Universitat Autònoma de Barcelona

Metallothionein family: the multipurpose protein. Influence of Mt1 in the Tg2576 mouse model of Alzeimer's disease

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PHD. THