improved motor function and survival, and delayed disease onset; however, after development of clinical symptoms the drug effect was not prominent (Ahn et al., 2012). JGK-263, an oral GSK3β inhibitor, also promoted promising effects in SOD1 mice (Ahn et al., 2014). Concerning lithium, although in an initial study using both a mouse model for ALS and human patients, the drug was shown to slow disease progression (Fornai et al., 2008), in more recent and larger clinical trials, lithium failed to show a significant beneficial effect (Chio and Mora, 2013; Group et al., 2013). Nevertheless, a combined treatment with lithium and valproate, also a mood stabilizer, showed promising effects in a mouse model of ALS in delaying the onset of motor dysfunction, prolonging survival-time and reducing neurological deficits (Feng et al., 2008). The effect of the cotreatment with lithium and valproate was attributed to a pronounced inhibition of GSK3β. Interestingly, similar positive effects were obtained with lithium and valproate cotreatment in mouse models for HD and PD (Castro et al., 2012; Chiu et al., 2011; Li et al., 2013). Clinical trials to examine the effect of lithium, given alone or with valproate, were initiated in HD and in AD patients, although without results reported yet (ClinicalTrials.gov identifier NCT00095355), (ClinicalTrials.gov identifier NCT00088387).

In summary, there is intriguing preclinical data concerning GSK3β inhibition as an effective pharmacotherapy for neurodegeneration. However, further results from clinical studies are required to conclude the benefits from using GSK3β inhibitors in neurodegenerative diseases. Major concerns on targeting GSK3β are related with the complex regulation of the kinase, its activity on multiple substrates, and the fact that agents that inhibit GSK3β activity are not entirely specific and simultaneously affect other signaling pathways. Besides GSK3β, another kinase with the ability to phosphorylate tau, which is hyperactivated in AD brains, is Cdk5 (Tseng et al., 2002). Cdk5 has been identified has a relevant target for the development of therapeutic drugs for AD. One molecule presenting promising effects was TFP5, a modified truncated 24-aminoacid peptide derived from the Cdk5 activator p35 (Zheng et al., 2010). *In vivo* studies with an AD mouse model demonstrated that by inhibiting Cdk5 hyperactivation TFP5 rescued cognitive and motor deficits present in the AD mice, reduced levels of hyperphosphorylated tau and of amyloid plaques, and increased the life span of the animals (Shukla et al., 2013). The TFP5 had the advantage to cross the blood brain barrier being administrated by intraperitoneal injection and did not demonstrate any sign of toxicity. Moreover, TFP5 is highly specific for Cdk5 (Zheng et al., 2010). All these properties classify the peptide as a strong candidate for AD therapeutics.

As mentioned above, Cdk5 phosphorylation of tau and CRMP2 is required for the subsequent phosphorylation of these substrates by GSK3β. As such, modulation of Cdk5 might impact on the regulation of GSK3β activity towards the substrates that require a prior phosphorylation by a "priming kinase", what might be relevant in neurodegeneration conditions resultant from either tau or CRMP2 hyperphosphorylation.

4. Unexplored avenues in drug targeting of the neuronal cytoskeleton

Our overview of the current drugs with ability to interfere with cytoskeleton functions in neurodegenerative diseases showed that the major options include: compounds interfering with MT stability, compounds that target enzymes involved in posttranslational modifications of tubulin, and drugs targeting protein kinases that regulate cytoskeleton-associated proteins. In our perspective, drug targeting of molecules that directly interact with cytoskeleton components, namely MAPs, ABPs, and motor proteins, would be crucial in terms of therapeutics for cytoskeleton damage.

4.1. CRMP2: a promising therapeutic target

In the case of MTs, drugs targeting specific MAPs should be the focus of future research. Several studies have been performed with

tau due its relevance in AD. MAP1B dysregulation was related to AD (Ulloa et al., 1994) and ALS (Coyne et al., 2014) but additional studies regarding the involvement of this MAP in neurodegenerative disorders are required to determine its putative relevance as a therapeutic option. CRMP2 is also a crucial MAP that is emerging as a critical and druggable target in neurodegeneration. CRMP2 hyperphosphorylation has been mainly associated with AD as phospho-CRMP2 accumulation occurs in NFTs of AD patients and both in the cortex and hippocampus in AD animal models (Cole et al., 2007; Yoshida et al., 1998). CRMP2 was recently also implicated in PD, (Fang et al., 2015) and similarly to tau, it might be implicated in additional neurodegenerative disorders. Regarding therapeutic targeting of CRMP2, several kinases phosphorylate it, namely GSK3β, Cdk5 and ROCK. However, inhibition of kinases as a therapeutic option was discussed above and, as highlighted, it has some major weaknesses. In this respect, curcumin, a drug with anti-inflammatory properties, was tested in an induced rat model of AD where it completely reversed the spatial memory deficits by inhibiting CRMP2 hyperphosphorylation (Wang et al., 2013). These results highlight CRMP2 phosphorylation as a potential therapeutic target for AD. Nevertheless, there is no evidence that curcumin is acting directly on CRMP2. In this respect, LKE a naturally occurring metabolite of the amino acid lanthionine, orally bioavailable, brain-penetrating and non-toxic was shown to bind CRMP2 (Hensley et al., 2010a). Moreover, LKE induced an increase in neurite outgrowth of cultured dorsal root ganglia neurons, an effect suggested to be mediated by CRMP2 (Hensley et al., 2010b; Hensley et al., 2011). *In vivo*, LKE showed beneficial effects in a mouse model of multiple sclerosis which was associated with a decrease in the levels of phospho-CRMP2 (Dupree et al., 2015). LKE was also associated with beneficial effects in a mouse model of AD; however the drug rescued the observed decreased levels of CRMP2 phosphorylation in the used mouse model (Hensley et al., 2013), what raises the question of the LKE effect on CRMP2 phosphorylation. Concerning ALS, LKE slowed disease progression in SOD1G93A mice, although results were not correlated with CRMP2 (Hensley et al., 2010b). Accordingly, *in vivo* overexpression of CRMP2 in wild-type animals had no effect on motor neurons, discarding a possible role of CRMP2 in ALS. Nevertheless, CRMP4 was suggested to be implicated in the pathophysiology of ALS by inducing neurodegeneration (Duplan et al., 2010). CRMP4 displays contradictory functions in different neuronal types. In embryonic motor neurons CRMP4 overexpression decreased axonal growth (Duplan et al., 2010). Similarly, in chick dorsal root ganglion neurons, CRMP4 was identified as a regulator of axon growth inhibition (Alabed et al., 2007). Contradictorily, in both cortical and hippocampal neurons, CRMP4 was shown to promote an increase in neurite outgrowth (Khazaei et al., 2014; Quinn et al., 2003). These results raise questions concerning the modulation of CRMP4 as a therapeutic option. Recently, CRMP2 and CRMP4 were shown to interact in neuronal growth cones of hippocampal neurons to regulate axonal outgrowth by linking microtubule and actin dynamics: CRMP2 regulates axonal development *via* CRMP4-mediated actin interaction, and CRMP4 regulates axonal development *via* CRMP2-mediated tubulin interaction (Tan et al., 2015). These results suggest that targeting CRMP2 might have an effect in CRMP4 function, and vice-versa. LKE interaction with CRMP2 was determined in brain lysates in which this is the most abundant CRMP (Hensley et al., 2010a) but it was not investigated whether LKE targets other CRMPs

Based on the clear role of CRMP2 on axonal growth promotion, further investigation on LKE and other drugs targeting specifically CRMP2 in the context of neurodegeneration is mandatory. Moreover, the relevance of the other CRMPs, namely CRMP4, in neurodegenerative disorders should also be addressed due to the role of this family of proteins in the regulation of the neuronal cytoskeleton.

4.2. The missing targeting of actin binding proteins

As discussed in this review the majority of the compounds targeting cytoskeleton damage rely on affecting the regulation of MT dynamics. Nevertheless, actin cytoskeleton disruption is also critical for the process of neurodegeneration and in this respect, inhibitors of the RhoA/ROCK pathway emerged as therapeutic approaches. The majority of the compounds targeting the actin cytoskeleton and modulating its dynamics are natural toxins and can be divided into two groups depending on their effect on the actin cytoskeleton: i) those that enhance the formation and stabilization of actin filaments, and ii) those that destabilize filaments or prevent the assembly of the microfilaments. The most recognized filament forming/stabilizing compounds are phalloidin and jasplakinolide. Phalloidin is an actin filament-stabilizing compound that is not able to freely enter into cells and is generally used coupled with a fluorophore to stain actin filaments (Chazotte, 2010). Jasplakinolide is another actin filament stabilizer that in addition is a potent inducer of actin polymerization (Bubb et al., 1994). The most common filament-blocking/

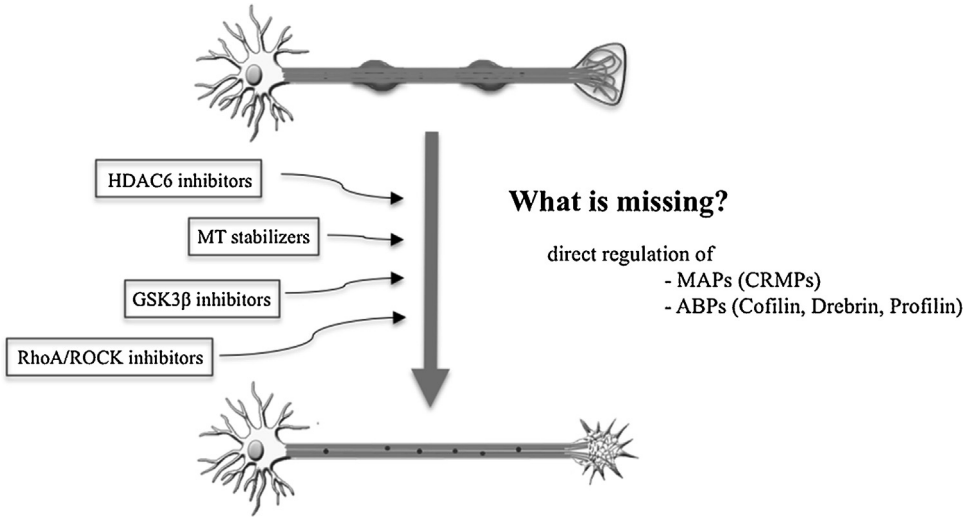

Fig. 2. Schematic representation of the explored and unexplored therapeutic strategies targeting the neuronal cytoskeleton.

destabilizing agents are the latrunculins and the cytochalasins (Cooper, 1987; Spector et al., 1983). Although used in *in vitro* studies, these compounds are ineffective as pharmacological treatments due to poor uptake into cells or toxic effects. As such, unlike MT stabilizers/destabilizers, actin-targeting drugs aiming to correct actin cytoskeleton defects in models of neurodegenerative disorders await further investigation.

An important step for the development of new treatments for neurodegenerative disorders is the investigation of the modulation of ABPs, namely cofilin, drebrin and profilin1, which are now seen as perturbed in an increasing number and variety of neurodegenerative diseases. In respect to cofilin its dysregulation not only affects actin dynamics but is also involved in the formation of cofilin-actin rods in AD, and suggested to be implicated in other neurodegenerative disorders, namely HD and PD (Schonhofen et al., 2014). Drebrin function in dendritic spines is involved in cognitive decline which is characteristic of several neurodegenerative disorders (Zhao et al., 2006). Moreover, drebrin links actin and MTs which is crucial for the maintenance of the neuronal cytoskeleton. Profilin1 mutations have now been identified in familial ALS (Wu et al., 2012). Based on the described studies not only both cofilin, drebrin and profilin1 might be further studied as therapeutic targets in neurodegenerative disorders, but it is also critical to increase our understanding on cofilin-actin rods across different neurodegenerative disorders and on how their manipulation might have therapeutic relevance. In this respect, the development of new mouse models with functional mutations in ABPs would be crucial to address the cytoskeletal abnormalities that lead to neurodegeneration and help in drug discovery for neurodegenerative diseases. Being the maintenance of a dynamic state in the actin cytoskeleton crucial for neuronal activity, the concentrations of these ABPs must be fine tuned for balanced actin dynamics. Moreover, being ABPs important in different cellular mechanisms crucial for several cell types, neuronal targeting of these proteins should be a goal when considering their use in therapeutic strategies.

5. Conclusions and future perspectives

This review focuses on the interesting observation that common alterations in the neuronal cytoskeleton, namely regarding MT stability, axonal transport, and actin dynamics, occur in several unrelated neurodegenerative disorders. Although in some cases it is not clear whether cytoskeleton defects are causative or occur as a consequence of neurodegeneration, as presented in this review, several therapeutic strategies have a high impact both on cytoskeleton correction and on amelioration of disease symptomatology.

One common strategy that is being used in several neurodegenerative disorders is the use of MT stabilizers. However, the analysis of the dynamic state of MTs among different neurodegenerative disorders is relevant, as an equilibrium of MT dynamics is critical to maintain neuronal integrity. As referred to above, pathology may derive in one hand from hyperstabilization of MTs, as described for HSP, or in the other hand, from accentuated MT destabilization which affects axonal transport and synaptic contacts, as observed in AD. MT stabilizers were initially thought to be applied in disorders where tau aggregation occurs namely AD and related tauopathies and now these are also presenting beneficial effects in tau-unrelated neurodegenerative disorders such as ALS and PD. Regarding the use of HDAC6 inhibitors, caution should also be taken due to their dual effect on MT stability, and consequently on axonal transport, and on protein aggregation.

We also addressed the modulation of signaling molecules that regulate cytoskeleton dynamics, namely RhoA/ROCK and GSK3β. Targeting these upstream molecules presents two major disadvantages: the lack of selectivity for the specific kinase, and the fact that they target not only cytoskeleton but also non-cytoskeleton proteins. In this respect, although inhibiting RhoA/ROCK and GSK3β signaling in neurodegenerative disorders might have a positive effect, a direct impact on the neuronal cytoskeleton is not shown.

The most striking observation is that the field is much less explored in terms of targeting actin dynamics when compared to MT stability and axonal transport. The fact that the major regulators of actin dynamics, cofilin, drebrin and profilin1 are dysregulated in several neurodegenerative disorders, suggests that further investigation on the impact of these proteins on neurodegenerative contexts is imperative. Moreover dysregulation of actin dynamics leads to the formation of toxic structures to the cells, the cofilin-actin rods, which although mainly characterized in AD might be a common pathogenic hallmark on other neurodegenerative diseases. As such investigation on cofilin-actin rods should be extended to other conditions, and the identification of drugs with the ability to revert the formation of these structures should be a major focus of research. Another major goal on the design of strategies to revert cytoskeleton damage should be based on targeting molecules that directly interfere with the cytoskeleton proteins namely MAPs, ABPs, specifically in neurons.

In summary, although several promising results are being observed in animal models and are being tested in clinical trials, research in the targeting the neuronal cytoskeleton during degeneration has mainly addressed the MT component whereas the actin cytoskeleton awaits further investigation. Moreover, it will be crucial to design therapeutic strategies acting directly on the cytoskeleton components to avoid the side effects of acting on upstream regulators. In Fig. 2 a schematic summary of the explored *versus* the unexplored therapeutic strategies targeting the neuronal cytoskeleton is depicted.

Conflict of interest

The authors declare that they have no conflict of interest.

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The role of transthyretin on cell biology: a critical point to prevent pathology

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Abstract

Transthyretin (TTR) is an extracellular protein mainly produced in the liver and choroid plexus, with a well-stablished role in the transport of thyroxin and retinol throughout the body and brain. TTR is prone to aggregation, as both wild type and mutated forms of the protein can lead to the accumulation of amyloid deposits, resulting in a disease called TTR amyloidosis. Recently, novel roles for TTR in cell biology have emerged, from cellular fate determination, regulation of proliferation and metabolism, to neuronal health preservation in both central and peripheral nervous systems. Having a complete overview of TTR impact in different cell types and tissues within the human body is critical for understanding its protective and pathogenic sides, and potential therapeutic opportunities for different diseases. Here, we will review the novel literature regarding TTR new roles in cell biology and discuss the importance of these new findings for human pathologies.

Keywords: Transthyretin, neuroprotection, neuronal health, cell metabolism, proliferation.

Declarations

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Conflict of interest

The authors declare no conflict of interest.

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JM and JE performed the literature search and wrote the manuscript, JM and MAL conceived the structure and content. MAL critically revised the work.

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Introduction

Transthyretin (TTR) is a 55kDa homotetrameric protein, with each monomer composed by 127 aminoacids [1] and approximately 14kDa of weight. The TTR tetramer forms a narrow cylindrical hydrophobic channel running through the centre of the molecule [2,3] which carries two symmetrical binding sites, being able to accommodate two molecules of thyroxin (T4) molecules, one of the major TTR physiological ligands. Besides T4, TTR is also the main carrier of retinol (vitamin A), by establishing a 1:1 molar complex with retinol binding protein (RBP), with binding being limited to a maximum of two RBP molecules per TTR tetramer [4]. Accordingly, the main acknowledged physiological function of TTR is the transport of thyroxin and retinol within the body and brain. The prime sites for TTR synthesis are the liver, from where TTR is released to the blood, and the choroid plexus, which is the origin of cerebrospinal fluid (CSF) TTR [5]. In addition, TTR is also expressed in the endothelial cells of Islets of Langerhans [6], ciliary pigment epithelia [7], retinal pigment epithelium [8,9], pancreas [10], intestine [11], visceral yolk sac [12], and, in fairly minor amounts, stomach, heart, skeletal muscle and spleen [5]. One study also reported expression of TTR in the meninges [13] and, more recently, TTR synthesis has been demonstrated in the human placenta [14]. Importantly, TTR expression has also been suggested to occur in Schwann cells [15], and different neuronal types dorsal root ganglia [16], hippocampal neurons [17], motor neurons [18] and cerebellar granule cells [19].

Besides its original role of transporting retinol and T4, TTR has, through the years, also been described to have other cellular roles, including a role in the pathogenesis of cardiac amyloidosis and familial amyloid polyneuropathy (FAP) [20]. Destabilization of TTR due to single amino acid mutations precipitates misfolding and aggregation, which leads to TTR amyloid deposition and tissue dysfunction. TTR amyloidosis is a systemic disorder, resulting in different clinical manifestations, such as, polyneuropathy, autonomic neuropathy, cardiomyopathy, and often a mixture of these. Wild-type (WT) TTR can also destabilize, particularly in the elderly, and aggregate causing non-familial cases of TTR amyloidosis [20]. Additionally, TTR was also shown, many years ago, to act as a metalloprotease, being able to cleave the C-terminus of ApoA-I [21], reducing its ability to promote cholesterol efflux and increasing its amyloidogenic potential [22], being relevant for the development of atherosclerosis. Years ago, a neuroprotection function was also assigned to TTR, as it was shown to decrease amyloid Aβ peptide (Aβ) deposition, by cleaving not only soluble, but also aggregated forms of Aβ *in vitro* [23], diminishing its fibrillogenesis and toxicity [24] and promoting neuroprotection *in vitro* [25]. Accordingly, TTR has also been shown to be neuroprotective in Alzheimer´s Disease (AD) *in vivo* [26].

Recently, new research has revealed TTR is responsible for other effects at the cellular level. In the following sections, we will discuss these novel roles for TTR in cell biology and cell metabolism, with emphasis on the molecular pathways activated within different cell types.

Impact on cell fate and proliferation

In the central nervous system (CNS), TTR was originally described to be produced by epithelial cells in the choroid plexus and secreted to the cerebrospinal fluid CSF, with the role of facilitating distribution of thyroid hormones throughout the CNS [27]. Besides being present in the CSF, TTR was also observed to be synthesised in different neuronal populations, such as hippocampus and cortex [17], as well as in motor neurons [18] and cerebellar granule cells [19]. The expression of TTR in different neuronal populations raised the possibility of other roles for TTR in the brain.

The subventricular zone (SVZ), which covers the ventricular walls, is a stem cell niche that sustains lifelong *de novo* generation of neurons and oligodendrocytes, and is in close contact with CSF that carries TTR. Hence, a role for TTR in neural differentiation in the SVZ region was hypothesised. As such, the brain of *ttr* knockout (KO) mice was analysed, and it was shown that the absence of TTR hampers the neuronal differentiation in the SVZ region, resulting in a shift towards oligodendrogenesis at the expense of neurogenesis [28,29] with the increase in oligodendrocyte number and proliferation leading to hypermyelination of the *ttr* KO mouse brain [28]. Oligodendroglial cell proliferation, survival, and differentiation rely on the coordinated transduction of phosphatidylinositol-3-phosphate kinase (PI3K)/AKT and extracellular signal-regulated protein kinases 1 and 2 (ERK1/ERK2), processes that are essential to CNS myelination [30,31]. In transgenic mice overexpressing the PI3K/AKT signalling pathway, myelin thickness is increased [32], whereas disruption of the Erk1/2 expression in oligodendrocytes reduces myelin thickness and induces an overall degradation of axonal integrity [33]. How TTR regulates oligodendrocyte maturation and proliferation remains to be elucidated; however, TTR absence was shown to potentiate AKT phosphorylation in oligodendroglial lineage cells, regulating oligodendrogenesis, and consequently myelination [29]. Whether this regulation of CNS myelination by TTR relates to the observation that TTR null mice present delayed CNS development [34], memory impairment [35] and inferior cognitive performance [36], needs clarification.

TTR was also recently found to a have a role in cell proliferation, having an impact in the regulation of angiogenesis, the process of formation of new blood vessels. Angiogenesis

is a normal process during growth, development, and in wound healing; however, it is also a fundamental step in tumour progression to malignancy [37]. TTR was shown to increase proliferation of tumour cells both *in vitro and in vivo,* by stimulating the formation of capillary-like tubes and increasing the proliferation of endothelial cells [38]. Additionally, another study revealed that incubating endothelial cells with recombinant TTR V30M, a mutant prone to aggregate, leads to down-regulation of several pro-angiogenic genes involved in endothelial proliferation, survival, and migration, negatively modulating angiogenesis compared to the TTR WT. The authors of this study suggested that TTR regulates angiogenesis through the direct regulation of endothelial gene expression [39]. The fact that the mutant TTR V30M is not able to induce proliferation and angiogenesis, as TTR WT is; and knowing that the TTRV30M mutant is less stable that TTR WT, perhaps the stability of TTR is important for its role in regulating angiogenesis.

Besides stimulating tumour growth by inducing endothelial cells proliferation, TTR has also shown the ability to regulate tumour environment by regulating differentiation and proliferation of myeloid cells in the bone marrow [38]. The myeloid lineage cells are the precursors of the granulocytes, macrophages, dendritic cells, and mast cells of the immune system [40]. Hence, it is interesting that the injection of recombinant TTR in mice leads to an increase in cell differentiation in the myeloid compartment of the bone marrow [38], suggesting a potential role of TTR in immune system regulation. Overall, TTR has been described as having a role in tumour progression through both direct regulation of endothelial cells proliferation, and regulation of the immune cells surrounding the tumour.

Controlling cell metabolism

The same study that presented TTR as a regulator of myeloid cells differentiation and proliferation, also showed that TTR was able to induce changes in the metabolism of those cells. Besides activating Akt1/mTOR pathway, ERK, p38, and NF-κB and p65 molecules, TTR enhanced the production of reactive oxygen species (ROS), enabling these cells to become functional myeloid-derived suppressive cells, highly effective in suppressing T-cell response. Thus, the authors suggested an undescribed role for TTR: the control of the metabolic reprogramming of myeloid lineage cells, via the Akt1/mTOR pathway [38].

Recently, Zawiślak et al. found that neuron-derived TTR stimulates astrocyte metabolism, *in vitro*, in a process independent of TTR ligands. They showed that TTR induces the expression of glycolysis regulators, phosphofructokinase P (PFKP), and pyruvate kinase

M1/M2 isoforms (PKM1/2) in astrocytes, increasing their ATP synthesis [41]. TTR seems to be involved in the activation of glycolytic enzymes engaged in glycogen degradation (PKM1/2 and PFKP), not affecting glucose uptake, as TTR does not appear to affect enzymes of that pathway, such as hexokinase 1 (HK1) [41]. Interestingly, TTR is here described to have an antagonistic effect on the PI3K/AKT pathway [41], which is associated with glucose uptake, whereas previously the PI3K/AKT pathway in neurons was described to be stimulated by TTR [42]. This novel report by Zawiślak et al, implicates TTR in astrocytic energy metabolism, and by consequence in brain energetics, glia-neuron interactions, and even neuronal plasticity.

When considering TTR and cellular metabolism, it is of importance to refer the insulin-like growth factor I (IGF-I) pathway, which is well-known for regulating proliferation and differentiation, as well as controlling metabolic activities, being linked, among others, to cancer, neurodegenerative disorders, and metabolic disorders [43]. The IGF-1 receptor controls the activation of two downstream pathways, the MAPK/Ras-Raf-Erk and PI3K/Akt/mTor pathways, which control various cellular responses, such as differentiation, proliferation, and protection from apoptosis [44]. Years ago, Vieira *et. al*. revealed TTR as an inducer of the IGF-I receptor pathway in the CNS. In that study, TTR KO mice presented decreased levels of IGF-I receptor, while, in cultured hippocampal neurons, exogenous TTR was shown to regulate IGF-I receptor expression [42]. These observations reinforce the notion that TTR may have a pivotal role in metabolic regulation in different cell types and in maintaining cellular health and function.

Impact on neuronal health and function

TTR is expressed in different types of cells of the CNS [17-19] and of the PNS [15,16]. Additionally, TTR has been shown to have other roles in the brain, independently of its role as a transporter, namely, being neuroprotective [26,45,46]. Moreover, in the PNS, accumulation of pathological forms of TTR cause FAP [47], and more importantly, soluble physiological TTR is present in the PNS and is associated with increased nerve regeneration [48]. Hence, together, these observations both in the CNS and PNS are suggestive of a role for TTR in neuronal biology. In this section, we will explore the most recent reports on TTR function in neuronal health, both in the CNS and PNS.

Neuroprotection in CNS

As described in the previous sections, in the brain, besides being expressed in the epithelial cells of the choroid plexus, TTR is also expressed in hippocampal, cortical,

motor and cerebellar granule neurons [17-19]. Besides being an hormone transporter in the brain, TTR has also been shown to have other independent roles, namely in pathological conditions, such as neuroprotection in Alzheimer´s Disease (AD) [25,26,45] and in cerebral ischemia [46,49]. Moreover, TTR is decreased in the CSF of amyotrophic lateral sclerosis (ALS) patients, and its expression decreased in the degenerating motor neurons [50], suggesting a role of TTR in ALS pathology.

The molecular mechanisms by which TTR exerts its neuroprotective effects in CNS are not yet fully, however, new reports elucidating these mechanisms are emerging. TTR was found to be determinant for hippocampal neuronal survival and neurite outgrowth and preservation, in *in vitro* excitotoxic conditions, and in a mouse model of permanent middle cerebral artery occlusion (pMCAO), a model of cerebral ischemia [46]. In support of this, in hippocampal neurons, TTR was found to enhance neurite survival and outgrowth by interacting with the receptor megalin and activating MAP kinase, ERK, Akt, and Src, ultimately leading to the upregulation of the CREB transcription factor, favouring the expression of anti-apoptotic Bcl2 protein family members [46]. TTR was also shown to increase intracellular calcium through NMDA receptors, in a Src/megalin-dependent manner, which the authors suggest that, by itself, might explain the ability of TTR to enhance neurite outgrowth [46].

Besides binding to megalin, TTR also interact with the IGF-I receptor and regulates this pathway in hippocampal neurons, as described in a previous section [42]. The IGF-I receptor pathway is involved in neurogenesis, neurodegeneration and neuroprotection [51]. As hippocampal neurogenesis decline with age, so does IGF-I signalling, whereas restoration of IGF-I levels improves not only hippocampal neurogenesis but also cognition [52]. Moreover, IGF-I was found to be altered in different neurodegenerative disorders, such as AD [53] and PD [54]. Other studies also show a neuroprotective role for IGF-I in ALS, where restoring IGF-I levels ameliorates disease progression [51]. At the cellular level, IGF-I receptor controls the activation of two downstream pathways, the MAPK/Ras-Raf-Erk and PI3K/Akt/mTor pathways, which control various cellular responses such as differentiation, proliferation, and protection from apoptosis [44]. In neurons, in particular, the IGF-I pathway controls neuronal plasticity, synaptic activity and neurite outgrowth [55,56]. The observation that TTR binds IGF-I receptor and regulates this pathway [42] may, in part, explain the neuroprotective effects described for TTR.

Recently, another important receptor in the CNS, the GABA (γ-aminobutyric acid) A receptor ($GABA_A$-R), was described to be regulated by TTR [19]. GABA receptors play a critical role in mediating neural network activity, in particular, in inhibiting neural activity [57]. Deficits in $GABA_A$-R-mediated neurotransmission are involved in epilepsy, anxiety,

depression, schizophrenia, and autism [58]. Using yeast two hybrid screening, TTR was identified as an interacting partner of the $GABA_A$ receptors' δ subunit (δ-$GABA_A$-Rs). More importantly, TTR was found to interact with δ-$GABA_A$-Rs in primary cerebellar granule neurons, in primary cortical neurons, and in mouse cerebellum extracts. In the same study, TTR was described to regulate δ-$GABA_A$-Rs, as TTR knockdown in cultured cerebellar granule neurons significantly decreased the receptor expression, whereas overexpressing TTR in cortical neurons increased it. *In vivo* analysis using TTR knockdown mice revealed a significant decrease in surface expression of δ-$GABA_A$-Rs in cerebellar granule neurons, confirming TTR as a regulator of these receptors in the brain [19]. This study uncovered the possible role of TTR in mediating neuronal activity through δ-$GABA_A$-Rs, which can contribute to the neuroprotective role described for TTR.

TTR impact on nerve biology

TTR is well-known for its association with familial amyloidotic polyneuropathy, as pathological aggregated TTR accumulates in the peripheral nerves causing neurodegeneration. However, in physiological conditions, TTR is also present in the peripheral nervous system (PNS). Originally, it was found in the nerve endoneurial fluid, and was proposed to gain access to the PNS through the peripheral blood, crossing the blood-nerve barrier (BNB). Another potential source of TTR in the PNS is the choroid plexus, due to the communication between the subarachnoid and endoneurial spaces [59]. The expression of TTR by the dorsal root ganglia (DRG) is a controversial topic, as Sousa *et al.* showed that TTR is not expressed in DRG [60], while Murakami, *et al*, reported that TTR is expressed in the glia satellite cells of the DRG [16] and in the Schwann cells [15]. Regardless of its local or systemic origin, TTR was described years ago, as neuroprotective in the PNS, enhancing nerve regeneration [48]. This ability was linked to a neuritogenic activity of TTR in DRG neurons [48] via its interaction with megalin [61], similarly to what was observed in the hippocampal neurons [46]. Moreover, TTR KO mice present sensorimotor impairment [48] and DRG from TTR KO mice show compromised retrograde transport, which can account for the decrease in neurite length [61]. Thus, in the PNS, just like in CNS, TTR is neuroprotective by maintaining neurite health.

TTR aggregation and cellular toxicity

TTR destabilization due to single amino acid mutations in the TTR gene precipitates misfolding and aggregation, which leads to TTR amyloid deposition and tissue dysfunction. TTR amyloidosis is a systemic disorder that can result in polyneuropathy,

autonomic neuropathy, or cardiomyopathy, and often a mixture of these clinical manifestations [47]. Ageing is associated with increased protein aggregation [62], and TTR is no exception to this rule: WT TTR can destabilize and aggregate, particularly in the elderly, causing non-familial cases of TTR amyloidosis [20]. Aggregated TTR has vastly been associated with cytotoxicity and cellular degeneration [47]. The mechanism by which extracellular TTR modulates intracellular molecular pathways is not yet fully understood, but TTR was shown to bind to receptors such as the Receptor for Advanced Glycation End Products (RAGE) [63]. Recently, it has been suggested that TTR aggregates can be internalized for degradation [64]. Regardless of how TTR activates intracellular pathways, it is well established that in FAP, extracellular aggregated TTR induces endoplasmatic reticulum stress [65], proteosome dysfunction [66], apoptosis [67], inflammation [68,69] and oxidative stress [70-72].

FAP is characterized by a dying-back axonal degeneration, which might suggest a disturbance of the distal cytoskeleton as consequence of the TTR deposition. Interestingly, a study using a FAP Drosophila model expressing TTRV30M in the fly retina, reported a novel genetic interaction between TTRV30M and members of Rho GTPase-regulated pathways, well known regulators of actin cytoskeleton. The authors have shown that downregulation of Rac1/2, Cdc42, Pak and LIMK suppress TTRV30M-induced axonal defects on the retina of the flies [73]. All these proteins are major players in actin dynamics and implicated in several other neurodegenerative disorders [74], which raised the possibility that Rho GTPase and the actin cytoskeleton are involved in FAP pathology.

The actin cytoskeleton is closely associated with endocytosis, with actin dynamics being critical to remodel the cell surface and vesicular movement [75]. Noticeably, aggregated TTR was found to disrupt the endocytic transport within the cell, as the presence of aggregated TTR decreases transferrin endocytosis in human carcinoma cells and primary liver cells [76]. This observation also adds to the idea of a possible link between TTR aggregation and disturbance in the actin cytoskeleton. In the future, it would be important to clarify if TTR aggregates induce a disturbance in neuronal actin regulators and in cytoskeleton dynamics, and whether that cytoskeleton disturbance leads to axonal defects.

The deregulation of calcium homeostasis has also been associated to FAP pathology, as the amyloidogenic mutant form of TTR, L55P was shown to induce calcium influx into the growth cones of DRG neurons, whereas WT TTR had no significant effect. [77]. In another study, performed in cardiomyocytes, aggregated TTR was shown to cause cytotoxicity by deregulating cytoplasmic Ca^{2+} levels, modifying mitochondrial potential and increasing oxidative stress, leading to the prolongation of the action potential, which may contribute

to the development of cellular arrhythmias and conduction alterations often seen in patients with cardiac TTR amyloidosis [78]. The TTR-induced alterations of calcium levels within cells and consequent cytotoxicity may also be a major contributor to the pathophysiology associated with TTR aggregates.

The role of Schwann cells, the PNS glia cells, in FAP has been the focus of recent studies. In different neurodegenerative disorders, the glia, has been described to have a role in inflammation and in the clearance of aggregates. Thus, in FAP, where extracellular aggregates of TTR cause pathology and inflammation was described, is logic to propose that glia may also have role in this disease. As such, the impact of TTR aggregates was analysed in Schwannoma cells, and TTR was found to stimulate ROS production and to decrease the levels of endogenous antioxidants, decreasing the overall cellular antioxidant capacity [70]. Additionally, PNS glia (satellite cells and Schwann cells) have been reported uptake and clear extracellular TTR aggregates, via lysosomal degradation, in a FAP mouse model and in PNS human tissue [79]. Corroborating this idea, TTR aggregates were found in the cytoplasm of Schwann cells and satellite cells of TTR transgenic mice carrying the human Met30 *TTR* gene [15]. The effect of TTR in Schwann cells health and function surely also have an impact on neuronal health and survival, as glia cells support neurons and maintain their environment. To address this, conditioned media from Schwann cell bearing mutant aggregation-prone TTR was shown to inhibit neurite outgrowth of DRG neurons [15], suggesting that Schwann cells have a critical role in the neurodegeneration of sensitive neurons. Another study, supports this idea as sural nerve biopsy specimens from patients with FAP (TTR Val30Met mutation) were shown to have disrupted blood-nerve barrier, with loss of the tight junctions and the fenestration of endothelial cells and atrophy of Schwann cells [80]. Together, the accumulation of aggregated TTR in Schwann cells, the disruption of clearance mechanisms, and the cytotoxicity triggered in Schwann cells are probably major contributors to the neurodegeneration of sensitive neurons observed in TTR amyloidosis patients.

Discussion

The physiological function of TTR has been addressed for many researchers along the years, and led to the development of the TTR KO mouse model [81]. These mice are viable, fertile, phenotypically normal, and have a normal life span, suggesting that TTR is not essential for the organism to survive. However, it is clear that the physiological functions of TTR are yet to be fully understood, as reports of different roles for TTR keep

emerging. In the previous sections, we gathered the most recent reports on new functions attributed to TTR, specifically its effect on cellular health and function. Next, we will discuss the implications of these new roles for human pathology.

Impact in Cancer

TTR has been recently described as a biomarker for lung [82] and ovary [83] cancer, as its levels are elevated in the serum of patients. The biological connection between these types of cancer and TTR is not yet understood, as it is not clear whether the changes in TTR levels are a cause or simply a consequence of the cancer itself. However, lung cancer cells are reported to express TTR, in contrast to normal lung cells [84]. Moreover, TTR was found to regulate proliferation of endothelial and myeloid cells in lung tumours via the AKT pathway and to control immune cell function by modulating myeloid cell differentiation [38]. Since immune regulation and the activation of the AKT/proliferation pathways are pivotal in cancer progression, TTR may be an important molecule to take in consideration in cancer research, and further studies are needed to broaden the knowledge of TTR role in cancer.

TTR and diseases of the CNS

TTR has been associated to the preservation of the health and function of neuronal cells of the CNS. In hippocampal neurons TTR regulates the expression and interacts with both the IGF-I receptor [42], which is involved in neuronal plasticity, synaptic activity, and neurite outgrowth [55], and megalin [46], which controls neurite outgrowth and synaptic plasticity [85]. Additionally, TTR was found to modulate GABA receptors, [19], which are critical to regulate neuronal activity [57]. This role in regulating receptors that contribute for neuronal health and function can explain the neuroprotective role of TTR in neurodegenerative diseases, such as AD [26,46]. Interestingly, in TTR KO mice, the absence of TTR accelerates memory deficits usually associated with ageing [86], and aged memory-impaired rats present decreased TTR expression [35]. The hippocampus plays a crucial role in the formation of memories, and the fact that TTR absence is linked to memory impairment and that TTR can bind to receptors in the hippocampal neurons that regulate neuronal function, makes TTR a molecule potentially crucial for diseases that involve memory decline, such as AD and other types of dementia. Additionally, novel reports indicate that TTR regulates astrocyte metabolism and energy production, by stimulating glycolysis in astrocytes [41]. Knowing that astrocytic degradation of glycogen is a prerequisite for neuron plasticity and memory formation [87], this also supports the

idea of TTR being an important molecule for neuronal function/plasticity and memory formation.

Furthermore, the recent observation that TTR potentiate oligodendrogenesis leading to a hypermyelination phenotype in TTR null mice [29] may be of great importance to understand the signalling cascades that lead to demyelinating diseases and have implications for future strategies for the development of therapeutics for diseases of demyelination, such as multiple sclerosis. Additionally, since TTR null mice present delayed CNS development [34] and memory impairment [35,86] it would be interesting to understand how hypermyelination relates to these phenotype alterations seen in the TTR null mice.

TTR and diseases of the PNS

TTR is involved in a peripheral neuropathy, FAP. A disease characterized by a preferential deposition of aggregated TTR in the peripheral nervous system (PNS) of the patients, leading to cytotoxicity and axonal loss. Along the years, studies focused on how TTR aggregates are cytotoxic for neuronal cells [47], but, as described in the previous sections another hypothesis has recently been proposed, namely, that Schwann cell damage and dysfunction leads to the disease [15]. Since Schwann cells are dysfunctional due to the presence of aggregated TTR, and neuron-glia interactions are critical for maintaining neuronal function is reasonable to propose that Schwann cells are contributors for the neurodegeneration. Still, it is probably the combination of both the direct damage to the peripheral neurons [47] and the damage to the peripheral glia [15] and consequent impact in glia-neuron interactions, the drivers for the TTR aggregation associated pathology.

As FAP is a dying-back axonal degenerative disease, it is possible that the disturbance of the distal cytoskeleton is involved in the pathology. The idea of a link between TTR and actin cytoskeleton comes from the evidence that TTR interacts with regulators of the actin cytoskeleton [73] and the fact that TTR disrupts the endocytic transport pathway [61,76], which is closely interconnected with the actin cytoskeleton [75]. Whether, TTR aggregates induce disturbances in neuronal actin regulators and in cytoskeleton dynamics in the distal axons is still unknown. But, as cytoskeleton disturbances would be an early event that precedes the degeneration of distal axons in peripheral nerves, this would be a new pathological pathway with great potential to be a therapeutic target for FAP.

Additionally, is important to mention here the physiological role of TTR in neurite outgrowth [61,48] and axonal transport in the PNS, as the mutated and consequent

instable TTR, can undergo loss of function, impairing neuronal health in the PNS, contributing for the pathology observed in FAP.

In this review, we summarized the novel evidences of TTR impact on cell biology, hoping to contribute to a better knowledge of the pathophysiological roles of TTR within cells (Fig.1). Understanding both the TTR-associated pathology and the biological role of TTR at the cellular level is of great importance to find new therapies for TTR-related diseases, to use TTR as a protector of cell health in other disease and to anticipate consequences of TTR targeting therapies for FAP and other diseases.

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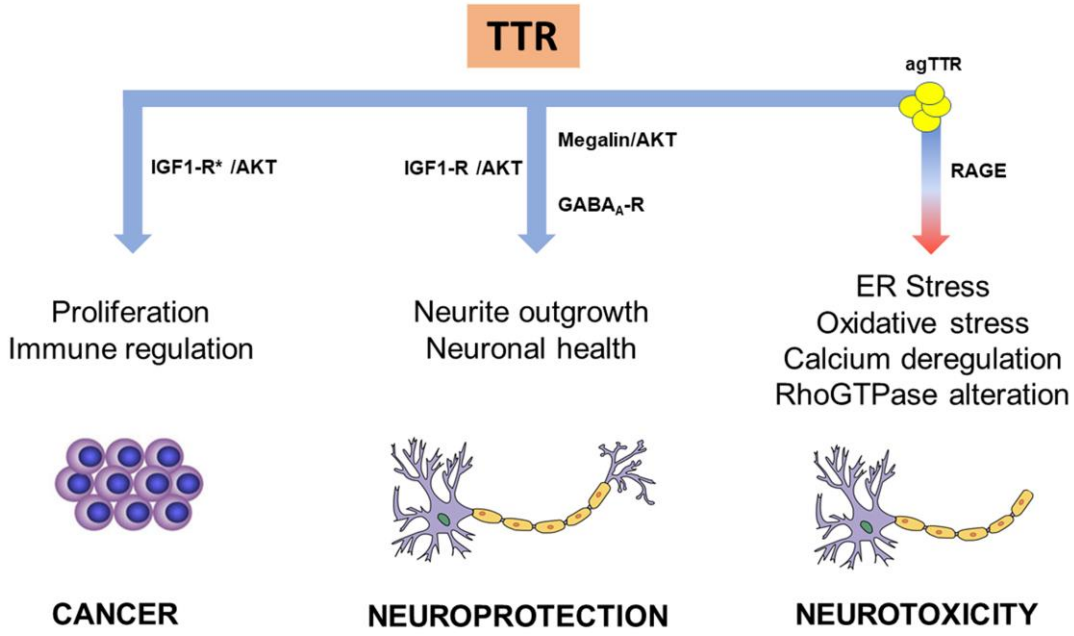

Figure 1. Summary of the identified impact of TTR in cells. TTR was described to regulate proliferation and immune cells, via AKT, having a potential impact in cancer; TTR controls neuronal outgrowth in PNS and CNS and neuronal function in CNS, having a neuroprotective role. Nevertheless, TTR also induces neurotoxicity when it aggregates (agTTR), causing ER stress, oxidative stress, calcium deregulation and Rho GTPases alterations. The potential (*) and the known TTR receptors are described in the figure; however other receptors might be involved in the pathways impacted by extracellular TTR.

RESEARCH AIMS

The main focus of this thesis was to unravel the cellular and molecular mechanisms underlying the dual role of TTR in nerve biology. In order to achieve this purpose, a number of studies were performed in order to:

- In **Chapter I**, dissect the impact of TTR proteolytic activity in Aβ fibrillogenesis inhibition, Aβ fibril cleavage, and Aβ induced toxicity in neurons;

- In **Chapter II**, determine whether physiological TTR promotes axon elongation and regeneration by modulating MT dynamics;

- In **Chapter III**, elucidate the mechanisms behind neuronal death in FAP proposing cytoskeletal components as main targets for TTR-induced neurodegeneration;

- In **Chapter IV**, identify molecular mechanisms underlying TTR impact on the neuronal cytoskeleton using a quantitative proteomics assay.

CHAPTER I

Transthyretin neuroprotection in Alzheimer's disease is dependent on proteolysis

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ABSTRACT

The deposition of amyloid β peptide (Aβ) in the hippocampus is one of the major hallmarks of Alzheimer's disease (AD), a neurodegenerative disorder characterized by memory loss and cognitive impairment. The modulation of Aβ levels in the brain results from an equilibrium between its production from the amyloid precursor protein and removal by amyloid clearance proteins, which might occur via enzymatic (Aβ-degrading enzymes) or nonenzymatic (binding/transport proteins) reactions. Transthyretin (TTR) is one of the major Aβ-binding proteins acting as a neuroprotector in AD. In addition, TTR cleaves Aβ peptide *in vitro*. In this work, we show that proteolytically active TTR, and not the inactive form of the protein, impacts on Aβ fibrillogenesis, degrades neuronal-secreted Aβ, and reduces Aβ induced toxicity in hippocampal neurons. Our data demonstrate that TTR proteolytic activity is required for the neuroprotective effect of the protein constituting a putative novel therapeutic target for AD.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive memory deficits and cognitive decline (Blennow, de Leon, & Zetterberg, 2006). The 2 major hallmarks of AD are the intraneuronal presence of neurofibrillary tangles constituted by tau and the extracellular accumulation of senile plaques consisting of amyloid-β peptide (Aβ) (Kosik, Joachim, & Selkoe, 1986). Aβ results from the cleavage of the amyloid precursor protein (APP) by β- and γ-secretases (De Strooper, Vassar, & Golde, 2010). Lowering Aβ levels is a major therapeutic goal in AD, which might be achieved by interfering with the production, aggregation, or degradation of the peptide. Proteases have an important role in controlling Aβ homeostasis, and several Aβ-degrading enzymes were described, among them neprilysin and insulin-degrading enzymes have a well-established role in the regulation of Aβ levels (Iwata et al., 2000; Kurochkin & Goto, 1994).

Transthyretin (TTR), a protein with metalloprotease activity (Liz et al., 2012), was shown to cleave Aβ *in vitro* (Costa, Ferreira-da-Silva, Saraiva, & Cardoso, 2008). TTR has been characterized as a neuroprotective protein in AD as: (1) TTR levels are decreased in the cerebrospinal fluid (CSF) of AD patients (Riisoen, 1988); (2) overexpressing human TTR wild type (WT) in an AD mouse model normalizes cognition and memory, and diminishes neuropathology and Aβ deposition (Buxbaum et al., 2008); and (3) *in vitro*, TTR reduces Aβ fibrillization (Costa, Goncalves, Saraiva, & Cardoso, 2008). TTR neuroprotection in AD has been mainly attributed to its ability to bind Aβ (Schwarzman et al., 1994), but the relevance of Aβ cleavage by TTR remains unknown.

We have characterized the TTR active site as a triad formed by the residues His88, His90, and Glu92 that bind to a catalytic zinc ion and by Glu72, which acts as the general base (Liz et al., 2012). The identification of TTR catalytic residues enables assessing TTR devoid of proteolytic activity by mutation of the above-mentioned residues, being therefore a powerful tool to study the relevance of TTR proteolysis. As such, in this work, we aimed to understand the impact of TTR proteolytic activity in AD by comparing the effect of TTR WT and TTR proteolytically inactive on Aβ aggregation, degradation, and neurotoxicity.

RESULTS

THE PROTEOLYTICALLY INACTIVE TTR MUTANT H90A HAS NO IMPACT ON Aβ FIBRILLIZATION

Mutation of the catalytic residues of TTR creates the possibility to characterize the relevance of TTR proteolysis. We previously demonstrated that TTR active site is constituted by His88, His90, and Glu92, and mutation of any of these 3 residues leads to inactive TTR (Liz et al., 2012). In this work, we assessed the effect of proteolytically competent TTR WT and the proteolytically inactive mutant TTR H90A in Aβ fibrillization, degradation and toxicity. To confirm the proteolytic activity of our TTR preparations towards Aβ, we assessed the cleavage of the fluorogenic peptide Abz-VHHQKL-EDDnp, which includes a 6-aminoacid sequence of the Aβ peptide containing one of the TTR cleavage sites previously described (Costa et al., 2008a). As expected TTR WT, but not TTR H90A, was able to cleave the fluorogenic peptide (Fig. 1A). In addition, we investigated whether TTR H90A binds Aβ; we performed TTR immunoprecipitation in reactions of Aβ incubated with either TTR WT or TTR H90A and observed that both variants coimmunoprecipitated with Aβ (Fig. 1B). This result shows that the comparison of the effect of TTR WT and TTR H90A on Aβ clearance is related with proteolysis and not to the ability to bind the peptide.

Previous studies demonstrated that TTR impacts on Aβ fibrillization. To ascertain whether this effect was related with TTR cleavage of Aβ, we compared the fibrillization of the peptide in the presence of either TTR WT or TTR H90A. Initially, we performed the ultrastructural analysis of soluble Aβ co-incubated with either TTR WT or TTR H90A in fibril formation conditions. Sample analysis by TEM revealed less abundant and shorter Aβ fibrils formed in the presence of TTR WT when compared with nontreated peptide, whereas TTR H90A did not affect Aβ fibril formation (Fig. 1C). We followed by analysing the preparations of Aβ-preformed fibrils where TTR, either WT or H90A, was added and further incubated at 37°C. We observed that while TTR WT was able to disrupt Aβ fibrils, TTR H90A had no effect (Fig. 1D). In both cases, the effect of TTR WT impacted on the length and number of fibrils, while their diameter was not altered. These results demonstrate that the effect of TTR on inhibition of Aβ fibril formation and on disruption of Aβ formed fibrils is dependent of TTR proteolysis.

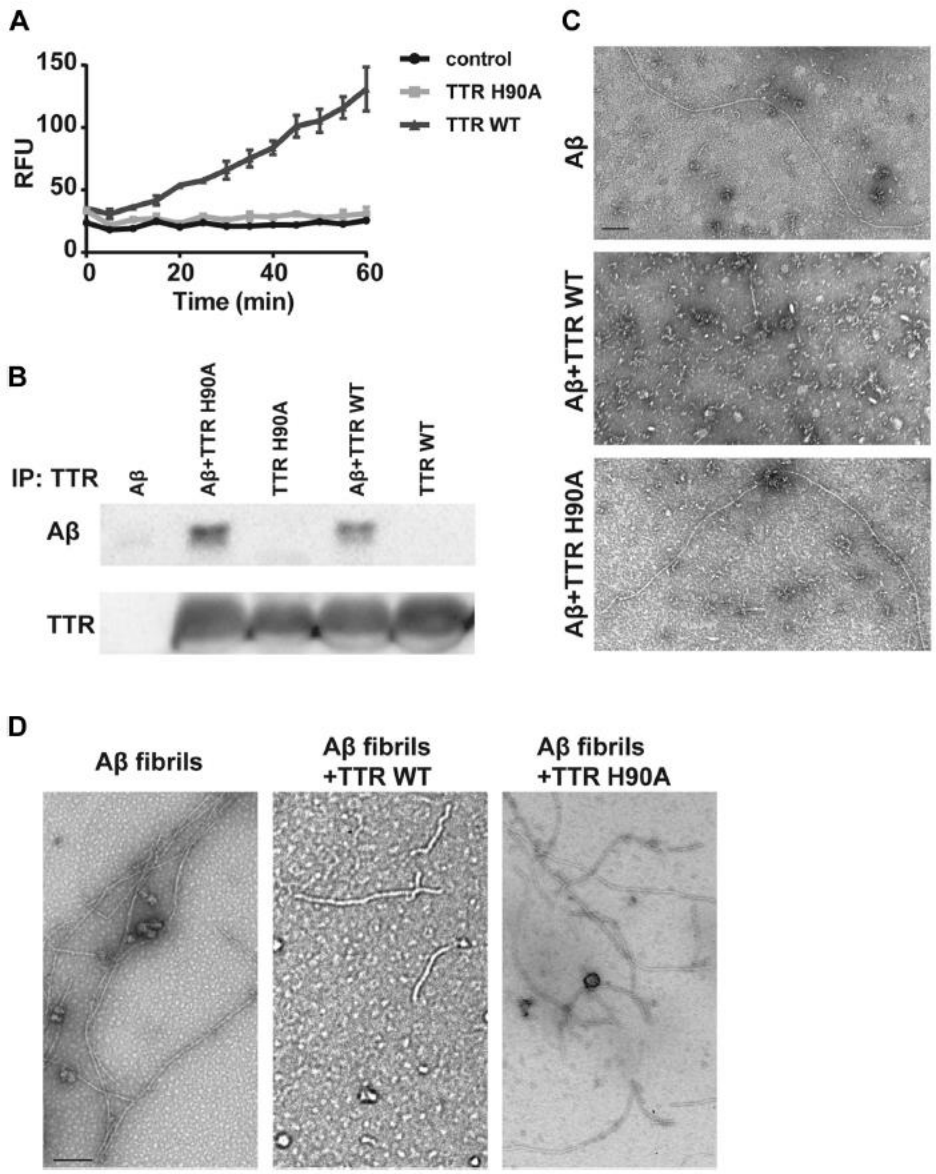

Figure 1. The proteolytically inactive TTR mutant H90A has no impact on Aβ fibrillization. (A) Hydrolysis of the fluorogenic peptide Abz-VHHQKL-EDDnp by TTR. The fluorescence of the peptide was measured in the absence (control) or in the presence of either TTR WT or TTR H90A. **(B)** Coimmunoprecipitation of TTR and Aβ. Aβ peptide was incubated without (Aβ) or with either TTR WT (Aβ+TTR WT) or TTR H90A (Aβ+TTR H90A), and the reactions were immunoprecipitated with an antibody against TTR. Control reactions with only TTR WT or TTR H90A were performed. Coimmunoprecipitations were detected by Western blot using anti-Aβ and anti-TTR antibodies. (**C)** TTR proteolysis inhibits Aβ fibril formation. Aβ peptide was incubated without (Aβ) or with either TTR WT (Aβ+TTR WT) or TTR H90A (Aβ+TTR H90A) and analysed by TEM at day 6 of incubation. **(D)** TTR proteolysis leads to a disruption of Aβ fibrils. Aβ-formed fibrils (Aβ fibrils) were incubated with either TTR WT (Aβ fibrils + TTR WT) or TTR H90A (Aβ fibrils + TTR H90A) and analysed by TEM at day 4 of incubation. Scale bar = 100 nm. Abbreviations: Aβ, amyloid β peptide; TEM, transmission electron microscopy; TTR, transthyretin.

TTR CLEAVAGE OF Aβ OCCURS IN CELL-BASED ASSAYS AND PROTECTS AGAINST Aβ-INDUCED NEUROTOXICITY

TTR proteolysis of Aβ was only characterized in *in vitro* experiments based on the incubation of the two proteins at the reported optimal conditions for TTR proteolytic activity (Costa et al., 2008a; Liz et al., 2004). To investigate if this effect was maintained in cellular assays, 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 a 2.4-fold reduction in Aβ levels as determined by ELISA of the cell supernatants (Fig. 2A). Addition of TTR H90A had no effect on Aβ levels when compared to control cells (Fig. 2A). To discard the hypothesis that TTR could be affecting the cell secretion of Aβ, we collected the cell supernatants from the control condition and then incubated with either TTR WT or TTR H90A; similar results to the ones obtained in cell culture were obtained showing that the decreased Aβ levels after treatment with TTR WT are related with proteolysis (Fig. 2A; cell-free assay). These results show that TTR proteolytic activity is maintained in cell-based assays and that TTR cleaves neuronal-secreted Aβ.

We followed by analysing whether TTR proteolysis impacts on Aβ-induced neurotoxicity. Hippocampal neurons have been widely used as a cellular model of Aβ toxicity, since these neurons are the most affected in AD. To evaluate the effect of TTR proteolytic activity on neuronal cell death induced by Aβ oligomers, primary cultures of mouse hippocampal neurons were incubated with 10 mM Aβ oligomers alone or plus either TTR WT or TTR H90A. Aβ-induced cell death was examined with the caspase 3 assay. After 24 hours of treatment, Aβ oligomers induced neurotoxicity as demonstrated by the 3.5-fold increased levels of caspase 3 activity (Fig. 2B, Table S1). TTR WT was able to decrease the toxic effect induced by Aβ oligomers by 1.4-fold (Fig. 2B, Table S1). However, the TTR-induced neuroprotective effect was not reproduced with the addition of TTR H90A, which did not alter the levels of caspase 3 induced by Aβ oligomers (Fig. 2B, Table S1). Treatment of cells with either TTR WT or TTR H90A (in the absence of Aβ) promoted an increase in caspase 3 levels when compared to the control, although less pronounced than the one of Aβ oligomers (Fig. 2B, Table S1). The increase in caspase 3 levels was similar between TTR WT and TTR H90A and might result from some aggregation of TTR in culture. This effect of TTR on caspase 3 activation might explain the fact that TTR WT did not totally rescue the effect of Aβ-induced neurotoxicity. We observed a decrease in the caspase 3 levels in the condition of cells treated with Aβ oligomers plus TTR WT when compared to cells treated with only Aβ oligomers; however,

the levels did not return to the ones of untreated cells, what probably results from TTR WT toxicity. In addition, in the condition of Aβ oligomers plus TTR H90A, a tendency (not statistically different, Table S1) for an increase in caspase 3 levels when compared to cells treated with only Aβ oligomers was observed, which might be explained by a cumulative effect of the toxicity of Aβ and of TTR H90A. Nevertheless, these results show that only proteolytically active TTR is able to reduce Aβ-induced cell death. 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 degradation and decreasing neurotoxicity.

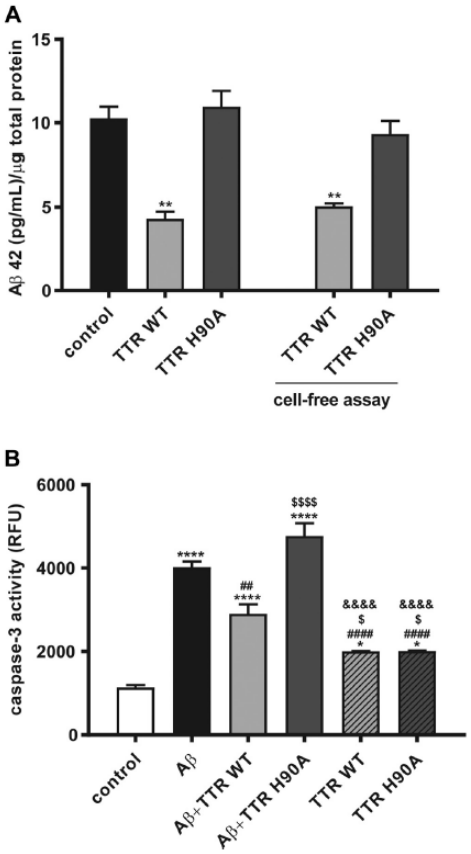

Figure 2. TTR cleavage of Aβ occurs in cell-based assays and protects against Aβ-induced neurotoxicity. (A) TTR degrades neuronal-secreted Aβ. N2A-APPswe cells were untreated (control) or treated with either TTR WT or TTR H90A, and cell supernatants were assessed for the levels of Aβ peptide by ELISA. In addition, ELISA was performed on cell supernatants from the control condition treated with either TTR WT or TTR H90A (cell-free assay). **(B)** TTR WT decreases Aβ-induced toxicity in hippocampal neurons. Hippocampal neurons (DIV = 5) were untreated (control) or treated with Aβ, Aβ plus TTR WT (Aβ+TTR WT) or TTR H90A (Aβ+TTR H90A), and TTR WT or TTR H90A. Subsequently, caspase 3 assay was performed on cell lysates. *Comparison with control, $^{\#}$comparison with Ab, $^{\$}$comparison with Aβ+TTR WT, $^{\&}$comparison with Aβ+TTR H90A). *$p < 0.05$. $^{\$}p < 0.05$. **$p < 0.01$. $^{\#\#}p < 0.01$. ****$p < 0.0001$. $^{\#\#\#\#}p < 0.0001$. $^{\$\$\$\$}p < 0.0001$. $^{\&\&\&\&}p < 0.0001$. Abbreviations: Aβ, amyloid β peptide; ELISA, enzyme-linked immunosorbent assay; TTR, transthyretin.

Discussion

TTR is mainly recognized as the transporter for thyroxin and retinol but is also a metalloprotease (Liz et al., 2012). In this respect, TTR was shown to cleave the Aβ peptide *in vitro* (Costa et al., 2008a), raising the unexplored hypothesis that TTR proteolysis could underlie TTR neuroprotective effect in AD. In this work, by using proteolytically competent versus proteolytically inactive TTR, we demonstrated that TTR cleavage of Aβ leads to inhibition and disruption of Aβ fibril formation. Moreover, using cell-based assays, which confirmed TTR cleavage of Aβ in physiological conditions, we showed that TTR proteolytic activity led to a reduction of Aβ levels and inhibition of Aβ-induced cell death. Our results clearly confirm TTR cleavage of Aβ and demonstrate that proteolysis is required for TTR neuroprotection in AD.

TTR was identified as a major Aβ-binding protein, and this function was suggested to underlie TTR neuroprotective effect as the protein could sequester Aβ preventing its aggregation and fibril formation (Schwarzman et al.,1994). In this work, we demonstrated that TTR proteolytic activity is required for the neuroprotective effect of the protein. However, we cannot state whether TTR binding to Aβ and TTR cleavage of Aβ are dependent or independent processes in TTR neuroprotection. In this respect, different TTR stabilizers were shown to have a distinct effect on TTR-Aβ binding and on TTR cleavage of Aβ (Ribeiro et al., 2012). The TTR stabilizer iododiflunisal was shown to increase TTR-Aβ interaction *in vitro*, and when administrated *in vivo* in a mouse model of AD led to a decrease in Aβ deposition and an improvement on cognitive function, although without an effect on TTR proteolytic activity (Ribeiro et al., 2012, 2014). In the future, it would be interesting to test *in vivo* compounds that increase TTR cleavage of Aβ.

Therapeutic strategies targeting Aβ degradation, by delivery of Aβ-degrading enzymes to the brain, are emerging as a valuable option for treating AD. One example is the case of neprilysin, as the intracerebral injection of this recombinant protein into AD mice led to a reduction of Aβ accumulation and amelioration of cognitive function (Park et al., 2013). In the case of TTR, it was demonstrated that overexpression of a WT human TTR transgene was ameliorative in the APP23 murine model of human AD (Buxbaum et al., 2008). Future work will assess the effect of the intracerebral injection of either TTR WT or TTR proteolytically inactive to validate our results *in vivo* and to demonstrate whether TTR proteolytic activity is therapeutically relevant in AD.

MATERIALS AND METHODS

TTR production, purification, and labelling. Recombinant TTR WT and TTR H90A (proteolytic inactive mutant) (Liz et al., 2012) were produced in BL-21 pLys *Escherichia coli* cells transformed with pETF1 carrying TTR complementary DNA (Goldsteins et al., 1997). Proteins were isolated and purified as previously described (Liz, Faro, Saraiva, & Sousa, 2004). Briefly, after bacterial lysis, protein extracts were run on DEAE-cellulose (Whatman) ion-exchange chromatography, and TTR was subsequently isolated in native Prosieve agarose (Lonza) gels. For cellular assays, proteins were detoxified using a high-capacity endotoxin removal resin (Thermo Scientific) and quantified using Lowry based DC Protein Assay (Bio-Rad Laboratories), following the manufacturer's protocols.

TTR proteolysis assay. TTR proteolytic activity was tested with the fluorogenic peptide Abz-VHHQKL-EDDnp (Genscript). TTR (5 μM total protein) was added to the substrate (5 μM) in a final volume of 100 μl of reaction buffer (50 mM Tris/HCl, pH 7.5). Hydrolysis of the peptide at 37°C was followed by measuring the fluorescence at λ_{em} = 420 nm and λ_{ex} = 320 nm in an F_{max} plate reader (Molecular Devices) for 60 minutes.

Production of Aβ species. Human Aβ peptide (1-42; Genscript) was dissolved in hexafluoroisopropanol (HFIP, Sigma) and incubated overnight at room temperature (RT). The hexafluoroisopropanol was removed under a stream of nitrogen, and the remaining residue was dissolved in dimethyl sulfoxide (DMSO, Sigma) at 2 mM. For oligomer formation, Aβ peptide was diluted to 100 μM in F12 medium (Lonza) and incubated for 5 days at 4°C. For fibril formation, Aβ (100 μM in F12 medium) was incubated at 37°C for 8 days. The formation of Aβ oligomers and of Aβ fibrils was confirmed by transmission electron microscopy (TEM).

Coimmunoprecipitation. Soluble Aβ peptide (1 μg) was incubated without or with either TTR WT or TTR H90A (5 μg) 1 hour at 37°C. Control reactions only with TTR were performed. Subsequently, the polyclonal rabbit antihuman TTR antibody (DAKO; 28.8 μg) was added to the Aβ-TTR reactions and incubated overnight at RT. To facilitate the immunoprecipitation, an equivolume of Polyethylene glycol 8000 (PEG 8000; 2.5% in 20 mM potassium phosphate, 0.85% NaCl, pH 7.4) was added, and the mixture was incubated 72 hours at 4°C. Reactions were washed with PEG 8000 (2.5% in phosphate-buffered saline [PBS]), centrifuged and resuspended with SDS sample buffer. Samples were run in a 13.5% SDS-PAGE gel and transferred to nitrocellulose membrane

(Amersham GE Healthcare, Protan 0.2 μm). The membrane was blocked and incubated overnight at 4°C in 3% non-fat dried milk with the following antibodies: mouse anti-Aβ (BAM-10; Sigma; 1:2000) and rabbit anti-TTR (DAKO, 1:2000). Incubation with horseradish peroxidase-labelled secondary antibodies was performed for 1 hour at room temperature. Blots were developed using the Clarity Western enhanced chemiluminescence (ECL) substrate (Bio-Rad) and visualized using a chemiluminescence detection system (ChemiDoc, Bio-rad).

Effect of TTR proteolytic activity in Aβ aggregation and fibril disruption. Soluble Aβ peptide (100 μM in F12 medium) was incubated with either TTR WT or TTR H90A (10 μM) and incubated at 37°C for 6 days. Alternatively, fibrillar Aβ (prepared as described above) was incubated with 10 μM TTR, either WT or H90A, at 37°C for 4 days. Samples were analysed by TEM. Experiments were performed 3 times.

Transmission electron microscopy (TEM). For TEM analysis, 5 μl sample aliquots were absorbed to carbon-coated collodion film supported on 200-mesh copper grids, for 5 minutes, and negatively stained with 1% uranyl acetate. The grids were exhaustively visualized with a JEOL JEM-1400 transmission electron microscope equipped with an Orious Sc1000 digital camera.

Cellular assays. Neuro 2A neuroblastoma cells stably expressing human APP carrying the K670N and M671L Swedish mutation (N2A-APPswe) were kindly provided by Dr Huaxi Xu (Sanford-Burnham Medical Research Institute, CA, USA). N2A-APPswe cells were plated at 50,000 cells/well in 24-well plates and maintained in 1:1 Dulbecco's Modified Eagle Medium (Sigma)/Opti-Minimum Essential Medium (Opti-MEM; Gibco) supplemented with 5% fetal bovine serum (FBS; Gibco) and 0.4% geneticine (Gibco). When 90% confluence was reached, medium was replaced by fresh medium without FBS containing 5 μM (the physiological concentration of TTR in the plasma) of either TTR WT or TTR H90A. After 24 hours of incubation, supernatants were collected for Aβ42 enzyme-linked immunosorbent assay (ELISA). Sandwich ELISA analyses of Aβ42 were performed using Human Aβ42 ELISA Kits (Invitrogen), according to the manufacturer's instructions. In addition, supernatants collected from untreated cells were incubated with 5 μM of either TTR WT or TTR H90A for 24 hours at 37°C and subsequently analysed by ELISA. Results were normalized by the total protein present in cell lysates, quantified by Bradford.
Hippocampal neuron cultures were performed as described (Kaech and Banker, 2006). Briefly, hippocampi were isolated from E17.5 mouse embryos and subsequently digested

with 0.06% porcine trypsin solution (Sigma) for 15 minutes at 37°C, triturated, and plated at 150,000 cells/well in 24-well plates containing glass cover slips coated with 20 μg/mL poly-L-lysine (Sigma). Neurons were cultured in Neurobasal medium (Invitrogen) supplemented with 1% penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco) and B27 (Gibco). After 5 days *in vitro* (DIV5), cells were treated with 10 μM Aβ oligomers plus 5 μM of either TTR WT or TTR H90A and maintained for 24 hours at 37°C. Cells were then lysed with 25 mM HEPES pH 7.4, 2.5 mM CHAP, 2.5 mM DTT, and used for caspase 3 assays. Activation of caspase 3 was measured using Caspase 3 Fluorimetric Assay Kit (Sigma), following the manufacturer's instructions.

Statistical analysis. Data are presented as mean ± standard error of the mean. Statistical significance was determined by Tukey's multiple comparisons test (1-way analysis of variance). All statistical analyses were performed with Prism 6 (GraphPad Software, San Diego, CA, USA).

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SUPPLEMENTARY TABLE

Table 1. Statistical Analysis of data presented in Figure 2B.

Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
control vs. Aβ	Yes	****	< 0,0001
control vs. Aβ+TTR WT	Yes	****	< 0,0001
control vs. Aβ+TTR H90A	Yes	****	< 0,0001
control vs. TTR WT	Yes	*	0,0287
control vs. TTR H90A	Yes	*	0,0274
Aβ vs. Aβ+TTR WT	Yes	**	0,0047
Aβ vs. Aβ+TTR H90A	No	ns	0,0639
Aβ vs. TTR WT	Yes	****	< 0,0001
Aβ vs. TTR H90A	Yes	****	< 0,0001
Aβ+TTR WT vs. Aβ+TTR H90A	Yes	****	< 0,0001
Aβ+TTR WT vs. TTR WT	Yes	*	0,0216
Aβ+TTR WT vs. TTR H90A	Yes	*	0,0226
Aβ+TTR H90A vs. TTR WT	Yes	****	< 0,0001
Aβ+TTR H90A vs. TTR H90A	Yes	****	< 0,0001
TTR WT vs. TTR H90A	No	ns	> 0,9999

CHAPTER II

Transthyretin impact on nerve biology is linked to regulation of microtubule stability by tubulin acetylation

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ABSTRACT

Transthyretin, (TTR) a protein initially described as a transporter for thyroxin and retinol, was assigned with a function on nerve biology. TTR was shown to increase axonal growth and impact on axonal transport in peripheral neurons, which was suggested to underly its capacity to promote nerve regeneration. In growing neurons, the microtubule (MT) cytoskeleton is crucial for axonal development and is finely tuned to create the optimal environment for guidance and outgrowth. In this work we investigated the hypothesis that wild type (WT) TTR promotes axon elongation and regeneration by modulating MT dynamics. Our *in vitro* results, demonstrated that TTR neuritogenic effect correlates with an impact of the protein on MT stability by increasing dynamics in the growth cone and decreasing it in the axon shaft. Accordingly, *in vivo*, WT-Thy1-EB3-YFP mice presented an increase in MT growth rates and EB3 comet density in growing axons after sciatic nerve injury, a process not observed in TTR KO-Thy1-EB3-YFP, which can be related to the decreased regenerative capacity of these animals. Additionally, we observed that in the absence of TTR, uninjured axons presented MT dynamic instability, which we show to be mediated by decreased levels of acetylated α-tubulin. In summary, we unravelled a new role for TTR on the modulation of MT stability by regulating α-tubulin acetylation which might underly TTR function on nerve biology.

INTRODUCTION

Transthyretin (TTR) is a homotetrameric protein predominantly synthesized in the liver and the choroid plexus and acknowledged to function as a thyroxin (Woeber & Ingbar, 1968) and retinol transporter (Goodman, 1984). TTR is highly associated with neurodegeneration since, when mutated, it aggregates and deposits in the peripheral nervous system (PNS), which results in a familial form of peripheral neuropathy – Familial amyloid neuropathy (Plante-Bordeneuve & Said, 2011). Besides causing neurodegeneration, a role of TTR on nerve physiology and repair was assigned by the phenotype of TTR KO mice that were shown to display sensorimotor impairment and present decreased regenerative capacity following sciatic nerve crush when compared to WT littermates (Fleming, Saraiva, & Sousa, 2007). Importantly, this phenotype was cell autonomous as it could be rescued when TTR was locally expressed or delivered in the nerve (Fleming, Mar, Franquinho, Saraiva, & Sousa, 2009; Fleming et al., 2007). TTR contribution to nerve regeneration was further suggested by its ability to increase neurite outgrowth of dorsal root ganglia neurons (DRG) and the observation that TTR KO mice exhibit impaired retrograde axonal transport (Fleming et al., 2009). However, the cellular and molecular details underlying the neuritogenic activity of TTR remain to be deciphered.

Microtubules (MTs) have an undisputable importance on neuronal health not only by providing structural support and establishing the tracks for axonal transport, but also because the modulation of MT dynamics is essential for proper axonal growth, either during development or upon nerve regeneration. After injury, PNS axons form in the distal tip a growth cone composed by dynamic microtubules while axonal shafts present more stable MT bundles (Erturk, Hellal, Enes, & Bradke, 2007). In addition to nucleotide binding state, tubulin isoforms and the action of MT associated proteins (MAPs), tubulin post translational modifications (PTMs) are important regulators of MT dynamics (Moutin, Bosc, Peris, & Andrieux, 2020). Among several tubulin PTMs, α-tubulin acetylation, a tubulin PTM associated with stable MTs, was recently shown to play an essential role in mechanosensory neurons (Yan et al., 2018), and increasing this modification by inhibiting the enzyme responsible for deacetylation, HDAC6 was shown to promote axon regeneration by increasing the number of stable microtubules in the axon shaft (Rivieccio et al., 2009).

In this study we investigated whether TTR neuritogenic activity in DRG neurons is related to its control of microtubule dynamics. We observed that during axonal growth TTR increased MT dynamics in the distal portion of growing axons while stabilizing MTs in the axonal shaft. Additionally, under physiologic conditions TTR regulates axonal MT stability by modulation of the levels of acetylated α-tubulin.

RESULTS

NEURITOGENIC ACTIVITY OF SOLUBLE TTR ON DRG NEURONS CORRELATES WITH INCREASED MICROTUBULE DYNAMICS IN THE GROWTH CONE

TTR was previously shown to increase neurite outgrowth of DRG neurons (Fleming et al., 2007) and here we set out to determine whether its neuritogenic activity was related to an impact of TTR on MT dynamics, as this is a critical process for axonal growth. First, we confirmed TTR neuritogenic activity in our experimental conditions, by showing that WT TTR added for 12h to primary cultures of 4-week-old mouse DRG neurons, 4h after plating, promoted neurite outgrowth as determined by a significant increase in the length of the longest neurite when compared to untreated neurons (Supplementary Fig. S1A-C). We tested whether this effect correlated with regulation of MT dynamics and found that in the growth cone of DRG neurons transfected with the MT plus-tip binding protein EB3, WT TTR induced a significant increase in EB3 growth rate (Fig. 1A-C) when compared to control neurons. The increase in polymerization rate was accompanied by an increase in comet growth length and decrease in growth duration, (Fig. 1D, E). Additionally, TTR increased the density of dynamic EB3 comets (Fig. 1A, B, F). These results suggest that TTR promotes neurite outgrowth by increasing MT plus end dynamics in the growth cone of DRG neurons.

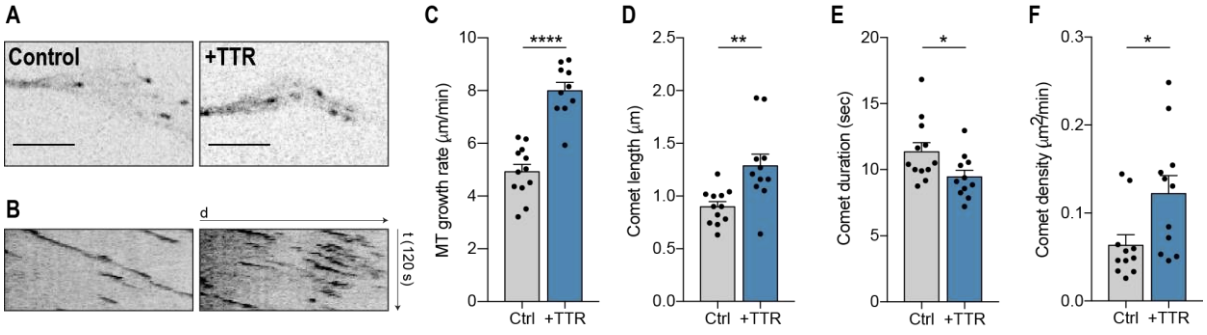


Figure 1. TTR addition to DRG neurons increases MT dynamics at the growth cone. (A, B) Representative still images of the growth cones from DRG neurons transfected with EB3-GFP either untreated (Control) or treated with WT soluble TTR (+TTR) and correspondent kymographs (B). **(C-F)** Quantifications of different MT dynamics parameters including MT growth rate (C), comet length (D), comet duration (E) and EB3 comet density (F) related to A and B. Results are plotted as mean ± SEM (n = 10-12 neurons/condition representative of 3 independent experiments). Statistical significance determined by Student's t-test: *P<0.05, **P<0.01, ****P<0.0001. Scale bars: 5 µm.

SOLUBLE TTR REDUCES DYNAMIC MT PLUS ENDS IN THE NEURITE SHAFTS OF DRG NEURONS

MT dynamicity is highly dependent on subcellular localization, especially in neurons, where MTs are not attached to the centrosome and MT plus ends are subjected to different stimuli depending upon whether their location is proximal or distal relative to the cell body. We investigated the effect of WT TTR on the dynamic state of MTs in the neurite shaft of DRG growing neurons and found that TTR had no impact on comet growth rate, comet length and in the growth duration (Fig. 2A-E). However, and in contrast to what was observed in the growth cone, TTR decreased EB3 comet density in the neurite shaft (Fig. 2F). We could not determine whether the reduction in comet density was caused by local inhibition of MT nucleation or loss of dynamic MT plus ends. Indeed, while a highly dynamic MT cytoskeleton is necessary in the growth cone for growth and orientation purposes, MTs residing in the axonal shaft need to be more stable even during axonal growth for proper maintenance of axonal transport and integrity of the axon. Altogether, our *in vitro* data correlate the promotion of neurite outgrowth induced by TTR with a dual impact on MT dynamics: an increase in MT dynamicity in the growth cone and loss of dynamic MT ends in the shaft.

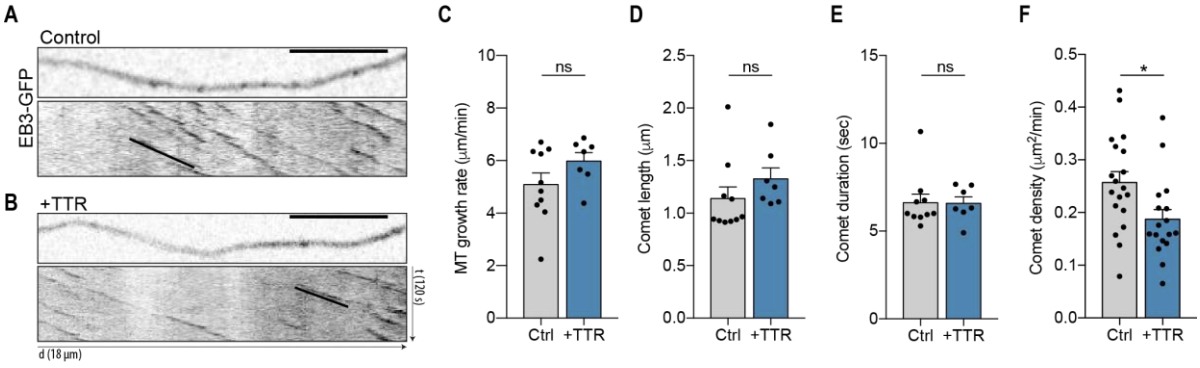


Figure 2. TTR decreases MT dynamics in the neurite the axonal shaft of DRG neurons. (A, B) Representative still images of the neurite shafts from DRG neurons transfected with EB3-GFP either untreated (Control, A) or treated with WT soluble TTR (+TTR, B) and correspondent kymographs. **(C-F)** Quantifications of different MT dynamics parameters including MT growth rate (C), comet length (D), comet duration (E) and EB3 comet density (F) related to A and B. Results are plotted as mean ± SEM (n = 7-10 neurons/condition for C, D and E; n = 17-19 neurons/condition for F, representative of 3 independent experiments). Statistical significance determined by Student's t-test: *P<0.05. ns, not significant. Scale bars: 5 µm.

TTR KO MICE FAIL TO MODULATE MT DYNAMICS IN RESPONSE TO SCIATIC NERVE CRUSH

Based on the observation that TTR modulates MT dynamics *in vitro* while inducing axon outgrowth, we investigated whether loss of TTR *in vivo* may affect MT dynamics during nerve regeneration after sciatic nerve crush (Fleming et al., 2007). For that, we analysed *in vivo* MT dynamics using TTR KO-Thy1-EB3-YFP and their control WT-Thy1-EB3-YFP littermates (crossings are detailed in the methods section). We performed sciatic nerve crush in 10 to 12-week-old mice, and at the 3rd day post injury we collected both the ipsilateral (crushed) and the contralateral (uninjured) nerves and performed *ex vivo* live imaging of EB3 comets (Fig. 3A). After crush, WT sciatic nerves, which display increased regeneration ability (Fleming et al., 2007), mounted a response to injury characterized by increased comet growth rate and density distally to the crush site, when compared with uninjured nerves (Fig. 3B-E). In the case of TTR KO mice no differences in comet growth speed or density were observed between injured and uninjured nerves (Fig. 3D, E). These results are in agreement with the increase in MT dynamics observed in the growth cones of growing neurons exposed to TTR *in vitro* (Fig.1) and demonstrate that in the absence of TTR the MT cytoskeleton fails to respond to nerve injury *in vivo*.

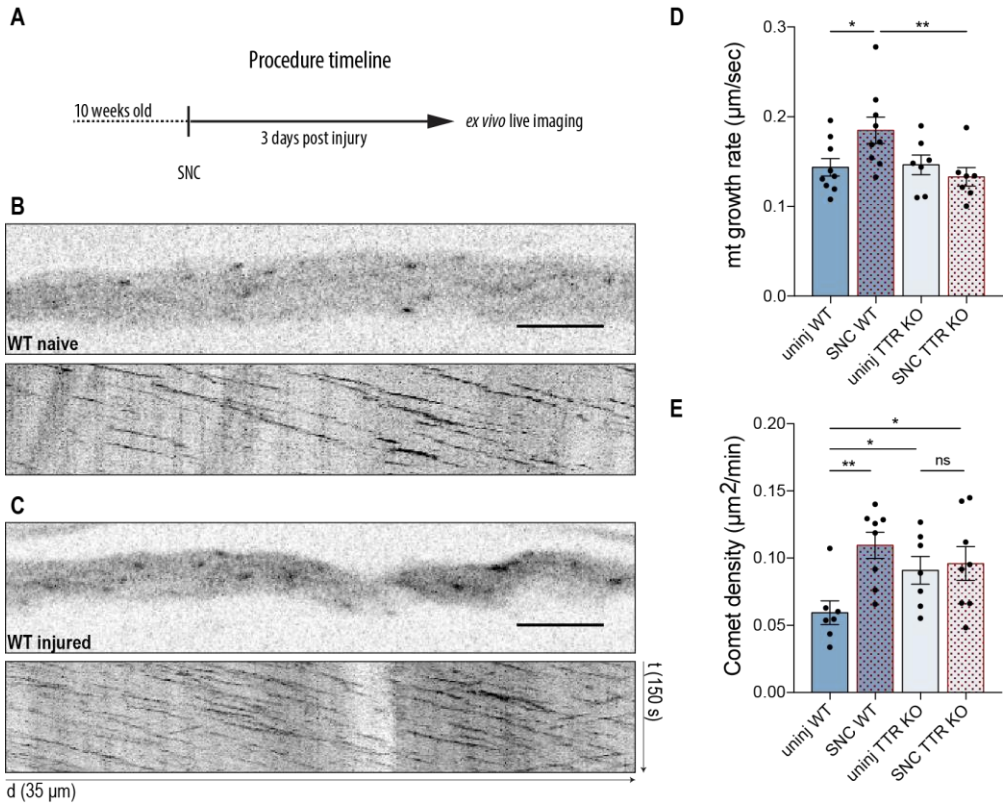


Figure 3. TTR KO mice fail to adjust MT dynamics in response to sciatic nerve crush. (A) Schematic representations of the procedure timeline of the experimental setup for MT dynamics assessment. **(B)** Representative still image of the axonal shaft from a WT-Thy1-EB3-YFP uninjured nerve and correspondent kymograph. **(C)** Representative still image of an axonal region distally to the lesion site from a WT-Thy1-EB3-

YFP crushed nerve and correspondent kymograph. **(D, E)** Quantifications of different microtubule (MT) dynamics parameters including MT growth rate (D) and EB3 comet density (E) from WT-Thy1-EB3-YFP and TTR KO-Thy1-EB3-YFP nerves either uninjured (uninj) or crushed (SNC). Results are plotted as mean ± SEM (n = 6-12 axons, 8-9 animals/condition). Statistical significance determined by Student's t-test: *P<0.05, **P<0.01. ns, not significant. Scale bars: 5µm.

AXONS IN THE SCIATIC NERVE OF TTR KO MICE HAVE MORE DYNAMIC MT PLUS ENDS

While analysing uninjured nerves, we found that TTR KO mice had higher EB3 comet density when compared to WT animals (Fig. 3E), indicating that loss of TTR either promoted MT nucleation or increased dynamicity by inducing plus end rescue events. To rule this out, we assessed the density of individual MTs by electron microscopy analysis of cross sections of WT and TTR KO sciatic nerves. Only axons with 2-4 µm were included in the analysis since those were also the ones that contributed to the differences in comet density observed by EB3 live-imaging. No differences were scored in MT density between WT and KO axons (Fig. 4A-C), indicating that in the absence of TTR, naïve axons in peripheral nerves display more dynamic MT plus ends and that this intrinsic increase in the number of dynamic MTs might account for their decreased ability to reorganize the MT cytoskeleton after injury in order to regenerate.

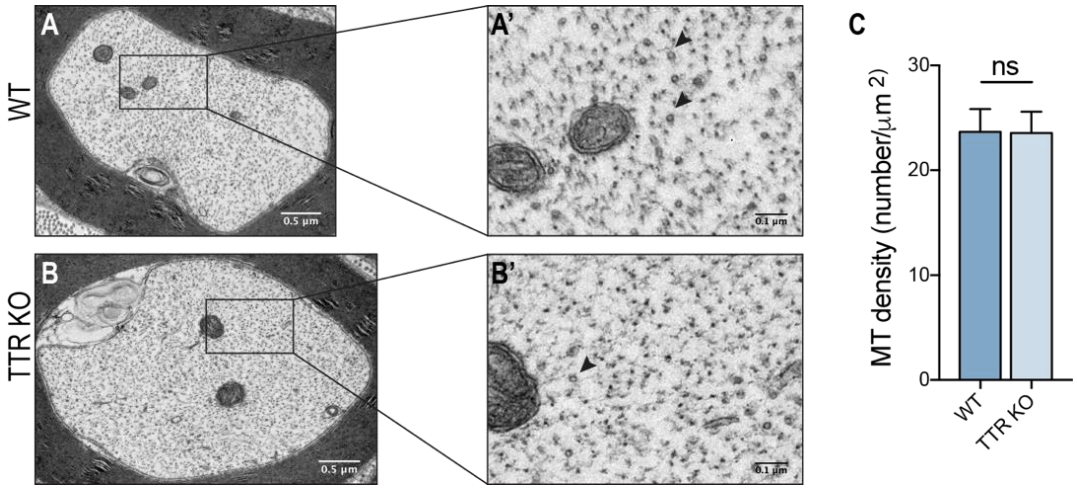
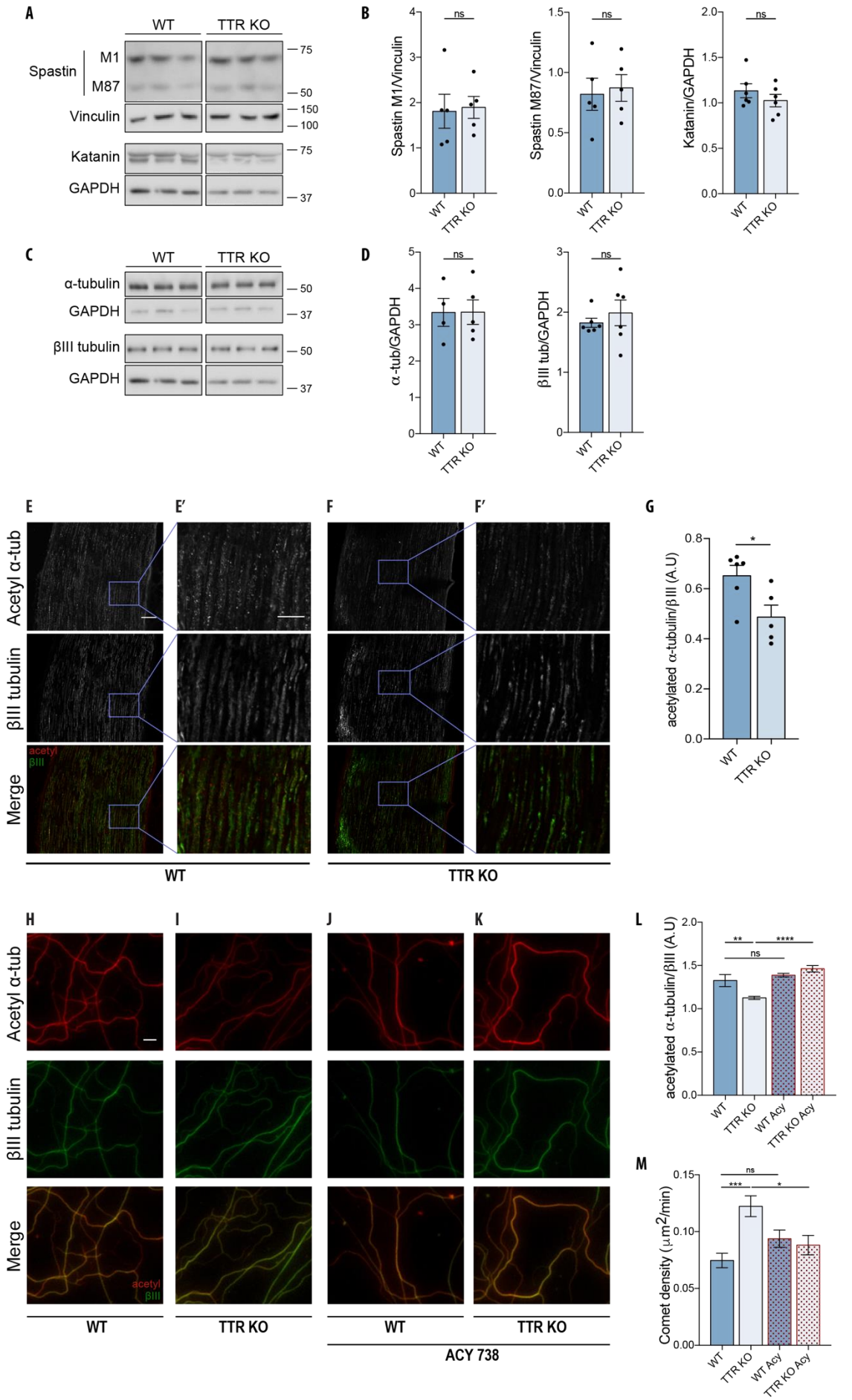


Figure 4. MT density assessment in WT and TTR KO sciatic nerves. (A, B) Representative electron microscopy images of individual axons from 10-week-old WT (A) and TTRKO (B) sciatic nerves and respective high magnification representations (A', B'). MTs are represented by black arrowheads. **(C)** Quantification of MT density from 2-4um axons from WT and TTR KO sciatic nerves. Results are plotted as mean ± SEM (n = 30-60 axons/animal, 6 animals/genotype). Statistical significance determined by Student's t-test: ns, not significant.

TTR IMPACTS MT DYNAMICS BY REGULATING TUBULIN ACETYLATION IN THE AXONAL SHAFT

To determine the molecular mechanisms underlying axonal MT instability in peripheral nerves of TTR KO mice, we hypothesized whether this was related to differences in the levels of MT severing enzymes (Zhang, Fishel, Bertroche, & Dixit, 2013). However, western blot analysis showed no difference in the levels of both spastin and katanin on protein extracts from TTR KO and WT naïve sciatic nerves (Fig. 5A, B). We analysed the total levels of α- and βIII-tubulins and found no difference between WT and KO nerves, a result in agreement with similar MT density scored between the two genotypes by electron microscopy analysis (Fig. 5C, D). Given the impact of acetylated α-tubulin on MT stability, we analysed this PTM by immunohistochemistry, and quantification of acetylated α-tubulin on βIII-tubulin labelled axons revealed a relative decrease in α-tubulin acetylation in the sciatic nerves of TTR KO mice (Fig. 5E-G). Acetylated α-tubulin accumulates onto previously stabilized MTs but its presence has been further associated to an increase in MT stability given the enhanced ability of acetylated MTs to resist breakage (Xu et al., 2017). Altogether, our data strongly support the notion that loss of acetylated α-tubulin in TTR KO mice is related to an increase in dynamic MTs and suggest that in TTR KO mice these defects are both related to the inability of their nerves to regenerate.

To investigate whether loss of acetylated α-tubulin in TTR KO nerves underlies the increase in the number of dynamic MT ends, we evaluated the consequences of exposing WT and KO DRG neurons to the HDAC6 inhibitor ACY-738 to increase tubulin acetylation levels. To this end, DIV4 DRG neuron cultures isolated from adult WT and KO mice were conducted and levels of acetylated tubulin by semi quantitative immunocytochemistry were evaluated in these neurons. First, we confirmed that the neurite shafts of TTR KO had decreased levels of acetylated α-tubulin when compared to WT controls (Fig. 5H, I, L). More importantly, while the addition of ACY-738 did not impact WT neurons, it normalized loss of α-tubulin acetylation in the neurite shafts from TTR KO neurons (Fig. 5J, K, L). We analysed whether the increase in α-tubulin acetylation promoted by ACY-738 in TTR KO axons had an impact on the number of dynamic MTs by measuring EB3 comet density in in WT-Thy1-EB3-YFP and TTR KO-Thy1-EB3-YFP neurons. Indeed, EB3 live imaging confirmed an increase in comet density in TTR KO neurite shafts when compared to WT controls (Fig. 5M) and similarly to what was observed *in vivo*. Strikingly, this effect was completely reverted through treatment with ACY-738 (Fig. 5M), strongly suggesting that the regulation of axonal MT density by TTR is mediated by its role in modulating α-tubulin acetylation levels.


A
WT
TTR KO
Spastin
M1
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Vinculin
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Spastin M1/Vinculin
Spastin M87/Vinculin
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TTR KO
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α-tubulin
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α-tub/GAPDH
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WT Acy
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ACY 738

Figure 5. TTR impacts on MT dynamics by regulating tubulin acetylation in the axonal shaft. (A, B) Western blot (A) and respective quantification (B) showing MT severing enzymes levels in sciatic nerves of 10-week-old WT and TTR KO mice. Vinculin was used as control for Spastin and GAPDH was used as a control for Katanin. Data represent mean ± SEM (n = 5-6 animals/condition). ns – not significant by Student's t test. **(C, D)** Western blot (C) and respective quantification (D) showing α- and βIII-tubulin levels in sciatic nerves of 10-week-old WT and TTR KO mice. GAPDH was used as a control. Data represent mean ± SEM (n = 4-6 animals/condition). ns - not significant by Student's t test. **(E, F)** Representative images of 10-week-old WT (E) and TTR KO (F) sciatic nerves immunostained for acetylated α-tubulin and βIII-tubulin. Scale bar: 50 μm (E', F') Zoomed in regions from E and F, respectively. Scale bar: 20 μm **(G)** Quantification of the relative values of acetylated α-tubulin over βIII-tubulin. Data represent mean ± SEM (n = 45-57 axons/animal, 5-6 animals/genotype). Statistical significance determined by Student's t-test: *P<0.05. **(H-K)** Representative images of acetylated α-tubulin (red) and βIII-tubulin (green) immunostaining of WT and TTR KO 4DIV DRG cultures untreated (H, I) and treated with ACY-738 (J, K) Scale bar: 10 μm. **(L)** Quantification of the relative values of acetylated α-tubulin over βIII-tubulin. Data represent mean ± SEM (n = 50 axons/condition, representative of 2 independent samples from 2 independent experiments). Statistical significance determined by Tukey's multiple comparisons test: **P<0.01, ****P<0.0001, ns - not significant. **(M)** Quantification of comet density in the neurite shafts from DIV4 WT-Thy1-EB3-YFP and TTR KO-Thy1-EB3-YFP DRG neurons untreated and treated with ACY-738. Data represent mean ± SEM (n = 9-17 neurons/condition, representative of 2 independent experiments). Statistical significance determined by Tukey's multiple comparisons test: *P<0.05 ***P<0.001, ns - not significant.

DISCUSSION

Transthyretin is a protein with a very particular feature as it is involved both in neurodegeneration (Saraiva, Costa, & Goodman, 1983) and nerve biology (Fleming et al., 2007). The intriguing roles assigned to TTR in neurite outgrowth and retrograde axonal transport, with an impact in nerve regeneration (Fleming et al., 2007), associated with the pivotal importance of cytoskeleton modulation during axonal elongation and regeneration processes were the main aspects we took into consideration when executing this work which had as main goal to acquire new mechanistic insights for the impact of TTR on nerve biology.

In this work we were able to establish a correlation between the modulation of MT dynamics with the neuritogenic effect of TTR. EB3 comet density serves as a general marker of MT dynamics and of ongoing remodelling of axonal arbours (Kleele et al., 2014). Our *in vitro* results show that TTR, in conditions that promote neurite outgrowth, has a compartmentalized impact on the neuronal MT cytoskeleton, regulating MTs differently in the growth cone and in the axonal shaft. While it promotes increased MT plus ends density in the growth cone, which translates into a more dynamic MT cytoskeleton, it seems to stabilize MTs in the shaft unravelling a pro-growth function to TTR. In fact, during neurite extension, stable MTs are needed in the axonal shaft to drive growth forward, while in the growth cone MTs must be highly dynamic to support growth and respond to extracellular stimuli (Bradke, Fawcett, & Spira, 2012). Further studies should explore how TTR promotes this compartmentalized function.

The impact of TTR as a MT dynamics modulator observed *in vitro* was recapitulated *in vivo*. While WT-Thy1-EB3-YFP animals responded efficiently to sciatic nerve injury shown by increased MT dynamics in growing axons, TTR KO-Thy1-EB3-YFP animals failed to modulate MTs after injury. Furthermore, uninjured TTR KO-Thy1-EB3-YFP nerves displayed an inherent increased MT instability determined by increased comet density values in axonal shafts from these nerves but maintaining the normal values of MT total mass. These observations might underlie the decreased ability of TTR KO animals to reorganize the MT cytoskeleton after injury in order to regenerate.

MT severing enzymes generate internal breaks in the MT polymer and are known to interfere in the number of growing plus tips. The absence of differences in MT severing enzymes in TTR KO animals suggested that the MT instability observed *in vivo* could be a consequence of alterations in tubulin post translational modifications. In this respect, we observed decreased levels of acetylated α-tubulin in axonal shafts in the absence of TTR, which we demonstrated to be the cause of the decreased MT stability when performing assays with the HDAC6 inhibitor ACY-738 that rescued acetylated α-tubulin levels and MT

stability. Acetylated α-tubulin is associated with stable MTs and recent studies have shown that loss of acetylated α-tubulin led to increased MT debundling and increased MT plus-end dynamics along the axon with consequences in CNS neuronal development (Dan et al., 2018). In mouse peripheral sensory neurons acetylated α-tubulin was found to be enriched in submembranous bands in the soma rather than in the cytoplasmic MT network (Morley et al., 2016). This acetylated α-tubulin enrichment in the submembranous band is thought to tune the mechanical properties of the membrane and, in its absence, cells are less elastic and require more force to trigger the mechanosensitive channels and, in fact, recent studies have shown the importance of acetylated α-tubulin in maintaining touch sensitivity in mechanosensory neurons (Yan et al., 2018). These findings present a particular relevance since TTR KO mice present a sensorimotor impairment that starts at 6 months of age (Fleming et al., 2007).

It would be important to dissect how TTR modulates acetylated α-tubulin levels in axons. The expression levels of some players involved in tubulin acetylation should be explored, namely the acetylase ATAT1 and the deacetylases HDAC6 and Sirtuin2. In fact, TTR was previously reported as acting as a transcription factor (Sousa, Yan, Stern, & Saraiva, 2000) which could be a hypothesis for the mechanism underlying the regulation of the expression of the referred enzymes.

Overall, our results pinpoint TTR as a MT dynamics regulator by modulating the acetylated α-tubulin levels. This fine tuning of α-tubulin acetylation and the consequent modulation of MT stability might underly the impact of TTR on nerve biology, which will be validated with studies addressing the impact of the HDAC6 inhibitor ACY-738 on the decreased axonal growth of TTR KO neurons.

MATERIALS AND METHODS

Animals. Mice were handled according to European Union and National rules. WT and TTR KO (Episkopou et al., 1993) littermates (in the Sv/129 background), were obtained from the offspring of heterozygous breeding pairs. Thy1-EB3-YFP (Kleele et al., 2014) animals were crossed with TTR KO mice. The resultant TTR KO(+/-).Thy1-EB3-YFP were intercrossed generating WT-Thy1-EB3-YFP and TTR KO-Thy1-EB3-YFP. All animals were maintained under a 12 h light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. Genotypes were determined from ear extracted genomic DNA. For DRG neuronal cultures, animals with 4-8 weeks of age were used. For sciatic nerve crush experiments, electron microscopy assessment of MT density, Immunoblotting and Immunohistochemistry experiments, sciatic nerves from 12-16-week-old WT and TTR KO mice were used. For sciatic nerve immunostaining analysis, mice were perfused with PBS for 5 min followed by 4% paraformaldehyde (PFA, pH 7.4) in PBS (40ml). Sciatic nerves were collected and maintained in 4% PFA for 24h and then cryopreserved in 30% sucrose for 48h.

Recombinant TTR production and purification. Recombinant WT TTR was produced using a pETF1 vector carrying human WT TTR (Goldsteins et al., 1997) and used in the transformation of competent *Escherichia coli* BL21(DE3) cells. The protein was expressed as described in (Silva et al., 2017) and purified through successive ionic exchange, hydrophobic interaction and size exclusion chromatographies. For cellular assays, recombinant TTR was detoxified using a high-capacity endotoxin removal resin (Thermo Scientific) and quantified using the Lowry based DC Protein Assay (Bio-Rad Laboratories).

Primary DRG neuronal cultures and cell treatment. Mice were humanely euthanized through CO_2 inhalation and DRG neurons were collected and cultured as described in (Liz et al., 2014). For neurite outgrowth experiments, DRG were plated in 20 µg/ml PLL + 5 µg/ml Laminin coated 24-well plates at a density of 5000 cells per well in DMEM/F12 (Sigma-Aldrich, D8437) supplemented with 1× B27 (Gibco), 1% penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 50 ng/mL NGF (Millipore, 01-125) at 37ºC and 5% CO_2. 4h after plating, DRG were treated with recombinant WT TTR (equal volume of PBS for the control condition) at a concentration of 300 µg/ml and incubated for 12 h at 37ºC and 5% CO_2 before fixing. For EB3-GFP transfection experiments, the 4D Nucleofector Amaxa system (Lonza, Barcelona, Spain, CM#137 program) was used and cells were transfected at a density of at least 200.000 cells/condition with a plasmid

encoding EB3-GFP (0,5 µg; (Liz et al., 2014). Cells were left in suspension for 24 h in complete medium at 37ºC and 5% CO_2. Cells were then plated in 20 µg/ml PLL + 5 µg/ml Laminin coated 35 mm µ-dishes (iBidi) at a density of 15000 cells per dish in phenol-free DMEM/F12 with its supplementation. 4 h after plating, DRG were treated with recombinant WT TTR at a concentration of 300 µg/ml and incubated for 12 h at 37ºC and 5% CO_2 for 12 h before performing live imaging. For HDAC6 inhibition experiments with ACY-738, WT-Thy1-EB3-YFP and TTR KO-Thy1-EB3-YFP DRG neurons were used. Neurons were plated in 20 µg/ml PLL + 5 µg/ml Laminin coated 24-well plates at a density of 5000 cells per well for acetylated α-tubulin and βIII-tubulin staining and in 8 well µ-dishes (iBidi) at a density of 8000 cells per well for EB3 live imaging of MT dynamics in DMEM/F12 (Sigma-Aldrich, D8437) supplemented with 1× B27 (Gibco), 1% penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 50 ng/mL NGF (Millipore, 01-125), 60 µM 5-Fluoro-2'-deoxyuridine (FluoU) and 100 nM of ACY-738 in DMSO (equal volume of DMSO for the control condition) at 37ºC and 5% CO_2. At DIV3 half the medium was changed to new medium supplemented with 2x the concentration of ACY-738 and FluoU. At DIV 4, 2 h before fixing, medium was changed supplemented with ACY-738. For live imaging experiments, 2 h before imaging, medium was changed to phenol-free DMEM/F12 with its supplementation and imaging was started in the ACY-738 treated conditions to minimize the effect of its short life in plasma (Jochems et al., 2014).

Sciatic nerve crush. 12-week-old mice were anesthetized with isoflurane and a 4-mm-long incision was made in the shaved thigh skin. For nerve crush, the sciatic nerve was exposed and crush was performed using Pean forceps, three times during 10 s. To standardize the procedure, the crush site was maintained constant for each animal at 5 mm distally to the notch. A single skin suture, immediately above the crush site, served as an additional reference. After surgery, animals were allowed to recover for 3 days after which, mice were sacrificed in the CO_2 chamber and the sciatic nerves (injured and contralateral naïve nerves) were subsequently collected for live cell imaging.

EB3 live imaging for the analysis of the MT dynamics. For the analysis of the different MT dynamics parameters transfected DRG neurons were recorded for 2 min (60 frames total) in phenol-free DMEM/F12 supplemented as mentioned above, at 37°C and 5% CO_2, on a Spinning Disk Confocal System Andor Revolution XD with an iXonEM+ DU-897 camera and an IQ 1.10.1 software (ANDOR Technology). For the *ex vivo* imaging, sciatic nerves from WT-Thy1-EB3-YFP and TTR KO-Thy1-EB3-YFP were collected from the animals 3 days post injury and placed in 35 mm µ-Dish (iBidi) with phenol-free DMEM/F12 and recordings were performed as described above. For the quantification of the different EB3

dynamics parameters, kymographs were made using the Fiji KymoResliceWide plugin (distance, x axis; time, y axis). Starting and end positions of the traces were defined using the Fiji Cell Counter plugin generating comet length, which is the comet movement length in micrometres, comet duration, which is the comet lifetime in seconds, and growth rate, which is the comet length/comet duration. Comet density at the growth cone is the number of comets in 30 consecutive frames divided by the number of quantified frames and growth cone area. Comet density at the axonal shaft is the number of comets per micrometre squared per minute.

Sciatic nerve ultrastructure preparation and MT density analysis. For ultrastructure analysis of MT density, 12-16-week-old WT and TTR KO mice were sacrificed using a CO_2 chamber and sciatic nerves were collected and fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 5 days and processed for ultrathin sections as previously described (da Silva et al., 2014). For MT density analysis 30-60 axons of 2-4 µm of diameter were analysed using Photoshop CS3 for image processing and mounting.

Immunoblotting. Protein lysates from 12-week-old quick frozen sciatic nerves from WT and TTR KO mice were prepared in ice-cold RIPA lysis buffer (1% Triton X-100, 0.1% SDS, 140 mM NaCl, 1x TE pH 8, 1x protease inhibitor Cocktail and 1mM Sodium orthovanadate), sonicated (2x10 cycles, Output Power 50 Watts, Branson sonifier 250) and cleared by centrifugation at 15000 rpm for 10 min at 4^{o}C. 20 ug of protein extracts for analysis of MT severing enzymes or 5 ug of protein extracts for analysis of tubulin proteins were separated under denaturing conditions and transferred to Amersham Protran Premium 0.45 µm nitrocellulose membranes (GE Healthcare Life Sciences), and blocked in 5% non-fat dried milk in TBS-T for 1 h at room temperature. Membranes were probed overnight at 4^{o}C with the following primary antibodies (in 5% BSA in TBS-T): rabbit anti-katanin (1:500, Proteintech, 17560-1-AP), mouse anti-spastin (1:500, Santa Cruz Biotechnology, sc-81624), mouse anti-βIII-tubulin (1:10,000; Promega, G7121), mouse anti-α-tubulin (0.5 µg/ml; DSHB, 12G10), mouse anti-GAPDH (1:1,000; Santa Cruz Biotechnology, sc-166574) and rabbit anti-vinculin (3:10,000; ThermoFisher Scientific, 700062). Secondary antibodies were used in 5% non-fat dried milk in TBS-T for 1 h at room temperature. Secondary antibodies were mouse IgGκ light chain conjugated with horseradish peroxidase (HRP) (1:2,000; Santa Cruz Biotechnology, sc-516102) and goat anti–rabbit IgG conjugated with HRP (1:10,000; Jackson ImmunoResearch Labs, 111-035-003). Immunodetection was performed by chemiluminescence using ECL (Millipore, WBLUR0500) and quantified using ImageJ software.

Imunohistochemistry. Cryopreserved sciatic nerves from 12-week-old mice were embedded in Optimum Cutting Temperature (OCT) compound (ThermoFisher Scientific), frozen and cut longitudinally (Cryostat Leica CM3050S) in 12 µm thick sections. For acetylated α-tubulin and βIII-tubulin staining, sections were blocked with 5% normal donkey serum containing 0,3% Triton in PBS for 1 h at RT followed by incubation for two overnights at 4°C with the primary antibodies mouse anti-acetylated α-tubulin (1:500, Sigma-Aldrich, T7451) and rabbit anti-βIII-tubulin (1:500, Abcam, 1967-1) diluted in blocking buffer. Sections were then incubated with secondary antibodies: donkey anti mouse Alexa Fluor 568 (1:1000, Alfagene, A10037) and donkey anti rabbit Alexa Fluor 647 (1:500, Jackson ImmunoResearch, 711-605-152) diluted in blocking buffer for 1 h at RT, washed in PBS and mounted in iBidi mounting medium (iBidi, 50001).

Immunocytochemistry. For neurite outgrowth experiments control and TTR treated WT DRG neurons were fixed with 4% PFA, blocked with 5% normal donkey serum (NDS) containing 0,4%Tween for 1 h at RT, incubated with mouse anti–βIII-tubulin (1:2000; Promega, G7121) overnight at 4ºC followed by the incubation of the secondary antibody (donkey anti-mouse Alexa-Fluor 488, 1:1000 Alfagene, A21202) for 1 h at RT. For acetylated α-tubulin and βIII-tubulin staining WT DRG and TTR KO either untreated or treated with ACY-738 were fixed with cytoskeleton preservation fixative, PHEM fixative (4% PFA, 4% sucrose, 0.25% Glutaraldehyde, 0.1% Triton X-100, 300 mM PIPES, 125 mM HEPES, 50 mM EGTA and 10 mM Magnesium Chloride), permeabilized with 0.2% Triton X-100 for 5 min, quenched with 50 mM Ammonium Chloride for 5 min and blocked with 2% Fetal Bovine Serum (FBS), 2% BSA and 0,2% Fish Gelatine in PBS for 1 h at RT. Incubation of primary antibodies mouse anti acetylated α-tubulin (1:5000; Sigma-Aldrich, T7451) and rabbit anti-βIII-tubulin (1:500, Abcam, 1967-1) was performed in 10% blocking buffer overnight at 4ºC. Incubation of the secondary antibodies donkey anti mouse Alexa Fluor 568 (1:1000, Alfagene, A10037) and donkey anti rabbit Alexa Fluor 647 (1:500, Jackson ImmunoResearch, 711-605-152) was performed in 10% blocking buffer for 1 h at RT. Cells were mounted in Fluoromount (Southern Biotech).

Imaging and quantification. For neurite outgrowth experiments images were acquired using an epifluorescence microscope Zeiss AxioImager Z1 with an Axiocam MR3.0 camera and Axiovision 4.7 software. The longest neurite tracing analysis was performed using Fiji software and neuronJ plugin. For sciatic nerve acetylated α-tubulin/βIII-tubulin quantifications, WT and TTR KO slices were imaged in a laser scanning Confocal Microscope Leica TCS SP8, using the PL APO 10X objective. For acetylated α-tubulin/βIII-tubulin quantifications in DRG neurons, Leica DMI6000 FFW microscope with

an HCX PL Fluotar 20X objective was used with. LAS X software operating the navigator feature was used to acquire big regions of the slices and to perform the final merge of the imaged region. Ratiometric analysis of acetylated α-tubulin/βIII-tubulin in sciatic nerve slices and DRG neurons was performed by determination of regions of interest selecting stretches of axons, randomly, and using ImageJ software.

Statistics. All statistical tests were performed using GraphPad Prism 7 software. Data are shown as mean ± s.e.m. Unless otherwise stated, the Student's t test was used. Statistical tests and sample sizes are indicated in figure legends and significance was defined as *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns - not significant.

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SUPPLEMENTARY DATA

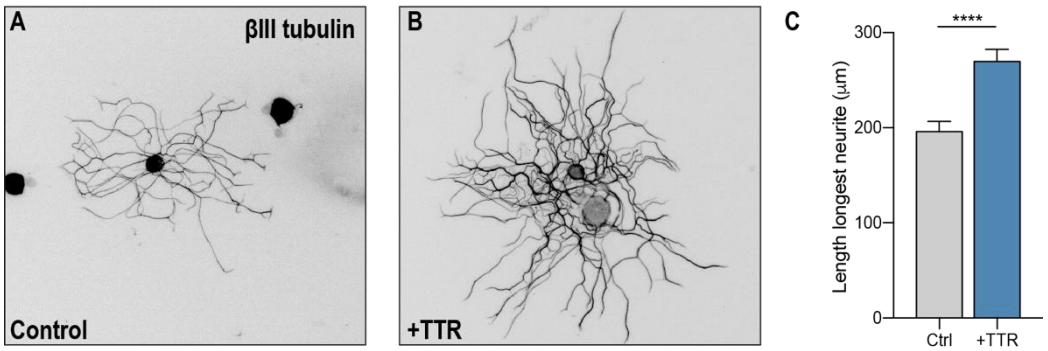

Figure S1. Exogenous TTR addition to DRG neurons increases neurite outgrowth. Representative anti-βIII-tubulin immunofluorescence images of control **(A)** and WT TTR treated **(B)** DRG neurons. **(C)** Quantification of the length of the longest neurite in DRG neurons treated with TTR. Data represent mean ± SEM (n = 101-127 neurons/condition, representative of 4 technical replicates, 2 independent experiments). Statistical significance determined by Student's t-test: ****P<0.0001.

CHAPTER III

Dissecting neuronal cytoskeleton alterations in Familial Amyloid Neuropathy

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ABSTRACT

Mutations in Transthyretin (TTR) are associated with familial amyloid polyneuropathy (FAP), a neurodegenerative disorder characterized by the deposition of insoluble TTR aggregates, particularly in the peripheral nervous system. However, the cellular and molecular mechanisms that underlie TTR toxicity in peripheral nerves are still unclear. Abnormalities in cytoskeletal organization are a common feature of many neurodegenerative disorders. Amyloidogenic proteins such as beta-amyloid peptide (Aβ) have been shown to mediate neurotoxicity by inducing major alterations in both microtubules and actin filaments. This work aimed at the elucidation of the mechanisms behind neuronal death in FAP proposing cytoskeletal components as main targets for TTR-induced neurodegeneration.

Our results show a complete reorganization of actin cytoskeleton in the growth cones of wild-type (WT) dorsal root ganglia (DRG) neurons treated with TTR aggregates, mediated by the receptor of advanced glycation end-products (RAGE) and with an involvement of Rac1. Actin dysfunction induced by TTR aggregates, was recapitulated using a FAP mouse model that carries the human TTR A97S mutation (hTTRA97S) and presents sensory nerve degeneration. DRG from hTTRA97S mice presented actin cytoskeleton alterations at the growth cone which was reverted upon Rac1 silencing. Importantly, actin dysregulation preceded *in vitro* neurodegeneration of hTTRA97S neurons, a process that was also reverted by upon Rac1 inactivation. Additionally, to the actin cytoskeleton dysregulation, *ex vivo* assessment of the peripheral roots from hTTRA97S DRG explants revealed impairment of axonal microtubule dynamics and mitochondrial transport.

Altogether, our findings unravelled impaired organization of the neuronal cytoskeleton in a FAP mouse model with a potential key impact in FAP pathophysiology. Further dissection of the impact of cytoskeleton disruption for neurodegeneration in FAP might constitute the basis for the development of new therapeutic strategies for the disease.

INTRODUCTION

Familial amyloid polyneuropathy (FAP) is an autosomal dominant neurodegenerative disorder characterized by the deposition of insoluble mutated transthyretin (TTR), in the form of oligomers, aggregates and amyloid fibrils, particularly in the peripheral nervous system (PNS). As a consequence of TTR deposition, a dying-back axonopathy develops and, in advanced disease stages it is accompanied by neuronal degeneration (Plante-Bordeneuve & Said, 2011). The Portuguese neurologist Corino de Andrade described FAP for the first time in 1952 as “a peculiar form of peripheral neuropathy” present particularly in fishermen kindreds in Póvoa de Varzim (Northern village in Portugal). Thus, although we have evidence of FAP hotspots in different parts of the world such as Sweden, Japan, Italy, Spain and Greece (Saraiva, Magalhaes, Ferreira, & Almeida, 2012), this major hotspot in the north of Portugal brands this disorder with particular relevance in this country. Liver transplantation slows disease progression in young onset patients. Other therapies include TTR stabilizers and silencing TTR gene expression (Plante-Bordeneuve, 2014). However, these can only be applied after disease onset (Plante-Bordeneuve, 2014), and are unable to revert neurodegeneration. Therefore, the characterization of cellular pathways and identification of new druggable targets involved in FAP-related axonopathy are highly demanded.

Besides being intimately related with neurodegeneration, TTR is also implicated in nerve regeneration (Fleming, Saraiva, & Sousa, 2007) and neuronal growth promotion. In fact, recent work has shown that the neuritogenic effect from TTR is mediated through the regulation of the microtubule (MT) cytoskeleton (Eira J *et al.*, 2021, *submitted*). Besides the suggestions that TTR might act as a neuronal cytoskeleton modulator, abnormalities in cytoskeletal organization are portrayed as a common feature of many neurodegenerative disorders. In the case of FAP, although the dying-back axonal degeneration might suggest an initial disturbance of the distal cytoskeleton as a consequence of TTR deposition, the analysis of cytoskeleton alterations was not previously addressed. Nevertheless, a *D. Melanogaster* FAP model in which TTR Val30Met is expressed in the photoreceptor cells, revealed that these flies displayed decreased axonal projection of photoreceptor neurons, with more compact growth cones lacking the spread distribution of filopodia and lamellipodia actin structures. Moreover, a genetic screen performed by crossing the TTR Val30Met-expressing flies with mutant fly lines for candidate genes whose function is associated with cytoskeleton organisation showed that Rho GTPases - the major regulators of actin dynamics - modulate TTR-induced neurodegeneration in *D. melanogaster* (M, Lopes, & Liz, 2020).

In this work we aimed at exploring the impact of cytoskeleton disruption in disease pathogenesis in FAP mouse models. We used a FAP TTR knock-in mouse model in which one allele of the *Ttr* locus was replaced with human wild-type TTR (hTTRWT) or human TTR with the A97S mutation (hTTRA97S). hTTRA97S mice present small fibre neuropathy, with degeneration of skin nerves and neuropathic pain, as well as large fibre sensory neuropathy (Kan et al., 2018).

Analysis on either an *in vitro* model of TTR-induced neurodegeneration using DRG exposed to TTR aggregates, or on DRG neurons from the hTTRA97S mouse model revealed actin cytoskeleton alterations with an involvement of Rac1 activity. Additional to actin damage, our *data* also point to dysregulation of the microtubular cytoskeleton as well as to an impairment in mitochondrial transport.

Altogether, this study characterizes a cascade of events behind the TTR-induced cytoskeleton defects with a particular emphasis on the identification of novel therapeutic strategies targeting cytoskeleton dysfunction in FAP.

RESULTS

EXOGENOUS ADDITION OF TTR LEU55PRO AGGREGATES TO DRG NEURONS RECAPITULATES NEURODEGENERATION PHENOTYPE *IN VITRO*

In order to investigate the impact of TTR deposition in the neuronal cytoskeleton remodelling, we used a robust methodology which consists in the addition of exogenous aggregated TTR to primary cultures of DRG neurons, a relevant cell type for FAP studies since mutant TTR accumulates close to the DRG. This methodology is useful in providing primary insights regarding mechanisms underlying TTR aggregates-induced neurotoxicity in FAP.

TTR L55P is a highly aggressive and amyloidogenic TTR mutation (Jacobson, McFarlin, Kane, & Buxbaum, 1992), and the pathway through which it dissociates from its tetrameric conformation and forms aggregates is efficiently accomplished, even under physiological conditions (Quintas, Saraiva, & Brito, 1997), very well characterized, and vastly used in cellular assays with the aim to assess amyloid-induced cytotoxicity in FAP.

Human recombinant TTR L55P was produced, purified and, subsequently, subjected to aggregation. TTR L55P aggregates formation was initially confirmed by transmission electron microscopy (TEM) and thioflavin T (Th-T) fluorescence assessment, a method that specifically detects the presence of β-sheet structures arranged in a cross-β conformation characteristic of amyloid (Cardoso, Pereira, Damas, & Saraiva, 2000). TEM analysis confirmed the formation of TTR L55P aggregates (Fig. 1A, A'). Th-T results demonstrated a typical bell-shaped curve with a peak in fluorescence at approximately 435nm in the protein solution of TTR L55P aggregates condition, when compared to a solution with WT soluble TTR, further confirming that TTR L55P formed amorphous aggregates (Fig. 1B).

To validate *in vitro* neurodegeneration, we evaluated the impact of TTR L55P on the proportion of DRG neurons presenting axonal swellings, neurodegeneration related structures characterised by disorganization of the MT cytoskeleton and accumulation of organelles, namely mitochondria. We observed that 12h of TTR L55P aggregates incubation to DIV0 DRG neurons induced a 1.7-fold increase in the percentage on neurons with axonal swellings when compared to untreated control cells, while the treatment with WT soluble TTR did not have any effect in the proportion of neurons with swellings (Fig. 1E). These results validated our cell-based system as a model of TTR-induced neurite degeneration.

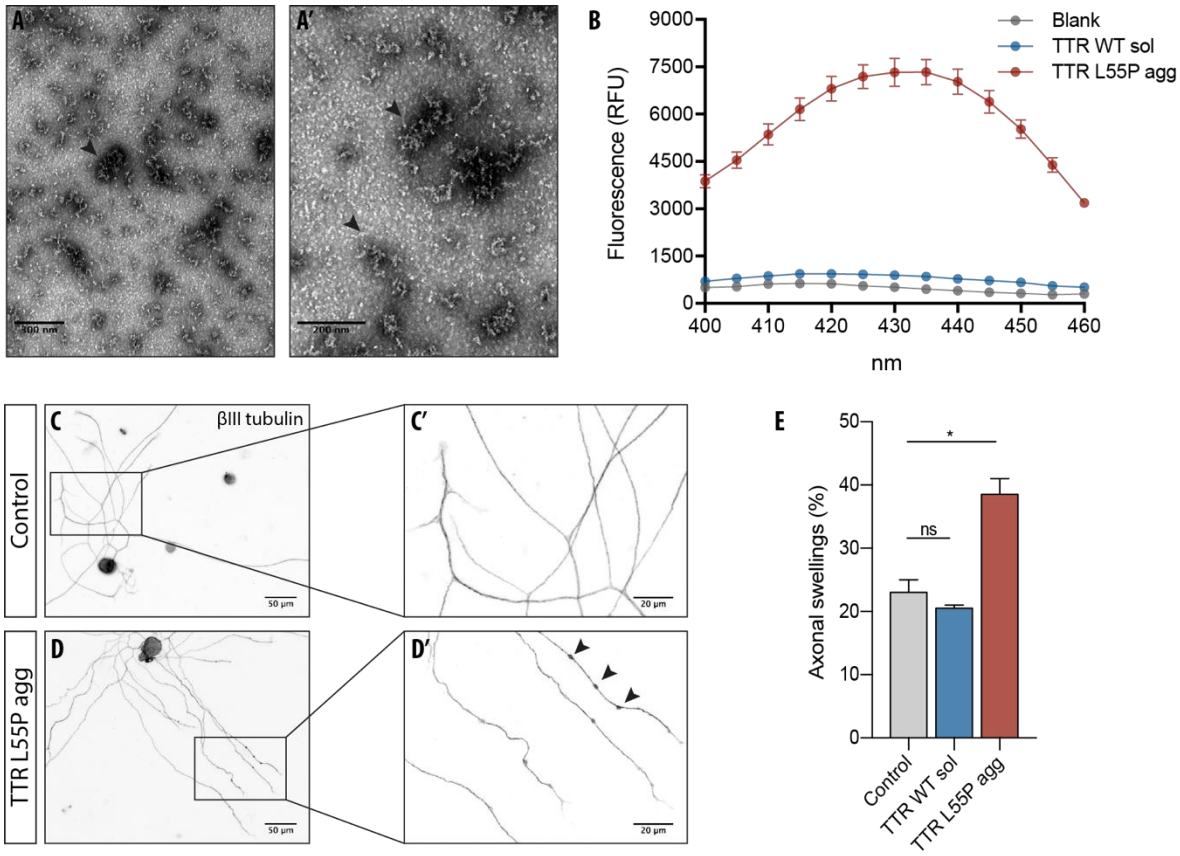


Figure 1. TTR L55P forms amorphous aggregates and induces neurodegeneration in DRG neurons. (A, A') Transmission electron microscopy images of TTR L55P aggregates at low (A) and high (A') magnifications. TTR L55P protein aggregates depicted with black arrowheads. A – 60000x; A' – 120000x. **(B)** Analysis of TTR L55P aggregates through Th-T assay. WT soluble TTR (TTR WT Sol) was used as a negative control. RFU: relative fluorescence units. **(C, C', D, D')** Representative images of DRG neurons immunolabelled with βIII-tubulin, either untreated (Control) (C, C') or treated with TTR L55P aggregates (TTR L55P agg) (D, D'). (C', D') Higher magnification of neurites in C and D, respectively. Arrowheads indicate axonal swellings. **(E)** Quantification of the percentage of neurons presenting axonal swellings in control and TTR L55P aggregates treated DRG neurons. Results are plotted as mean ± SEM. 273-299 neurons/condition, representative of 2 technical replicates. Statistical significance determined by Student's t-test: *P<0.05; ns, not significant.

TTR LEU55PRO AGGREGATES PROMOTE ACTIN CYTOSKELETON DYSREGULATION IN DRG NEURONS

The growth cone has a cytoskeleton organization with a distinctive distribution of MTs and actin (Lowery & Van Vactor, 2009). In order to evaluate the impact of TTR L55P aggregates in growth cone cytoskeleton organization, we used our established *in vitro* model of mouse DRG neurons either untreated or treated with WT soluble TTR or TTR L55P aggregates. We evaluated the growth cone morphology of these neurons and assessed both, the MT and actin cytoskeleton patterns. We divided the MT pattern into

two categories: the normal (methods section, Fig. 6A-C), in which MTs display either a splayed or bundled conformation and are able to protrude into the growth cone, and the dystrophic pattern, in which MTs form a dystrophic bulb at the tip of the axon (methods section, Fig. 6D, E). The actin category was also divided into two categories: the splayed pattern containing the typical lamellipodia and filopodia structures, organized in a star-shaped morphology (methods section, Fig. 6A, B) and the retracted pattern characterized especially by the absence of lamellipodia as well as growth cones with dystrophic actin morphologies, such as actin patches (methods section, Fig. 6C-E).

Qualitative analysis of the growth cone morphology in DRG neurons demonstrated that while TTR L55P aggregates did not alter MTs cytoskeleton organization at the growth cone (Fig. 2A), they did impact in the growth cone actin cytoskeleton promoting a 1.4-fold increase in the proportion of retracted growth cones, when comparing with control neurons (Fig. 2B, C). The presence of WT soluble TTR did not have an effect on the organization of either MTs or actin patterns in the growth cone (Fig. 2A, C).

Actin retrograde flow - the dynamic movement of actin filaments promoted by myosin and a pushing force from the cellular membrane as actin polymerizes - is an essential process for growth cone guidance and other types of cell motility (Lin, Espreafico, Mooseker, & Forscher, 1996). Since TTR L55P aggregates promote the remodelling of the actin cytoskeleton in the growth cone of DRG neurons, we hypothesized that actin dynamics, such as actin retrograde flow, would also be impaired in the presence of the aggregated protein. While no alterations in actin dynamics were observed in the presence of WT soluble TTR (Fig. 2D, E), TTR L55P aggregates promoted a significant reduction in the actin retrograde flow in the growth cone of DRG neurons, when comparing with control neurons (Fig. 2D, E), suggesting that mutant aggregated TTR has an impact in actin dynamics that might influence in its remodelling at the growth cone.

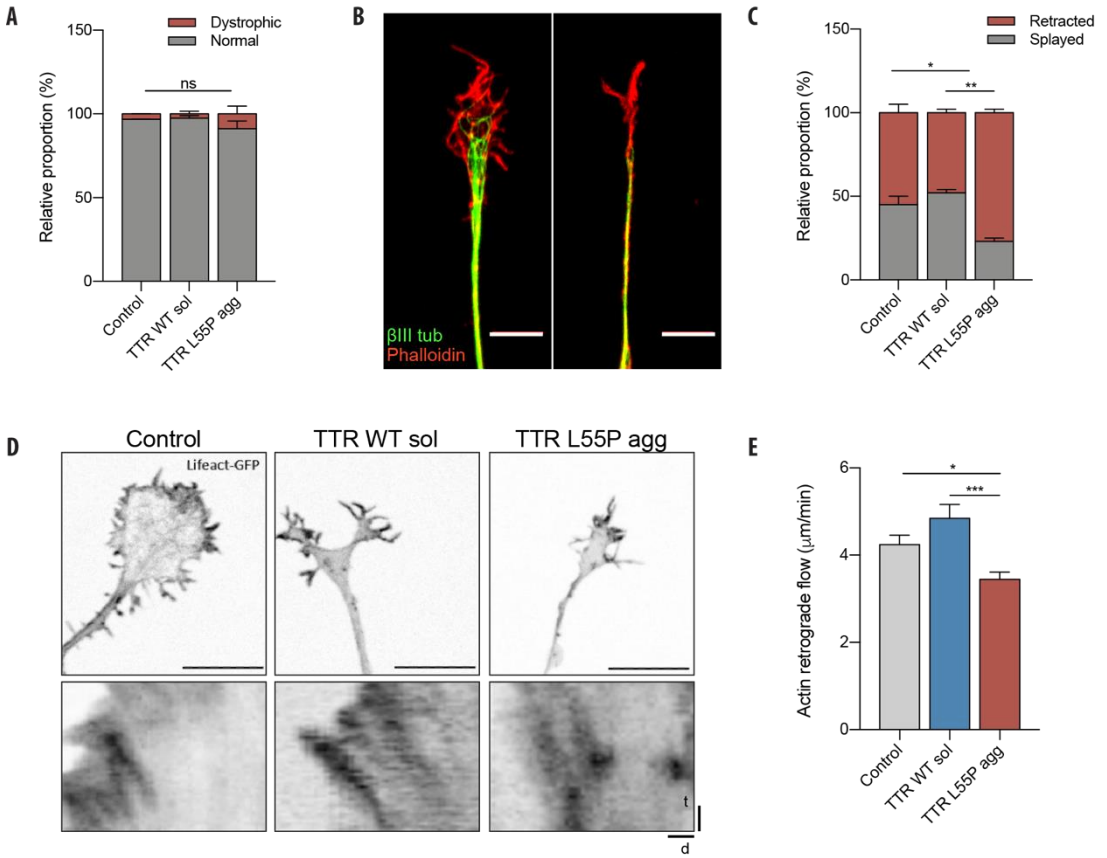


Figure 2. TTR L55P aggregates induce disorganization of the actin cytoskeleton in the growth cone of DRG neurons. (A) Quantification of the relative proportions of normal and dystrophic MT patterns in growth cones from DRG incubated with both WT sol TTR and TTR L55P aggregates. Results are plotted as mean ± SEM. n=2. Statistical significance determined by Two-Way ANOVA, followed by Tukey's multiple comparisons test: ns - not significant **(B)** Representative images of growth cones of DRG neurons double-labelled with βIII-tubulin (green) and phalloidin (red), either displaying a splayed (left) or retracted (right) actin pattern. Scale bar: 5 μm. **(C)** Quantification of the relative proportions of splayed and retracted actin patterns in growth cones from DRG incubated with both WT sol TTR and TTR L55P aggregates. Results are plotted as mean ± SEM. n=2. Statistical significance determined by Two-Way ANOVA, followed by Tukey's multiple comparisons test: *P<0.05, **P<0.01. **(D)** Representative images of growth cones of DRG neurons transfected with Lifeact-GFP, either untreated (Control) or treated with WT soluble TTR (TTR WT sol) and TTR L55P aggregates (TTR L55P agg) and respective kymographs of actin retrograde velocity, respectively. Scale bar: 10 μm. **(E)** Quantification of actin retrograde flow relative to D. Results are plotted as mean ± SEM. Statistical significance determined by One-Way ANOVA, followed by Tukey's multiple comparisons test: *P<0.05, ***P<0.001.

RAGE AND RHO GTPASES AS MEDIATORS OF TTR-INDUCED ACTIN CYTOSKELETON DISRUPTION

In order to explore the molecular mechanism behind the effect of TTR aggregates on the actin cytoskeleton we investigated the involvement of RAGE receptor, previously reported to bind to TTR aggregates mediating neurotoxicity in FAP (Sousa, Yan, Stern, &

Saraiva, 2000). Until now, RAGE is the only receptor described to be responsible for the binding to TTR aggregates, triggering an inflammatory response involving ERK and NF-kB, and mediating neurotoxicity in FAP (Sousa et al., 2000). Soluble RAGE (sRAGE) is an isoform of the receptor, containing only the extracellular domain, which is composed of three immunoglobulin-like domains (V, C and C'), and lacking the transmembrane and intracellular ones (Fig. 3A). It functions as a decoy receptor sequestering TTR aggregates, blocking their binding to RAGE receptors in the membrane and, consequently, inhibiting their effect (Bucciarelli et al., 2002) (Fig. 3A). Additionally, RAGE was previously described to control actin cytoskeleton remodelling (Xiong et al., 2011), therefore we hypothesized that the impact of TTR L55P aggregates on the organization of the actin cytoskeleton would be mediated by RAGE. We took, thus, advantage of sRAGE and analysed its effect on the actin pattern of the growth cone of DRG neurons, either untreated or treated with TTR. In accordance to previous results, we observed neurons incubated with TTR L55P aggregates presented a 1.4-fold increase in the proportion of retracted growth cones (Fig. 3B). Co-treatment with sRAGE completely reverted TTR L55P aggregates-induced alterations on actin organization in the growth cone (Fig. 3B), confirming that the disruption of the actin cytoskeleton induced by these aggregates is mediated by RAGE.

Besides being described as interfering in actin cytoskeleton remodelling, RAGE was also shown to have a downstream signalling function in modulating Rho GTPases, namely activating Rac1 and Ccd42 (Hudson et al., 2008). Additionally, work in *D. melanogaster* suggested the involvement of Rho GTPases in TTR-induced neurodegeneration (M et al., 2020). In an attempt to assess whether Rho GTPases were involved in the TTR L55P aggregates-induced actin cytoskeleton remodelling, we transfected DRG neurons with Rac1 FRET probe (Nakamura, Aoki, & Matsuda, 2005; Nakamura, Kurokawa, Kiyokawa, & Matsuda, 2006) and assessed its activation upon addition of TTR L55P aggregates. Our *data* show a transient Rac1 activation in DRG growth cones in the presence of TTR L55P aggregates, noticeable 10 minutes after the addition of the protein, which returned to normal levels 40 minutes after TTR L55P aggregates addition (Fig. 3C, D).

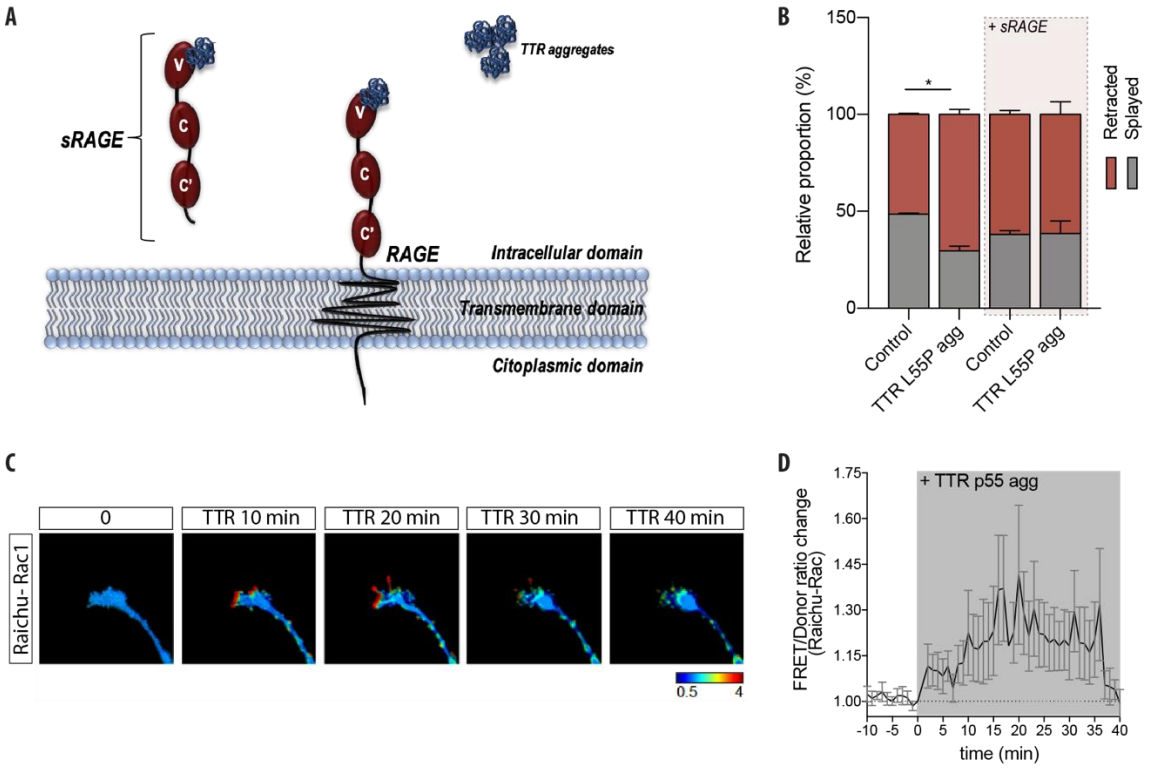


Figure 3. The effect of TTR L55P aggregates on the actin cytoskeleton is mediated by RAGE. (A) Schematic representation of the structure of RAGE and its isoform, sRAGE, and their relationship with TTR aggregates. **(B)** Quantification of the relative proportions of the splayed and retracted actin patterns in DRG neurons treated with TTR L55P aggregates (TTR L55P agg) in the absence and presence of sRAGE. Results are plotted as mean ± SEM. Statistical significance determined by Two-Way ANOVA, followed by Tukey's multiple comparisons test: *P<0.05. **(C-D)** Rac1 activation assessment in the growth cones of DRG neurons treated with TTR L55P aggregates. (C) Representative images of a TTR L55P aggregates treated growth cone in a time-lapse FRET/Donor ratio change images panel, coded according to the pseudo-colour ramp. Pseudo-colour ramps represent min/max CFP/FRET ratios. (D) Graph showing a time-lapse FRET/Donor ratio change images coded according to the pseudo-colour ramp (n =10 cells pooled from 2 different experiments).

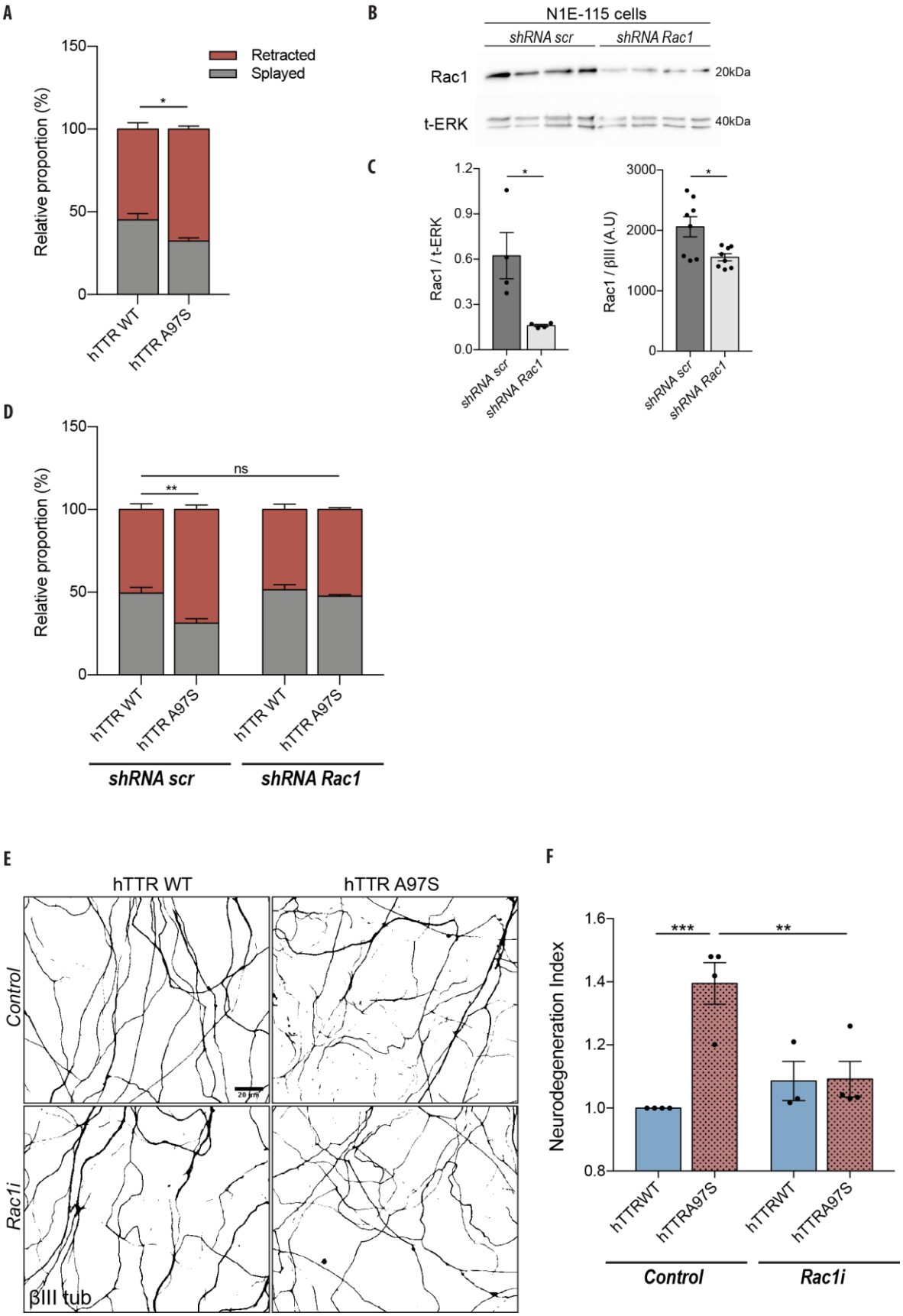
RAC1 MEDIATED TTR-INDUCED ACTIN DYSFUNCTION IS RECAPITULATED IN THE HTTRA97S MOUSE MODEL

Recently, a mouse model carrying the human TTR A97S mutation was generated. These mice replicate what has been observed in the early stage of FAP, including TTR deposition in the PNS, small fibre neuropathy, with degeneration of skin nerves (Kan et al. 2018). This mouse model represents, thus, a valuable tool to validate the results obtained with the *in vitro* addition of TTR L55P aggregates and to correlate actin dysfunction with neurodegeneration.

We started by assessing whether DRG neurons from these animals presented actin cytoskeleton alterations at the growth cones. Adult DRG neurons from hTTRA97S mice present an approximately 1.25-fold increase in the proportion of retracted growth cones

when compared to neurons from hTTRWT mice (Fig. 4A), recapitulating the experiments with addition of TTR aggregates. To test the hypothesis of Rac1 involvement in TTR-induced actin cytoskeleton remodelling, Rac1 was silenced in cultured adult DRG neurons by transfection with shRNA against Rac1. Initially, we validated Rac1 silencing by immunoblotting of a neuronal cell line or immunohistochemistry of DRG neurons transfected with the shRNAs (Fig. 4B, C). We followed by analysing the effect of Rac1 silencing on actin cytoskeleton organization of hTTRWT and hTTRA97S DRG neurons. Control scrambled shRNA transfected hTTRA97S neurons displayed, the typical shift towards the increase in the proportion of retracted growth cones (1.4-fold increase) when compared to scrambled shRNA transfected hTTRWT neurons (Fig. 4D). Rac1 knockdown efficiently reverted the actin cytoskeleton phenotype of the growth cone of hTTRA97S DRG neurons, while not affecting growth cones from hTTRWT neurons (Fig. 4D). These results show that Rac1 mediates growth cone actin cytoskeleton remodelling in DRG neurons and corroborates the observed TTR interaction with actin regulators shown previously in the *D. Melanogaster* FAP model (M et al., 2020).

To test whether hTTRA97S mediated actin cytoskeleton alterations precede neuronal damage, we assessed axonal fragmentation in hTTRWT and hTTRA97S 4DIV DRG neurons. Axonal fragmentation, as determined by neurodegeneration index measurements, in cultured hTTRA97S DRG neurons was significantly increased when compared with DRG neurons from hTTRWT animals (Fig. 4E, F). Importantly, inhibiting Rac1 activity in these neurons reverted the observed neurodegeneration phenotype *in vitro* (Fig. 4E, F), suggesting that Rac1 is a key mediator in TTR induced actin cytoskeleton remodelling which culminates in axonal degeneration.


A
Retracted
Splayed
Relative proportion (%)
150
100
50
0
*
hTTR WT
hTTR A97S
B
N1E-115 cells
shRNA scr
shRNA Rac1
Rac1
20kDa
t-ERK
40kDa
C
Rac1 / t-ERK
1.2
0.9
0.6
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shRNA Rac1
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Rac1i
20 µm
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1.4
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hTTRWT
hTTRA97S
hTTRWT
hTTRA97S
Control
Rac1i

Figure 4. Actin cytoskeleton dysregulation in A97S animals is Rac1 mediated. (A) Quantification of the relative proportions of splayed and retracted actin patterns in hTTRWT and hTTRA97S DRG growth cones. Results are plotted as mean ± SEM. n=3 independent experiments. Statistical significance determined by Two-Way ANOVA, followed by Tukey's multiple comparisons test: *P<0.05. **(B, C)** Rac1 silencing validation through immunoblotting and immunofluorescence. Western blot (B) and respective quantification (C, left) of total cellular extracts from the mouse neuroblastoma cell line N1E-115 transfected either with shRNA scrambled or shRNA Rac1. Results are plotted as mean ± SEM (n = 4 experimental replicates). Statistical significance determined by Student's t-test: *P<0.05. Quantification of fluorescence levels (C, right) of βIII-tubulin and Rac1 stained DRG neurons transfected either with shRNA scrambled or shRNA Rac1. Results are plotted as mean ± SEM (n = 8 neurons). Statistical significance determined by Student's t-test: *P<0.05. **(D)** Quantification of the relative proportions of splayed and retracted actin patterns in hTTRWT and hTTRA97S DRG growth cones either transfected with scrambled shRNA (shRNA scr) or transfected with shRNA Rac1. Results are plotted as mean ± SEM. n=3 independent experiments. Statistical significance determined by Two-Way ANOVA, followed by Tukey's multiple comparisons test: **P<0.01, ns - not significant. **(E)** Representative immunofluorescence images of βIII-tubulin stained hTTRWT and hTTRA97S DRG neurons 4DIV either untreated (Control) or treated with Rac1 inhibitor (Rac1i). **(F)** Axonal fragmentation quantification of Neurons treated in E. Scale bar: 20 μm.

TTR A97S MICE DISPLAY IMPAIRED MT DYNAMICS AND MITOCHONDRIAL AXONAL TRANSPORT

The MT cytoskeleton is often reported as one of the neuronal components altered before initial manifestations of neurodegeneration. Having seen a disruption in actin organization in the FAP mouse model and, since actin and MT components are closely interconnected, we decided to further evaluate the TTR-induced impact on the neuronal cytoskeleton, aiming specifically at assessing MT dynamics *in vivo* in hTTRA97S mice at an age where neurodegeneration was still undetected.

We used Thy1-EB3-YFP mice which we crossed with either hTTRA97S or hTTRWT mice in order to obtain hTTRA97S and hTTRWT mice expressing EB3-YFP exclusively in neurons. We performed *ex vivo* live imaging of the peripheral root of DRG explants from these animals (Fig. 5A) since it is a major place of TTR amyloid accumulation in disease (Sousa, Cardoso, Fernandes, Guimaraes, & Saraiva, 2001). Live imaging of EB3 comets from 6 months old DRG explants showed that hTTRA97S axons presented decreased EB3 comet growth length with no alterations in comet growth rate and duration, and in EB3 comet density (Fig. 5D-H) when compared to hTTRWT animals.

Since axonal transport is intimately dependent on MT cytoskeleton stability, we decided to assess this parameter in the hTTRA97S animals focusing on mitochondrial transport. Live imaging of the peripheral root of DRG explants from hTTRA97S mice labelled with MitoTracker®, a dye that stains mitochondria in live cells, displayed

generalized impairment in axonal transport when compared with hTTRWT animals as revealed by the decrease in anterograde (Fig. 5I) and retrograde transport velocities (Fig. 5J), as well as, the decrease in total percentage of motile mitochondria (Fig. 5K). These observations suggest that the actin cytoskeleton defects observed in hTTRA97S mice are transversal to different cytoskeleton components, in which MT alterations were observed in parallel with the axonal transport defects.

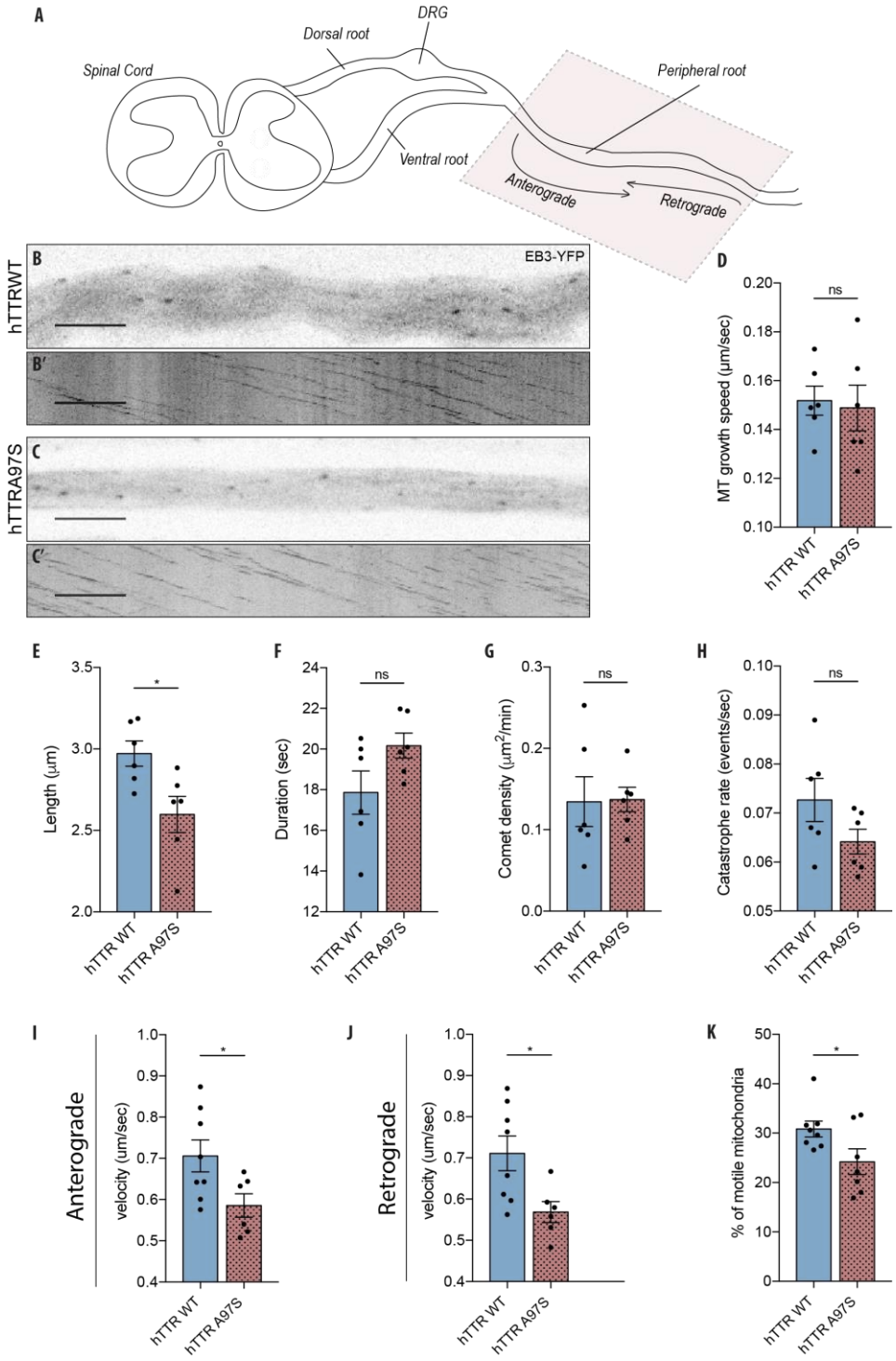

Figure 5. TTR A97S mice display impaired MT dynamics and mitochondrial axonal transport. (A) Schematic representation of the spinal cord with its dorsal, ventral and peripheral roots. Highlighted is the region in which live imaging was performed. **(B-C)** Representative still images of the peripheral root from 6-month-old hTTRWT-Thy1-EB3-YFP (B) and hTTRA97S-Thy1-EB3-YFP (C) mice and correspondent kymographs (B', C'). Scale bars: 5µm. **(D-H)** Quantifications of different microtubule (MT) dynamics parameters including MT growth rate (D), comet length (E), comet growth duration (F), EB3 comet density (G), and EB3 catastrophe frequency (H) from hTTRWT-Thy1-EB3-YFP and hTTRA97S-Thy1-EB3-YFP animals. Results are plotted as mean ± SEM (n = 6 animals/genotype). Statistical significance determined by Student's t-test: *P<0.05. ns, not significant. **(I-K)** Quantifications of the different parameters of axonal transport namely anterograde mitochondria velocities (I), retrograde mitochondria velocities (J) and percentage of total moving mitochondria (K). Results are plotted as mean ± SEM (n = 6-8 animals/genotype. Statistical significance determined by Student's t-test: *P<0.05.

DISCUSSION

Neuronal cytoskeleton dysregulation is a relevant cellular phenomenon as a neurodegeneration precursor and a common feature of many neurodegenerative disorders (Eira, Silva, Sousa, & Liz, 2016). In the case of FAP, although the dying-back axonal degeneration might suggest an initial disturbance of the distal cytoskeleton as a consequence of TTR deposition, the analysis of cytoskeleton alterations has never been addressed before. This work shows for the first time a TTR-induced dysregulation of different neuronal cytoskeleton components in FAP that precede neurodegeneration.

Our findings clearly suggest that TTR promotes an alteration of the actin cytoskeleton in the growth cone of DRG neurons, both, with the exogenous addition of TTR aggregates and in hTTRA97S neurons. As the effect seen in the cultures from the mouse model was similar to the one of exogenous addition of TTR, and considering that in FAP TTR is present extracellularly, our results suggest that, *in vitro*, TTR is present in the culture medium either expressed by the hTTRA97S neurons in culture or by Schwann cells (Li, Masliah, Reixach, & Buxbaum, 2011; Murakami, Ohsawa, Zhenghua, Yamamura, & Sunada, 2010). Nevertheless, intracellular effects of TTR on FAP neurodegeneration should not be discarded.

Although being exclusively present in growing neurons, the growth cone is a very valuable cellular structure to study neuronal cytoskeleton rearrangements or dysregulations. Both the MT and actin cytoskeletons are very well characterized in the growth cone in which they form specific morphological structures essential to growth cue recognition and growth re-orientation which, when altered, have consequences for the neuron. However, we should point out that we evaluated neuronal cytoskeleton in the growth cone, which does not exist in mature neurons *in vivo*, where TTR accumulates and contacts with the axon. Thus, it would be important to analyse the actin cytoskeleton in the axon shaft, *in vitro*, by quantification of F-actin levels and evaluation of the actin/spectrin membrane-associated periodic skeleton (MPS) abundance and organization (Unsain et al., 2018).

Our results highlight that RAGE, the receptor for TTR aggregates contributing to the FAP-related neurotoxicity (Sousa et al., 2000), mediates TTR-induced actin cytoskeleton remodelling. This receptor was previously shown to impact on actin cytoskeleton (Xiong et al., 2011) and one of the signalling pathways downstream to RAGE leads to activation of Rho GTPases, namely Rac1 and Cdc42 (Hudson et al., 2008). In fact, we show that actin cytoskeleton phenotype at the growth cone is mediated by Rac1 and, although this Rho GTPase is highly acknowledged as a lamellipodia promoting factor in actin cytoskeleton remodelling, Rac1 was shown to promote Nox-mediated oxidative stress and neuronal cell

death (Wilson et al., 2016), which is in agreement with our growth cone collapse phenotype. We additionally report that actin cytoskeleton alterations precede neurodegeneration in our *in vitro* neurodegeneration index experiments and that Rac1 silencing by blocking actin damage halts development of neurodegeneration. FRET experiments using hTTRA97S neurons should be performed in order to assess Rac1 activation *in vitro* and pull-down assays from hTTRA97S nerves would be important in further disclosing Rac1 involvement in FAP. It will also be crucial to evaluate Rac1 downstream effectors, such as LIMK, Cofilin, SSH1, and, PAK1/2/3 and ERK1/2, to detail their molecular mechanism underlying actin damage in FAP. The modulation of altered Rac1 downstream targets *in vivo*, would be detrimental to assess its impact in reverting neurodegeneration observed in hTTRA97S mice and contribute to the development of novel therapeutic strategies to prevent neurodegeneration in FAP.

The alterations observed in MT dynamics and axonal transport allow us to conjecture that the impact of TTR in the neuronal cytoskeleton may be transversal to other cytoskeletal components. Interestingly, these alterations were observed prior to neurodegeneration onset in the hTTRA97S mice which provide some important clues regarding the “cause or consequence” question whether cytoskeleton alterations precede neurodegeneration in FAP. Further work is necessary to dissect MT cytoskeleton involvement in FAP, which will include the analysis of MT density and tubulin post translational modifications and might explain the effect on axonal transport. Additionally, it will be important to evaluate MT dynamics and axonal transport in distal peripheral nerves, namely the sural nerve where degeneration was reported in the hTTRA97S model.

Although Rac1 is mostly recognized as an actin cytoskeleton modulator, it is worth speculating about the connection between Rac1 activation mediated actin cytoskeleton dysfunction, and MT dynamics modulation. In fact, studies have shown that Rac1 promotes oriented MT growth in the leading edge of migrating cells (Nishimura, Applegate, Davidson, Danuser, & Waterman, 2012) and, during cell polarization, Rac1 was shown to mark special cortical spots which allowed the polarized MT array formation (Fukata et al., 2002). As such, it would be important to further explore this causative role of Rac1 in modulating actin cytoskeleton, MT cytoskeleton, as well as the axonal transport.

In summary, this work unravels cytoskeleton damage in the hTTRA97S FAP mouse model, with a potential key impact in FAP pathophysiology. In the future we aim to dissect the impact of cytoskeleton disruption on neurodegeneration in FAP, with the ultimate goal of establishing novel therapies targeting the neuronal cytoskeleton as means to halt the progression of the disease.

MATERIALS AND METHODS

Animals. Mice were handled according to European Union and National rules. All animals were maintained under a 12-hour light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. Genotypes were determined from ear extracted genomic DNA. Adult wild type (WT) mice in a 129/Sv background were used for experiments with exogenous addition of TTR. For MT dynamics experiments, Thy1-EB3-YFP animals were crossed with hTTRWT and hTTRA97S homozygous mice.

Recombinant TTR production, purification and TTR L55P aggregates production. Recombinant WT and L55P TTR were produced using a pETF1 vector carrying human WT TTR and L55P TTR (Goldsteins et al., 1997) and used in the transformation of competent *Escherichia coli* BL21(DE3) cells. The protein was expressed as described in Silva et al., 2017 and purified through successive ionic exchange, hydrophobic interaction and size exclusion chromatographies. For cellular assays, recombinant TTR was detoxified using a high-capacity endotoxin removal resin (Thermo Scientific) and quantified using the Lowry based DC Protein Assay (Bio-Rad Laboratories). To produce aggregates, recombinant TTR L55P was applied to a dialysis membrane of 1 kDa (Spectrum Labs) and dialysed against water overnight at 4ºC, with agitation. Aggregated insoluble protein was, then, centrifuged at top speed for 20 minutes, at 4ºC. The pellet was resuspended in pyrogen-free PBS and protein content was quantified using the Lowry method. TTR aggregates were aliquoted and stored at -80ºC in "single use" aliquots. Protein aggregation was assessed by transmission electron microscopy and fluorescence spectroscopy assays. Briefly, aggregated TTR L55P was placed on a nickel grid for 1 minute and after performing the negative staining with of 2% uranyl acetate, samples were visualized under the JEOL JEM-1400 transmission electron microscope, operating at 120 kV. Images were acquired at 50000x and 100000x magnification. For the fluorescence spectroscopy assay, 0.03 mM Thioflavin T (Th-T, Sigma-Aldrich), diluted in glycine (Merck Millipore) (Naiki, Higuchi, Hosokawa, & Takeda, 1989). Th-T was added to TTR, diluted in distilled water, and the fluorescent signal (emission: 482 nm; excitation: 400-460 nm) was measured in a Synergy Mx microplate reader (BioTek), using the Gen5 software.

Primary DRG neuronal cultures, transfection and treatments. Mice were humanely euthanized through CO_2 inhalation, and DRG neurons were collected and cultured as described in Liz et al., 2014. For axonal swelling evaluation and growth cone morphology assessment with exogenous addition of TTR, WT DRG neurons from 4-week-old mice were plated in 20 µg/ml PLL + 5 µg/ml Laminin coated 24-well plates at a density of 5000

cells per well in DMEM/F12 (Sigma-Aldrich, D8437) supplemented with 1× B27 (Gibco), 1% penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 50 ng/mL NGF (Millipore, 01-125) at 37ºC and 5% CO_2. 4 h after plating, DRG were treated either with WT soluble TTR or aggregated TTR L55P (equal volume of PBS for the control condition) at a concentration of 300 µg/ml and incubated for 12 h at 37ºC and 5% CO_2 before fixing. For the RAGE inhibition assays, the RAGE isoform, soluble RAGE (sRAGE) (Monteiro, Cardoso, Sousa, & Saraiva, 2006), was co-added with TTR at a concentration of 3 µg/mL, 4 h post-plating, for 12 h. sRAGE was kindly provided by the laboratory of Dr Maria João Saraiva (Molecular Neurobiology group, i3S/IBMC). For actin retrograde flow and Rac1 activation experiments, WT DRG neurons from 4-week-old mice were transfected using the 4D Nucleofector Amaxa system (Lonza, Barcelona, Spain, CM#137 program) and cells were transfected at a density of at least 200.000 cells/condition. For assessment of actin dynamics, DRG neurons were nucleofected with 750 ng of lifeact-GFP plasmid, which successfully stains filamentous actin (F-actin), without interfering with cytoskeletal dynamics (Riedl et al., 2008). For the FRET probe experiments, DRG neurons were transfected with 1 ug of Raichu Rac1 plasmid (Itoh et al., 2002). Cells were left in suspension for 24 h in complete medium at 37ºC and 5% CO_2, and were, subsequently, plated in 20 µg/ml PLL + 5 µg/ml Laminin coated 35 mm µ-dishes (iBidi) at a density of 15000 cells per dish in phenol-free DMEM/F12 with its supplementation. For lifeact experiments, 4 h after plating, DRG were treated either with WT TTR or with TTR L55P aggregates at a concentration of 300 µg/ml and incubated for 12 h at 37ºC and 5% CO_2 for 12 h before performing live imaging. For hTTRWT and hTTRA97S DRG cultures, 2-3-month-old animals were used. For growth cone morphology assessment, hTTRWT and hTTRA97S DRG neurons were cultured as described above and plated in 20 µg/ml PLL + 5 µg/ml Laminin coated 24-well plates at a density of 5000 cells per well for 24 h at 37ºC and 5% CO_2. For transfection-mediated Rac1 silencing, hTTRWT and hTTRA97S DRG neurons were transfected at a density of at least 200000 cells/condition with 0.75 µg of the shRNA Rac1 (TRCN00000551888, Sigma-Aldrich) or shRNA scramble (Sigma-Aldrich) and 1.125 µg of EGFP-C1 plasmid. Cells were left in suspension for 24 h in complete medium at 37ºC and 5% CO_2, and were, subsequently, plated in 20 µg/ml PLL + 5 µg/ml Laminin coated 24-well plates at a density of 5000 cells in supplemented DMEM/F12 for 24 h. For neurodegeneration index assessment, hTTRWT and hTTRA97S DRG neurons were plated in 20 µg/ml PLL + 5 µg/ml Laminin coated 24-well plates at a density of 10000 cells in DMEM/F12 supplemented with 1× B27, 1% penicillin/streptomycin, 2 mM L-glutamine, 50 ng/mL NGF, and 60 µM 5-Fluoro-2'-deoxyuridine (FluoU) at 37ºC and 5% CO_2. At DIV2 medium was changed and cultures were treated with 50 µM of Rac1 inhibitor NSC23766 (Tocris) and at DIV4 cells were fixed.

Immunocytochemistry. For axonal swelling assessment and neurodegeneration index evaluation, DRG neurons were fixed with 4% PFA, blocked with 5% normal donkey serum (NDS) containing 0,4% Tween for 1 h at RT, incubated with mouse anti–βIII-tubulin (1:2000; Promega, G7121) overnight at 4ºC followed by the incubation of the secondary antibody (donkey anti-mouse Alexa-Fluor 488, 1:1000 Alfagene, A21202) for 1 h at RT. For growth cone morphology assessment, DRG were fixed with cytoskeleton preservation fixative, PHEM fixative (4% PFA, 4% sucrose, 0.25% Glutaraldehyde, 0.1% Triton X-100, 300 mM PIPES, 125 mM HEPES, 50 mM EGTA and 10 mM Magnesium Chloride), permeabilized with 0.2% Triton X-100 for 5 min, quenched with 200 mM Ammonium Chloride for 5 min and blocked with 2% Fetal Bovine Serum (FBS), 2% BSA and 0,2% Fish Gelatine in PBS for 1 h at RT. Incubation of primary antibody mouse anti–βIII-tubulin (1:2000; Promega, G7121) was performed in 10% blocking buffer overnight at 4ºC. Incubation of the secondary antibody (donkey anti-mouse Alexa-Fluor 488, 1:1000 Alfagene, A21202) was performed in 10% blocking buffer for 1 h at RT. Actin was labelled with the probe-conjugated dye Rhodamine-conjugated Phalloidin (1:100, Life Technologies, R415), in parallel with secondary antibody. Cells were mounted in Fluoromount (Southern Biotech).

Image acquisition and image analysis. For axonal swellings assessment image acquisition of βIII tubulin stained DRG neurons was performed using a Leica DMI 6000B inverted microscope with an ORCA-Flash4.0 V2 C11440-22CU digital camera and Leica Application Suite Advanced Fluorescence (LAS AF) software, using a HCX PL APO 63X 1.30NA. Axonal swellings were assessed as swelled structures along the neurites. 72-201 neurons were assessed per condition and the proportion of cells with axonal swellings was measured. For growth cone morphology assessment, images were acquired using an epifluorescence microscope Zeiss Axio Imager Z1 microscope with an Axiocam MR3.0 camera and Axiovision 4.7 software, using a Plan-Apo 63X 1.4NA objective. Growth cones from DRG neurons labelled with βIII-tubulin and phalloidin were qualitatively categorized according to its MT and actin patterns. MT pattern was divided into two categories: the normal (Fig. 6A, B, and C), in which MTs display either a splayed or bundled conformation able to protrude into the growth cone, and the dystrophic pattern, in which MTs form a dystrophic bulb at the tip of the axon (Fig. 6D and E). The actin category was also divided into two categories: the splayed pattern containing the typical lamellipodia and filopodia structures, organized in a star-shaped morphology (Fig. 6A and B) and the retracted pattern characterized especially by the absence of lamellipodia as well as growth cones with dystrophic actin morphologies, such as actin patches (Fig. 6C, D and E). Approximately, 100 growth cones were analysed in each condition using

ImageJ software. For Neurodegeneration index analysis, βIII-tubulin labelled DRG neurons were used to acquire 15 representative images of each condition using an epifluorescence microscope Zeiss Axio Imager Z1 microscope with an Axiocam MR3.0 camera and Axiovision 4.7 software, using an EC Plan Neofluar 20X 0.50NA objective. To assess axonal fragmentation through neurodegeneration index quantification, the area occupied by the axons (total axonal area) and degenerating axons (fragmented axonal area) was analysed using the particle analyser algorithm of ImageJ (size of small fragments = 20–10,000 pixels). Degeneration index was calculated as the ratio between fragmented axonal area and total axonal area.

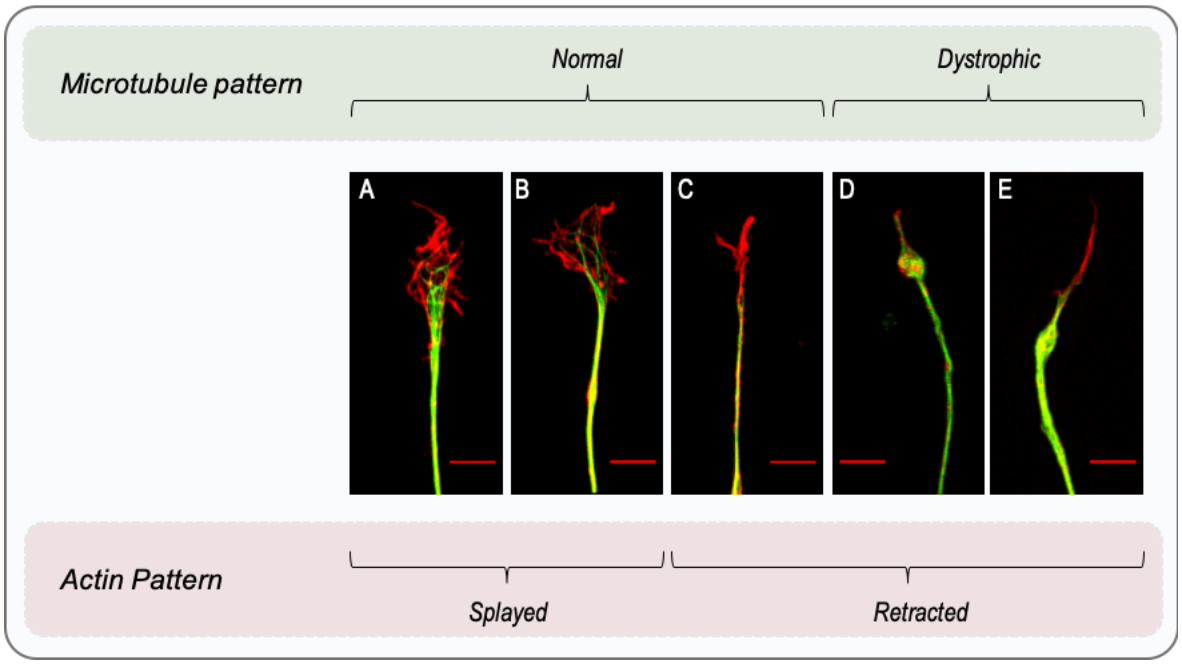

Figure 6. Growth cone morphology categorization for both MT and actin patterns. MT pattern was divided into two categories: the normal with splayed or bundled pattern in which MTs able to protrude into the growth cone, and the dystrophic pattern, in which MTs form a dystrophic bulb at the tip of the axon. The actin category was also divided into two categories: the splayed pattern containing the typical lamellipodia and filopodia structures, organized in a star-shaped morphology and the retracted pattern characterized especially by the absence of lamellipodia as well as growth cones with dystrophic actin morphologies, such as actin patches. Scale bars: 5 μm.

FRET experiments and image analysis. For live cell imaging, DRG neurons were plated on plastic-bottom culture dishes (μ-Dish 35 mm, iBidi). Before imaging, medium was changed to pre-warmed non-supplemented DMEM/F12 without phenol red. Imaging was performed using a Leica DMI6000B inverted microscope. High-speed low vibration external filter wheels (equipped with CFP/YFP excitation and emission filters) were mounted on the microscope (Fast Filter Wheels, Leica Microsystems). A 440-520 nm dichroic mirror (CG1, Leica Microsystems) and an HCX PL APO 63X 1.3NA glycerol

immersion objective were used for CFP and FRET images. Images were acquired with 2×2 binning using a digital CMOS camera (ORCA-Flash4.0 V2, Hamamatsu Photonics). Shading illumination was online-corrected for CFP and FRET channels using a shading correction routine implemented for the LAS AF software. At each time-point (1-minute timepoints for 50 minutes), CFP and FRET images were sequentially acquired using different filter combination (CFP excitation plus CFP emission (CFP channel), and CFP excitation plus YFP emission (FRET channel), respectively). 10 minutes after the initial acquisition, TTR L55P aggregates were added to the cells at a concentration of 300 µg/mL and acquisition was resumed for an additional period of 40 minutes. Quantification of biosensors was performed as described. Briefly, images were exported as 16-bit tiff files and processed in Fiji software. Background was dynamically subtracted from all frames from both channels. Segmentation was achieved on a pixel-by-pixel basis using a modification of the Phansalkar algorithm. After background subtraction and thresholding, binary masks were generated for the CFP and FRET images. Original CFP and FRET images were masked, registered and bleach-corrected. Ratiometric images (FRET/CFP for Raichu-Rac1) were generated as 32-bit float-point tiff images. Values corresponding to the mean gray values were generated using the multi calculation function in FIJI and exported.

Live imaging. For the analysis of actin retrograde flow, lifeact transfected DRG neurons were recorded for 2 min (60 frames total) in phenol-free DMEM/F12 supplemented as mentioned above, at 37°C and 5% CO_2, on a Spinning Disk Confocal System Andor Revolution XD with an iXonEM+ DU-897 camera, a IQ 1.10.1 software (ANDOR Technology) and using a UPLSAPO 100X 1.4NA objective. For the *ex vivo* imaging, DRG explants from hTTRWT-Thy1-EB3-YFP and hTTRA97S-Thy1-EB3-YFP were collected from 6 months old animals and placed in 35 mm μ-Dish (ibidi) with phenol-free DMEM/F12 and recordings were performed in the peripheral roots as described above. For both the quantification of the actin retrograde flow and the different EB3 dynamics parameters, kymographs were made using the Fiji KymoResliceWide plugin (distance, x axis; time, y axis). Starting and end positions of the traces, were defined using the Fiji Cell Counter plugin and coordinates were exported for subsequent analysis.

Rac1 silencing validation. For Rac1 silencing validation by Western Blot, N1E-115 cells (Sigma-Aldrich), an adrenergic cell line derived from the mouse neuroblastoma C1300 tumour, were grown in DMEM supplemented with 2 mM L-glutamine, 10% FBS and 1% P/S in uncoated 24-well plates at a density of 150000 cells/well. At DIV1, cells were transfected with 0.375 µg of the shRNA Rac1 (TRCN00000551888, Sigma-Aldrich) or

shRNA scramble (Sigma-Aldrich) and 0.25 µg of EGFP-C1 plasmid using lipofectamine 2000. At DIV3, protein extracts were prepared in lysis buffer (0.3% Triton X-100, 1x protease inhibitor Cocktail and 1 mM Sodium orthovanadate in PBS). 20 ug of protein extracts were separated under denaturing conditions in a 12% agarose gel, transferred to Amersham Protran Premium 0.45 µm nitrocellulose membranes (GE Healthcare Life Sciences), and blocked in 5% non-fat dried milk in TBS-T for 1 h at room temperature. Membranes were probed overnight at 4^{o}C with primary antibody mouse anti-Rac1 (1:2000, Abcam, ab33186) and with secondary antibody in 5% non-fat dried milk in TBS-T for 1 h at room temperature anti-mouse IgG-HRP 1:10000 (Jackson Research, 115-035-003). Immunodetection was performed by chemiluminescence using ECL (Millipore, WBLUR0500) and quantified using ImageJ software.

For Rac1 silencing validation by immunohistochemistry, shRNA scrambled and shRNA Rac1 transfected DRG were fixed in 4% PFA, permeabilized with 0.2% Triton X-100 for 5 min, quenched with 200 mM Ammonium Chloride for 5 min and blocked with 3% BSA in PBS for 1 h at RT. Incubation of primary antibody mouse anti-rac1 (1:250, ab33186, Abcam) and rabbit anti-βIII-tubulin 1:1000 was performed in 1% BSA in PBS overnight at 4ºC. Incubation of the secondary antibodies donkey anti-mouse Alexa-Fluor 647 and donkey anti-rabbit Alexa-Fluor 568 (1:1000, Alfagene) was performed in 1% BSA in PBS 1h at RT. Cells were mounted in Fluoromount (Southern Biotech). Images were acquired using an epifluorescence microscope Zeiss Axio Imager Z1 microscope with an EC Plan-Neofluar 40X 1.3NA objective.

MitoTracker® labelling, live imaging and data analysis. For live imaging, DRG explants from hTTRWT and hTTRA97S mice were collected from 9-month-old animals and incubated with 50 nM MitoTracker® orange (Thermofisher) in phenol-free DMEM/F12 for 30 min at 37ºC. Next, DRG explants were placed in a 35 mm µ-Dish (iBidi) with phenol-free DMEM/F12 and live imaging was performed. Acquisition was performed by confocal microscopy on a Leica TCS SP5 II with LAS AF software using a HC PL APO CS 40X 1.1NA objective. Recordings were performed in the peripheral roots for 5 min (150 frames total). Image analysis was performed using Fiji MtrackJ plugin. For mitochondria speed, only mitochondria that could be tracked for at least 5 consecutive time frames were analysed. A minimum of 50 mitochondria were analysed per condition.

Statistics. All statistical tests were performed using GraphPad Prism 7 software. Data are shown as mean ± s.e.m. Unless otherwise stated, the Student's t test was used. Statistical tests and sample sizes are indicated in figure legends and significance was defined as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns - not significant.

AUTHOR CONTRIBUTIONS:

Figure 1: Eira J performed the experiments and data analysis.

Figure 2: Eira J performed the experiments and data analysis.

Figure 3: Eira J performed the experiments. Eira J and Socodato R performed data analysis.

Figure 4: Magalhães J performed the experiments and data analysis.

Figure 5: Eira J and Magalhães J performed the experiments and data analysis.

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CHAPTER IV

Proteomic analysis: Identification of novel candidates underlying TTR impact on neuronal pathophysiology

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ABSTRACT

Transthyretin (TTR) regulates nerve biology and impacts on neurodegeneration. Under physiological conditions, TTR participates in nerve biology by increasing axonal transport, axon growth and regeneration, which we recently showed to be related to a role on modulation of MT dynamics. When mutated, TTR causes familial amyloid polyneuropathy (FAP), a neurodegenerative disease characterized by the deposition of TTR amyloid fibres, particularly in the peripheral nervous system (PNS), resulting in a dying-back axonopathy that culminates in neuronal death. The cytoskeleton is crucial for neuronal function including axonal transport and growth and, when disrupted, it is associated with neurodegeneration. In fact, recent findings from our group have shown that physiologically TTR modulates MT stability and that cytoskeleton damage occurs in a hTTRA97S FAP mouse model.

Aiming at dissecting the molecular mechanisms underlying TTR impact on the neuronal cytoskeleton, we performed a quantitative proteomics assay to pinpoint molecular targets that are differentially expressed in the presence of either WT soluble TTR or TTR L55P aggregates. Our results allowed the identification of different candidates, related not only with cytoskeleton regulation but also with molecular functions involved in catalytic processes, binding and transport activities.

Altogether, our findings may contribute in the future to determine possible molecular mechanisms in the impact of TTR on neuronal pathophysiology.

INTRODUCTION

Transthyretin (TTR), is a homotetrameric protein mainly synthesized in the liver and the choroid plexus of the brain that functions as a transporter of thyroxine (T4) and retinol (vitamin A), in the latter case by forming a complex with the retinol binding protein (RBP). TTR has also been assigned with a function in nerve biology and repair, enhancing neurite outgrowth of dorsal root ganglia (DRG) neurons *in vitro,* nerve regeneration *in vivo* (Fleming, Saraiva, & Sousa, 2007) and neuroprotection in Alzheimer's disease (AD) contexts (Costa, Ferreira-da-Silva, Saraiva, & Cardoso, 2008; Silva et al., 2017).

The enrichment in β-sheets provides TTR its intrinsic predisposition to self-aggregate, resulting in the formation of oligomers and fibrils, which deposit as amyloid in tissues (Westermark, Sletten, Johansson, & Cornwell, 1990) and reviewed in Saraiva, Magalhaes, Ferreira, & Almeida, 2012. Familial amyloid polyneuropathy (FAP) is an autosomal dominant neurodegenerative disease, first described in Portugal by Corino de Andrade (Andrade, 1952) and is characterized by the extracellular deposition of mutated TTR oligomers, aggregates and amyloid fibrils, mainly in the peripheral nervous system (PNS), resulting in a dying-back axonopathy and culminating in neuronal death (Plante-Bordeneuve & Said, 2011).

Our group obtained data suggesting an impact of TTR in the neuronal cytoskeleton that is related both to its neuritogenic activity and its neurodegeneration role in FAP. On one hand we unravelled a new role for TTR on the modulation of microtubule (MT) dynamics by regulating α-tubulin acetylation levels, and on the other hand, using DRG neurons treated with TTR aggregates or a FAP mouse model we disclosed a TTR-induced actin cytoskeleton dysregulation mediated by Rac1. However, the specific details underlying these pathways are still not fully clarified. Additionally, there might be additional mechanisms and molecular players by which TTR is exerting its effect in general nerve biology. In this respect, we performed quantitative proteomic analysis (tandem mass tag (TMT) proteomics) using a PNS cell line – N1E-115 – treated either with WT soluble TTR or TTR L55P aggregates. The candidates differentially regulated by either physiologic or pathologic TTR will be pinpointed as valuable targets for future investigation. This will open new avenues for the study of signalling partners of validated molecules and disclose new pathways in which TTR is involved.

RESULTS

N1E-115 CELL LINE: INNOVATIVE TOOL TO STUDY MOLECULAR PLAYERS IN TTR-INDUCED CYTOSKELETON REMODELLING

We used N1E-115 cells, a mouse-derived neuroblastoma cell line, either untreated or treated with WT soluble TTR or TTR L55P aggregates. Under differentiating conditions, N1E-115 cells start to flatten and to extend neurites, resembling the first stages of neuronal polarization and differentiation observed in primary hippocampal neurons (Sayas et al., 2015). This cell line is of significant relevance for our studies since cytoskeleton proteins are documented to be differentially expressed in undifferentiated and differentiated N1E-115 cells suggesting not only a rearrangement of the cytoskeleton during differentiation, but also that there is a concerted action of cytoskeleton proteins in the differentiation process (Oh et al., 2006). Furthermore, this neuronal cell line presents itself as a beneficial model compared to the primary neuronal cultures since it consists in a pure neuronal culture as opposed to the primary DRG cultures which are mixed cultures of both neuronal and glial cells, thus being less ideal for proteomic analysis. The N1E-115 neuronal cell line provides, also, a higher protein content in the total protein extracts after lysis and this cell culture does not require a BSA gradient, as in the DRG neuronal cultures, thereby minimizing protein contamination.

We started by examining the differentiation morphology of N1E-115 cells. N1E-115 cells differentiation is accomplished with two methods, either with overnight serum deprivation (Sayas et al., 2015) or with DMSO treatment (Oh et al., 2006). In this work we used the DMSO-mediated differentiation method. As shown in Figure 1, undifferentiated N1E-115 cells were differentiated from a round morphology (Figure 1A) into a morphologically neuronal phenotype with neurite outgrowth, labelled with the neuronal marker, βIII-tubulin (Figure 1B, C).

We further aimed to validate the impact of TTR in these differentiated cells by assessing, likewise to our model with DRG neurons, the growth cone morphology of differentiated N1E-115 cells incubated with TTRL55P aggregates and the MT dynamics of EB3-GFP transfected differentiated N1E-115 cells treated with WT soluble TTR. While no impact was observed in actin cytoskeleton remodelling at the growth cone of TTRL55P aggregates treated N1E-115 cells (data not shown), we were able to recapitulate the impact of TTR on MT dynamics with the increased EB3 growth rate at the growth cone (Figure 1D, E). It is important to mention that, although the differentiated N15-115 cells displayed a beautiful assembly of a growth cone with the typical MTs and actin patterns (Figure 1F), most neuronal immortalized cell culture models are derived from tumours and

are sometimes genomically abnormal. Thus, one cannot set aside that these cells may present features that by some reason makes the growth cone unsensitive to TTR aggregates but will respond to the TTR aggregates induced impact in the cytoskeleton or in neuropathology through other cellular responses.

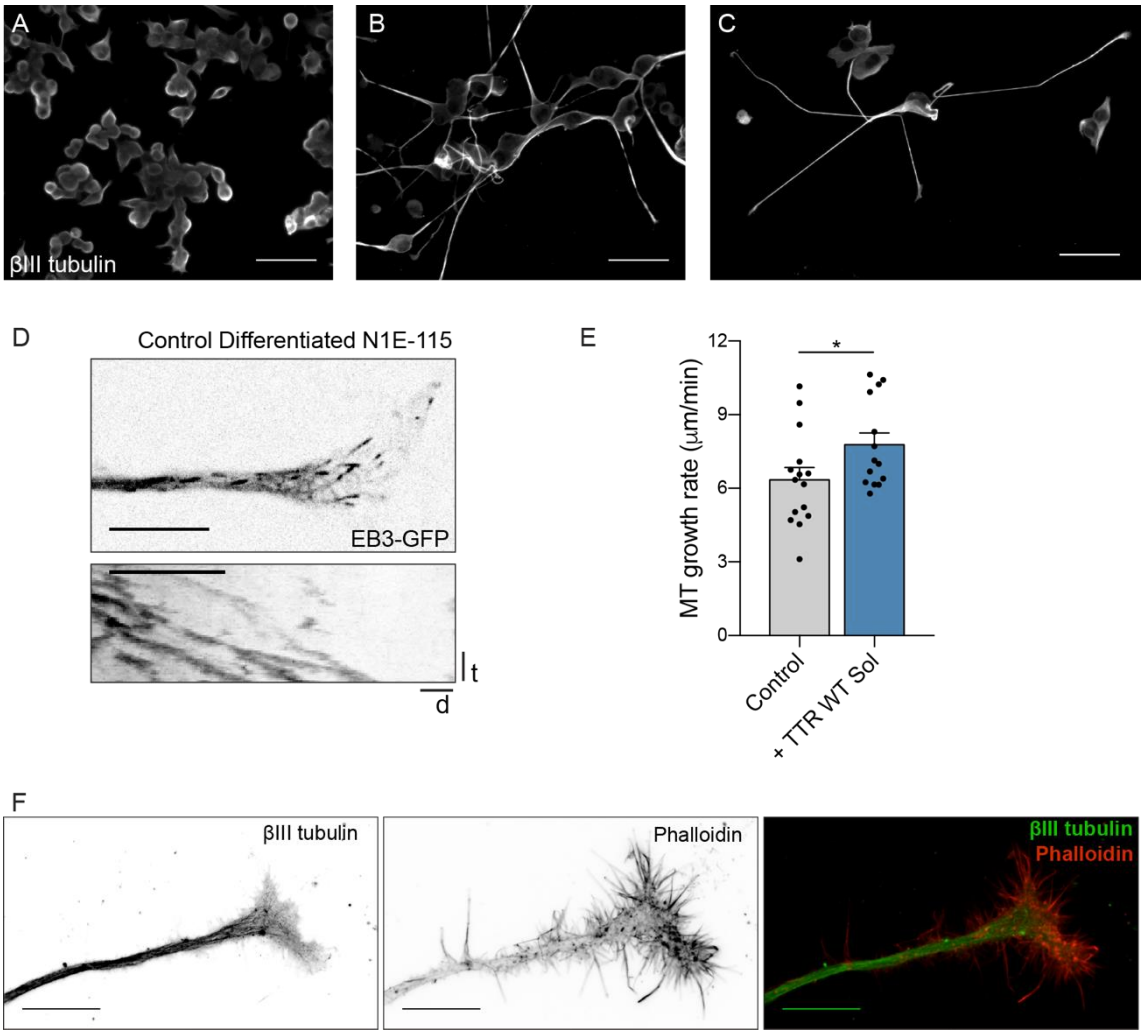

Figure 1. N1E-115 cells differentiate into βIII-positive neuron like cells sensitive to TTR on MT dynamics modulation. (A-C) Morphological difference between undifferentiated (A) and differentiated (B, C) N1E-115 cells. Scale bars: 100 μm. **(D)** Representative still images of the growth cones from a differentiated control N1E-115 cell transfected with EB3-GFP (top) and correspondent kymograph (bottom) **(E)** Quantification of MT growth rate related to D. Results are plotted as mean ± SEM (n = 14-15 neurons/condition representative of 2 independent experiments). Statistical significance determined by Student's t-test: *P<0.05, Scale bars: 10 μm in the still image, 5 μm in the kymograph. **(F)** Representative image of a growth cone from a differentiated N1E-115 cell immunolabelled for βIII tubulin and Phalloidin. Scale bars: 25 μm.

PROTEOMIC ANALYSIS REVEALS NOVEL MECHANISMS UNDERLYING THE IMPACT OF TTR ON NEURONAL CELLS

In order to identify novel molecular candidates and further unravel the mechanism behind the impact of TTR on neuronal cytoskeleton, we performed quantitative proteomic analysis performed at the Mass Spectrometry and Proteomics Core Facility of IRB Barcelona using tandem mass tag (TMT) proteomics. TMT, also known as isobaric tag, is a quantitative method based on the chemical labelling of peptides by incorporation of distinct isotopic labels, followed by tandem mass spectrometry (MS/MS) (Rauniyar & Yates, 2014; Thompson et al., 2003).

The TMT proteomics allows the identification of all the proteins differentially regulated in the presence of WT soluble TTR or TTR L55P aggregates, in a single assay. To select proteins that are mediating the effect promoted by WT soluble TTR, we selected candidates differentially regulated/expressed when compared to untreated cells and to cells treated with TTR aggregates, and to select proteins involved in the effect induced by TTR L55P aggregates, we selected candidates differentially regulated/expressed when compared to untreated cells and to cells treated with WT soluble TTR. Candidates were further selected according to the displayed differences (p-value<0.05; 0.7<fold change<1.3).

The results obtained from this proteomic assay, provided a list of new candidates, with molecular functions involved in catalytic processes, binding and transport activities. We used *PANTHER – gene list analysis* software (http://www.pantherdb.org) to divide our candidates according either to their molecular function or protein class (Appendix, Figures 2 and 3).

A total of 22 proteins were differentially regulated by TTR L55P aggregates, comparing with control condition and WT soluble TTR. Table 1 summarizes the proteins differentially regulated, in this case all of them were upregulated, in the presence of TTR L55P aggregates according to their protein class.

Signal transducer and activator of transcription 2 (STAT2) was the most upregulated candidate (5-fold) in our proteomics assay and appeared in the “gene-specific transcriptional regulator” category. This protein is a signal transducer and transcription activator that mediates signalling via type I interferons and a player of the JAK-STAT signalling pathway. STAT2 is the predominant signalling pathway used by cytokines, orchestrating adaptive immune mechanisms and, ultimately constraining inflammatory and immune responses (Benveniste, Liu, McFarland, & Qin, 2014; O'Shea & Plenge, 2012). Recent studies implicated STAT2 as a regulator of mitochondria dynamics as mutations in

STAT2 are associated to mitochondrial diseases with defects in mitochondria fission processes and mitochondria morphology defects (Shahni et al., 2015).

Transcription factor Dp-1, which is 3.65-fold upregulated and categorized in the "gene-specific transcriptional regulator" category, stimulates E2F-dependent transcription (Helin et al., 1993) and is involved in cellular processes such as NOXA activation and translocation to mitochondria, and oxidative stress-induced senescence.

BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3 (1.6-fold upregulation), which appears in the "scaffold/adaptor protein" category, is involved in protein ubiquitination pathways but has important roles as a negative regulator of Rho protein signal transduction (Chen et al., 2009). Still in the in the "scaffold/adaptor protein" category appears Sorting Nexin 17, which is 2-fold upregulated and is a protein involved in the recycling of numerous surface proteins, namely integrins and signal receptors (Steinberg, Heesom, Bass, & Cullen, 2012; Williams et al., 2004), namely LRP1 (Donoso et al., 2009). Additionally, Sorting Nexin 17 was shown to bind the APP cytoplasmic domain regulating APP endocytic trafficking and processing to Aβ (Lee et al., 2008).

Calcium-transporting ATPase type 2C member 1 came out upregulated (1.38-fold increase) and, despite mainly acknowledged by its ATPase activity, is associated to actin cytoskeleton reorganization processes in keratinocytes (Aronchik et al., 2003).

A small list of proteins remained unsorted according to its protein class. It is however important to mention two candidates that presented themselves more than 3-fold upregulated in the presence of TTR L55P aggregates. Transmembrane protein 164 is associated with neurologic conditions such as autistic disorder and Non-Syndromic X-Linked Intellectual Disability. Other protein significantly activated in the presence of TTR L55P aggregates (4-fold) is Rab-like protein 3 and is involved in the Ras protein signalling modulation (Nissim et al., 2019). Interestingly, Ras signalling has Rac1 as one possible downstream effector.

Table 1. List of proteins differentially regulated by TTR L55P aggregates selected from the TMT proteomics assay and categorized by PANTHER according its protein class. Proteins are selected based on their regulation levels in the presence of TTR L55P aggregates, compared with the control condition and with WT soluble TTR.

Candidates *Gene-specific transcriptional regulator*	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Transcriptional repressor CTCF	0,99	**1,33**

Transcription factor Dp-1	1,56	**3,65***
Signal transducer and activator of transcription 2	1,09	**5,07***
Retinoic acid receptor RXR-beta	0,96	**1,51**
Zinc finger protein 37	0,95	**1,42**
GON-4-like protein	1,10	**1,71**

Candidates <u>*Metabolite interconversion enzyme*</u>	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Glucosamine-6-phosphate isomerase 2	1,06	**1,58**

Candidates <u>*Chaperone*</u>	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Immunoglobulin-binding protein 1	1,02	**2,16**

Candidates <u>*Nucleic acid metabolism protein*</u>	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
DNA (cytosine-5)-methyltransferase 3B	0,94	**2,15**

Candidates <u>*Protein modifying enzyme*</u>	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Death-associated protein kinase 1	1,02	**1,36**

Candidates <u>*Scaffold/adaptor protein*</u>	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3	1,00	**1,67**
Sorting nexin-17	1,13	**2,09**

Candidates <u>*Transporter*</u>	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Calcium-transporting ATPase type 2C member 1	0,97	**1,38**
Zinc transporter ZIP6	1,01	**1,86**

Candidates <u>*Unsorted*</u>	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*

Luc7-like protein 3	1,01	**1,49**
E3 ubiquitin-protein ligase RNF25	0,95	**1,41**
Rab-like protein 3	0,99	**4,11***
Translocon-associated protein subunit delta	0,97	**1,56**
F-box/LRR-repeat protein 8	1,18	**2,10**
Transmembrane protein 164	1,07	**3,30***
Protein PET117 homolog, mitochondrial	0,99	**1,50**
Ropporin-1-like protein	0,95	**1,53**

A total of 8 candidates were found to be differentially regulated by WT soluble TTR, comparing with the control condition and TTR L55P aggregates. Table 2 summarizes the proteins differentially regulated, in the presence of WT soluble TTR according to their protein class.

Regarding upregulated proteins, Small G protein signalling modulator 3 (1.34-fold upregulated) was included in the “protein-binding activity modulator” category. This protein is mainly associated to its GTPase activator and Rab GTPase binding activities. Alterations in Rab GTPases, or the membrane compartments they regulate, are associated with virtually all neuronal activities in health and disease (Kiral, Kohrs, Jin, & Hiesinger, 2018).

HSPB1-associated protein 1 (HSPBAP1) (1.34-fold upregulated) binds to one of the small heat shock proteins, specifically hsp27. Hsp27 is involved with cell growth and differentiation. HSPBAP1 was found to be abnormally expressed in patients with intractable epilepsy, although how brain function is affected remains unknown (Xi et al., 2007).

Proteins that were found to be downregulated include Tyrosine-protein phosphatase non-receptor type 3 (0.65-fold downregulated) with a role in cytoskeletal protein binding acting at junctions between the membrane and the cytoskeleton, Mdm2-binding protein (0.66-fold downregulated), which regulates cell cycle arrest and regulates protein ubiquitination, and histidine protein methyltransferase 1 homolog (0.68-fold downregulated), which is a heat shock protein binder and presents histidine methyltransferase activity.

Table 2. List of proteins differentially regulated by WT soluble TTR selected from the TMT proteomics assay and categorized by PANTHER according its protein class. Proteins are selected based on their regulation levels in the presence of WT soluble TTR, compared with the control condition and with TTR L55P aggregates.

Candidates *Metabolite interconversion enzyme*	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Adrenodoxin, mitochondrial	**0,66**	0,98
Pyridine nucleotide-disulfide oxidoreductase domain-containing protein 1	**1,63**	1,20

Candidates *Protein modifying enzyme*	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Tyrosine-protein phosphatase non-receptor type 3	**0,65**	0,93

Candidates *Protein-binding activity modulator*	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Small G protein signalling modulator 3	**1,34**	0,91

Candidates *Transmembrane signal receptor*	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
Progestin and adipoQ receptor family member 3	**0,70**	0,97

Candidates *Unsorted*	*Fold change*	
	TTR WT sol / Control	*TTR L55P agg / Control*
HSPB1-associated protein 1	**1,34**	1,07
Mdm2-binding protein	**0,66**	1,15
Histidine protein methyltransferase 1 homolog	**0,68**	1,17

Ultimately, the differentially regulated molecules pinpointed by the proteomic analysis will be validated by immunoblotting and/or immunocytochemistry in DRG neuronal cultures. Particular attention will be given to the proteins having cytoskeleton-related function, but if other interesting hits are disclosed, these will be further analysed.

DISCUSSION

The proteomics assays allowed the identification of novel candidates that might be implicated in the mechanism behind the impact of TTR on neuroprotection and neurodegeneration. The results from TMT proteomics provided a list of all the proteins differentially regulated in the presence of TTR, included in categories such as “protein-binding activity modulator”, “scaffold/adaptor protein”, “transporter”, and “metabolite interconversion enzyme”.

Some candidates display functions indirectly linked to cytoskeletal regulation, which need further validation. Particularly, BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3 (BACURD3), which is upregulated in the presence of TTR L55P aggregates, belongs to a family of proteins responsible for the degradation of RhoA, by forming a complex with Cullin 3 (Cul3), controlling actin cytoskeleton structure and cell movement (Chen et al., 2009). This candidate is particularly interesting since we have observed altered actin cytoskeleton in neurons using a FAP mouse model and we have shown that these actin alterations are mediated by Rho GTPases.

STAT2 was the most upregulated candidate (5-fold) in our proteomics assay. It is a signal transducer of the JAK-STAT signalling pathway. The JAK-STAT pathway was already shown to be activated in α-synuclein exposed neurons and dysregulation of this pathway has pathological implications for neuroinflammatory diseases (Benveniste et al., 2014; O'Shea & Plenge, 2012). Recent work has shown in a α-syn-induced neuroinflammation and neurodegeneration model that inhibition of the JAK/STAT pathway exerts a beneficial effect in suppressing neuroinflammatory immune responses (Qin et al., 2016). Other studies have shown that STAT2 mutations lead to neurological disorders that display mitochondria dynamics deficits. These findings implicated STAT2 as a novel regulator of the mitochondrial fission protein DRP1 and opened a therapeutic avenue for neurological disorders such as Alzheimer’s, Huntington’s and Parkinson’s diseases, in which mitochondrial morphology has been implicated in disease pathogenesis. In this regard, and given our mitochondria dynamics alterations (Chapter III) in a FAP mouse model, we should give particular attention to this candidate.

An additional interesting candidate is sorting nexin-17, which is involved in protein transport by regulating the endosomal recycling of numerous receptors, channels and other transmembrane proteins, including the LRP multiligand receptor, more specifically LRP1 (Donoso et al., 2009; van Kerkhof et al., 2005). This is of particular interest since TTR binds to both, LRP1 and LRP2.

Rab like protein 3 is required for KRAS signalling regulation. KRAS signalling has as downstream effectors TAM1-Rac1. It will be important to further dissect this candidate

since this pathway may be modulating TTR induced actin cytoskeleton remodelling via Rac1 activation.

Regarding candidates differentially activated by WT soluble TTR, it is important to pinpoint HSPB1-associated protein 1, which was upregulated in the presence of TTR, and was found to be abnormally expressed in patients with intractable epilepsy. HSPBAP1 was found extensively in the cytoplasm of neurons and glial cells in all epilepsy specimens and suggested to play a role in the development of epileptic seizures in patients with intractable epilepsy (Xi et al., 2007).

The differentially regulated molecules pinpointed by the proteomic analysis will be validated by immunoblotting and/or immunocytochemistry in DRG neuronal cultures. Particular attention will be given to the proteins having cytoskeleton-related function, but if other interesting hits are disclosed, these will be further analysed. Our findings can, ultimately, represent an important contribution to determine possible molecular mechanisms in TTR-mediated impact on neuronal pathophysiology.

MATERIALS AND METHODS

Recombinant TTR production, purification and TTR L55P aggregates production. Recombinant WT and L55P TTR were produced using a pETF1 vector carrying human WT TTR and L55P TTR (Goldsteins et al., 1997) and used in the transformation of competent *Escherichia coli* BL21(DE3) cells. The protein was expressed as described in Silva et al., 2017 and purified through successive ionic exchange, hydrophobic interaction and size exclusion chromatographies. For cellular assays, recombinant TTR was detoxified using a high-capacity endotoxin removal resin (Thermo Scientific) and quantified using the Lowry based DC Protein Assay (Bio-Rad Laboratories). To produce aggregates, recombinant TTR L55P was applied to a dialysis membrane of 1 kDa (Spectrum Labs) and dialysed against water overnight at 4ºC, with agitation. Aggregated insoluble protein was, then, centrifuged at top speed for 20 minutes, at 4ºC. The pellet was resuspended in pyrogen-free PBS and protein content was quantified using the Lowry method. TTR aggregates were aliquoted and stored at -80ºC in "single use" aliquots. Protein aggregation was assessed by transmission electron microscopy and fluorescence spectroscopy assays.

N1E-115 Culture and differentiation. N1E-115 cells (Sigma-Aldrich), an adrenergic cell line derived from the mouse neuroblastoma C1300 tumour, were grown in DMEM supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 1% P/S and incubated in a humidified incubator with 5% CO_2 at 37ºC. At approximately 80% confluency, cells were plated at a density of 13500 cells/ well, in 24-well plates. After 24h, cells were differentiated for 3 days, replacing the "growing" medium by the "differentiation" medium - DMEM supplemented with 2 mM L-glutamine, 2% FBS, 1% P/S and 1.25% Dimethyl Sulfoxide (DMSO, Sigma-Aldrich). This step of differentiation allowed the extension of long and thin neurites, resembling neurons. At the 3rd day of differentiation, WT soluble TTR and TTR L55P aggregates were added to N1E-115 cells at a concentration of 300 µg/mL for 12 h.

Sample preparation. Cells were washed and detached with PBS, centrifuged at 1000 rpm, for 5 minutes, and resuspended in an appropriate volume of lysis buffer composed of 4% Sodium Dodecyl Sulfate (SDS, Sigma), 0.1 M Dithiothreitol (DTT, Sigma) and 0.1 M Tris-HCl pH 7.6 (Tris Base, NZYTech), in water. The lysates were, additionally, heated at 95ºC, for 5 min, for protein denaturation. Subsequently, lysates were sonicated (2x10 cycles, Output Power 30 Watts, Branson sonifier 250), and centrifuged at 16000 g for 5

min at RT. The supernatants were collected, and total protein was quantified using Lowry's method (DC Protein Assay Kit, Bio-Rad).

Proteomic analysis. Tandem Mass Tag (TMT) proteomics (Mass Spectrometry Core Facility in Institut de Recerca Biomèdica, Barcelona) is a quantitative proteomics method based on the chemical labelling of peptides by incorporation of distinct isotopic labels, followed by tandem mass spectrometry (MS/MS) (Thompson et al., 2003; reviewed in Rauniyar and Yates, III, 2014). This method allows the determination of the amount of proteins in different experimental conditions in a single assay.

Mass spectrometry core facility procedures

Mass Spectrometry Analysis

Sample preparation. Samples were delivered in ~150ml of lysis buffer (4% SDS, 0.1M DTT, 0.1M Tris-HCl, pH 7.5). First, they were quantified by PierceTM 660nm Protein Assay (Prod.#22662) and Ionic Detergent Compatibility Reagent (Prod.#22663). 140µg of each sample were digested with Trypsin following FASP protocol (Jacek R Winiewski, Universal
sample preparation method for proteome analysis Nature Methods 6, 359 - 362 (2009)). Digested samples were evaporated to dry and reconstituted in 100µl of 1% trifluoroacetic acid solution, followed by desalting and clean-up with PolyLC C18. From each sample elute two aliquots were taken, one for a check point (2µl) and the other one for peptide quantification (9µl). The rest of each sample was dried in speed vac and reconstituted in 100ml of TEAB 100mM (triethyl ammonium bicarbonate). The 2µl aliquots were dried, resuspended in 28µl of 1% formic acid in 3% acetonitrile and injected in the LC-MS/MS as a check point of the digestion. The 9ml aliquots were dried, resuspended in 63µl of water and used to quantify samples again by Colorimetric Peptide Assay (PierceTM Thermo, Prod.# 23275). A pool was made with 10µg of 3 samples (Control, WT and Agg). This mix and all the samples were labelled with TMT10plex (Thermo Scientific): 20µg of each sample were combined, taking into account peptide quantification. After combining, the TMT mix was dried in speed vac for desalting and cleaning-up first with PolyLC C18 and second with PolyLC SCX (cation exchange). The TMT mix was separated by High pH reversed-phase peptide chromatography. Sample fractionation was performed with a PierceTM column (ref. 84868), and 10 fractions were collected (F0-F9). Fractions were dried and reconstituted in 50µl in 1% formic acid in 3% ACN.

nanoLC conditions

Injected volume: 6 µL (600ng protein on column)

Chromatograph: Thermo Scientific Dionex Ultimate 3000 nano RSLC

Column: Trap loading, separation on column; Trap column: µ-Precolumn: 300µm i.d x 5 mm, C18 PepMap100, 5 µm, 100 Å (P/N 160454) (Thermo Scientific)

Analytical column: Analytical column: Acclaim PepMap® RSLC (75 µm × 75 cm, nanoViper, C18, 2 µm, 100Å) (Thermo Scientific)

Eluents: A. H2O 0.1 % formic acid; B. CH3CN 0.1 % formic acid

Gradient: 3% to 35% of B in 262 min + 35% to 50% in 5 min + 50% to 85% in 2 min

Flow rate: 200 nL/min

LC-MS coupling. LC-MS coupling was performed with the Advion Triversa Nanomate (Advion BioSciences, Ithaca, NY, USA) as the nanoESI source performing nanoelectrospray through chip technology. The Nanomate was attached to an Orbitrap Fusion Lumos™ Tribrid mass spectrometer and operated at a spray voltage of 1.7 kV and a delivery pressure of 0.5 psi in positive mode.

MS conditions (SPSMS3 method)

Mass spectrometer: Orbitrap Fusion Lumos™ Tribrid (Thermo Scientific)

Spray voltage: 1.6 kV

Ion transfer tube temp.: 275ºC

RF Lens: 30%

m/z range: 400-1600 a.m.u

Data acquisition method: SPSMS3: OT MS> CID IT MS2> SPS HCD OT MS3; MS1: full Orbitrap MS scan (Orbitrap); Parent ion isolation in Q1; MS2 scan: CID in the Ion Trap; SPS HCD FTMS3 scan; Synchronous Precursor Selection in the Ion Trap; HCD MS3, FTMS detection of MS3 fragments

Data processing

LTQFT Ultra: Xcalibur software vs 2.0SR2 (Thermo Scientific)

Software: Proteome Discoverer v2.1.0.81 software (Thermo Scientific) with SEQUEST HT algorithm

Database: SwissProt_MOUSE_canonical_2018_04.fasta and contaminants database

Enzyme: Trypsin (full) with 2 missed cleavages.

Static modifications: Carbamidomethyl in cysteine, TMT in N-term

Dynamic modifications: Methionine oxidation, TMT in lysine, Acetylation in protein N terminus

Precursor mass tol.: 10 ppm
MS/MS tolerance: 0.6 Da

Materials and methods

The nano-LC-MS/MS set up was as follows. Digested peptides were diluted in 3%ACN and 1% FA. Sample was loaded to a µ-Precolumn: 300µm i.d x 5 mm, C18 PepMap100, 5 µm, 100 Å, C18 Trap column (Thermo Scientific) at a flow rate of 15 µl/min using a Thermo Scientific Dionex Ultimate 3000 chromatographic system (Thermo Scientific). Peptides were separated using a C18 analytical column (Acclaim PepMap TM RSLC: 75 µm x 75 cm, C18 2 µm, nanoViper) with a 300 min run, comprising three consecutive steps with linear gradients from 1 to 35% B in 262 min, from 35 to 50% B in 5 min, and from 50 % to 85 % B in 2 min, followed by isocratic elution at 85 % B in 5 min and stabilization to initial conditions (A= 0.1% FA in water, B= 0.1% FA in CH3CN). The column outlet was directly connected to an Advion TriVersa NanoMate (Advion) fitted on an Orbitrap Fusion Lumos™ Tribrid (Thermo Scientific).

The mass spectrometer was operated in a data-dependent acquisition (DDA) mode. In each data collection cycle, one full MS scan (400-1600 m/z) was acquired in the Orbitrap (1.2 x 105 resolution setting and automatic gain control (AGC) of 2 x 105). The following MS2-MS3 analysis was conducted with a top speed approach. The most abundant ions were selected for fragmentation by collision induced dissociation (CID). CID was performed with a collision energy of 35%, 0.25 activation Q, an AGC target of 1 x 104, an isolation window of 0.7 Da, a maximum ion accumulation time of 50 ms and turbo ion scan rate. Previously analysed precursor ions were dynamically excluded for 30 s. For the MS3 analyses for TMT quantification, multiple fragment ions from the previous MS2 scan (SPS ions) were co-selected and fragmented by HCD using a 65 % collision energy and a precursor isolation window of 2 Da. Reporter ions were detected using the Orbitrap with a resolution of 60,000, an AGC of 1 x 105 and a maximum ion accumulation time of 120 ms. Spray voltage in the NanoMate source was set to 1.60 kV. RF Lens were tuned to 30%. The spectrometer was working in positive polarity mode and singly charge state precursors were rejected for fragmentation.

A database search was performed with Proteome Discoverer v2.1.0.81 software (Thermo Scientific) using Sequest HT search engine and SwissProt_MOUSE_canonical_2018_04.fasta and contaminants database. Search was run against targeted and decoy database to determine the false discovery rate (FDR).

Search parameters included trypsin, allowing for two missed cleavage sites, carbamidomethyl in cysteine and TMT peptide N-terminus as static modification and TMT in K, methionine oxidation and acetylation in protein N-terminus as dynamic modifications. Peptide mass tolerance was 10 ppm and the MS/MS tolerance was 0.6 Da in MS2 and 20 ppm in MS3. Peptides with a q-value lower than 0.1 and a FDR < 1% were considered as positive identifications with a high confidence level.

TMT reporter ions intensities were used for protein quantification. Unique peptides (peptides that are not shared between different protein groups) were considered for further quantitative and statistical analysis. Within each TMT experiment, peptide quantitation was normalized by summing the abundance values for each channel over all peptides identified within an experiment and then the channel with the highest total abundance was taken as a reference and all abundance values corrected in all other channels by a constant factor per channel, so that at the end the total abundance is the same for all channels. Protein quantitation was done by summing all peptide normalized intensities for a given protein. Proteins were only considered quantifiable if all quan channels have abundance values. DanteR [Taverner T et al. Bioinformatics. 2012 Sep 15;28(18):2404-6], by Pacific Northwest National Laboratory, was used to pre-process, visualize data (boxplots and principal component analysis) and perform relative quantification of proteins labelled with TMT. Protein quantitative measurements were log2 transformed and normalization across the four 10plex TMT experiments was performed using quantile normalization [Chick JM et al. Nature. 2016 Jun 15;534(7608):500-5]. Analysis of variance (ANOVA) was performed at protein level using a linear model. Weighting function was used to allow data variability to depend on data value. Comparisons considering condition or age were performed. Finally, p-values were adjusted for multiple testing using the Benjamini & Hochberg FDR correction. Differential expressed proteins were determined using a p-value cutoff of 0.05 and a fold change lower than 0.8 (down) or higher than 1.25 (up).

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APPENDIX

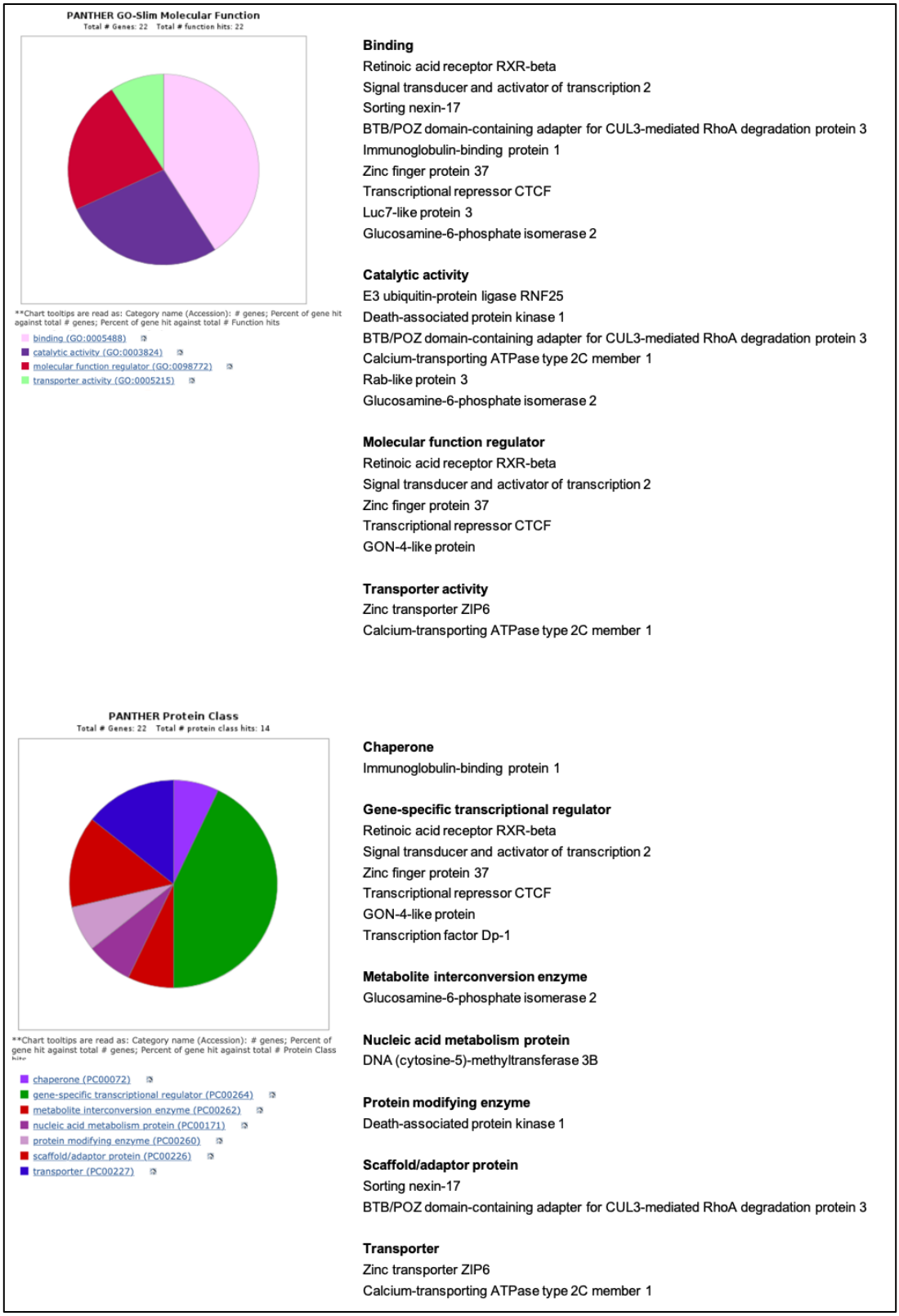

Figure 2. List of proteins differentially regulated by TTR L55P aggregates selected from the TMT proteomics assay and categorized by PANTHER according its molecular function or protein class.

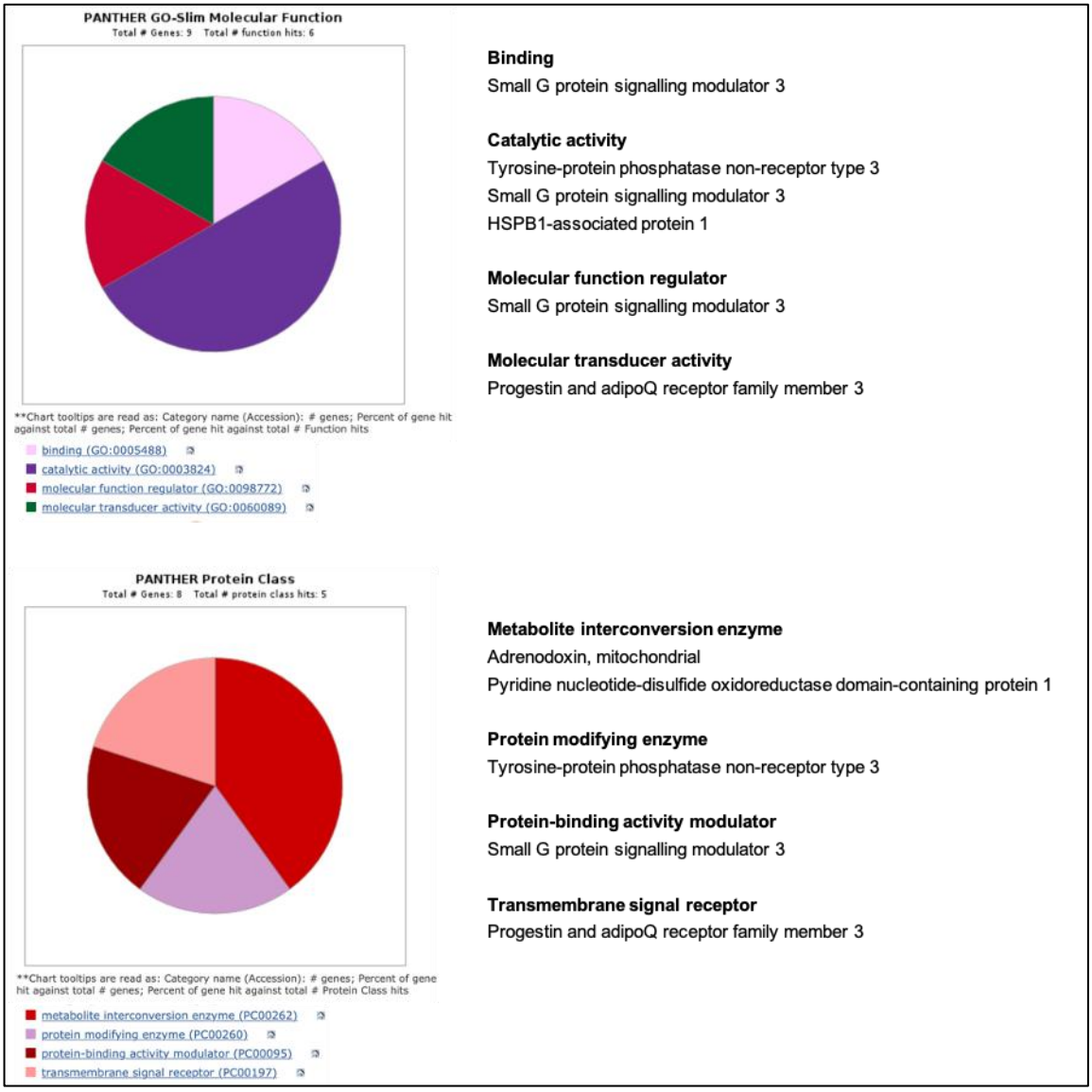

Figure 3. List of proteins differentially regulated by WT soluble TTR selected from the TMT proteomics assay and categorized by PANTHER according its molecular function or protein class.

CONCLUSIONS AND FUTURE PERSPECTIVES

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Transthyretin (TTR) is a protein with intriguing characteristics in the nervous system since it leads to neurodegeneration, on one hand, and on the other hand, it is associated to neuroprotection. The aim of this work was to dissect this dual impact of TTR with a specific focus in unravelling novel mechanistic insights in TTR-induced neuroprotection and neurodegeneration.

Over the last years, TTR has been suggested to have a protective role in Alzheimer's disease (AD) mostly due to its ability to bind amyloid β peptide (Aβ) and promote Aβ clearance. Knowing that TTR is able to cleave Aβ *in vitro*, in this work we further established the relevance of TTR as a metalloprotease in AD by demonstrating decreased Aβ-mediated neuronal toxicity, dependent on TTR proteolytic activity. Future studies should assess the effect in Aβ degradation and clearance of the intracerebral injection of either TTR WT or TTR proteolytically inactive in an AD mouse model, as similar experiments using neprilysin, also an Aβ targeting metalloprotease, were successful in reducing plaque burden and promoting behavioural performance in AD mice (Park et al., 2013). The outcomes of those studies will strengthen TTR as a therapeutic anti-Aβ agent.

In addition to the neuroprotective role in AD, TTR is also a nerve regeneration enhancer and promotes axonal growth *in vitro*. In this work we have shown that TTR is a regulator of neuronal microtubule (MT) dynamics by modulation of acetylated α-tubulin levels, which might be related to its neuritogenic activity. Future work should address how mechanistically TTR modulates tubulin acetylation and more importantly clearly demonstrate whether the impact of TTR on the MT cytoskeleton underlies its role on nerve biology. ACY-738 was successful in reverting the decreased levels of acetylated α-tubulin and altered MT dynamics in TTR KO DRG neurons. This potential should be further investigated in the assessment of peripheral nerve regeneration promotion in TTR KO, to clearly demonstrate that TTR modulation of MT stability is the mechanism underlying its role as an axonal growth and nerve regeneration enhancer. Furthermore, it is worth speculating about a more transversal role of TTR in neuronal MT modulation with an impact in other conditions. For instance, decreased levels of plasma TTR were shown in AD patients (Hansson et al., 2009) and TTR expression was shown to be decreased in aged memory-impaired rats (Brouillette & Quirion, 2008). It would be interesting to analyse whether the decreased presence of TTR, that different neurological conditions manifest, has an impact on neuronal health by dysregulating MT stability.

FAP is characterized by a distinctive axonopathy – the dying-back type – which is frequently associated to initial disturbances of the neuronal cytoskeleton. In this work, we were able to establish a link between cytoskeleton abnormalities and neurodegeneration in FAP by using a hTTRA97S FAP mouse model. In hTTRA97S DRG neurons we observed a Rac1-mediated dysregulation of the actin cytoskeleton preceding neurodegeneration. Importantly, Rac1 inhibition was successful in reverting neurodegeneration in hTTRA97S neurons. Rac1 signalling is implicated in several human diseases including cancer, cardiovascular diseases and neurodegenerative diseases. Therefore, targeting Rac1 in a clinical setting presents an interesting therapeutic opportunity. Rac1 is associated with both protective and harmful effects in neurodegenerative diseases, thus, studies modulating Rac1 include both, the activation and inactivation of the protein. In familial amyotrophic lateral sclerosis, expression of constitutively active Rac1 attenuated neuronal death induced by superoxide dismutase 1 mutants (Pesaresi et al., 2011). In an AD context, it was shown that Rac1 inhibition ameliorate cognitive defects and synaptic plasticity in AD animal models being effective as an AD-related memory loss treatment (Wu et al., 2019). In the future it would be important to characterize Rac1 as a novel therapy targeting actin cytoskeleton in FAP and assess its potential in halting disease progression.

Additionally to unravelling actin damage as a novel mediator of neurotoxicity in FAP, we obtained further data substantiating an impairment in MT dynamics and axonal transport in hTTRA97S mice. These observations require additional studies including exploring whether these cytoskeleton alterations are connected to actin dysfunction and result from Rac1 dysregulation.

Our group obtained data substantiating an effect of TTR on the neuronal cytoskeleton. However, TTR might be exerting its effects through other mechanisms and impacting on other molecular targets. In this respect, we performed a proteomics assay to obtain a general profile of candidates differentially expressed in the presence of TTR L55P aggregates which highlighted particularly STAT2, a signal transducer of the JAK-STAT pathway, implicated in several neurological diseases (Benveniste, Liu, McFarland, & Qin, 2014; O'Shea & Plenge, 2012; Shahni et al., 2015), and BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3 (BACURD3), involved in actin cytoskeleton structure modulation and cell movement (Chen et al., 2009). These candidates will be validated in the FAP mouse model, since their molecular functions overlay with TTR-induced actin cytoskeleton dysregulation and neuroinflammation in FAP (Sousa, Yan, Stern, & Saraiva, 2000), which brands them as promising candidates in the determination of novel molecular mechanisms in TTR-mediated impact on neuronal pathophysiology.

In conclusion, our work brings evidence to a novel role of TTR as a cytoskeleton modulator and, importantly, unravels cytoskeleton damage in FAP. The novel insights we obtained regarding the molecular mechanisms underlying TTR dual role in neuronal pathophysiology will be promising in opening new therapeutic avenues in FAP and potentially other neurological conditions.

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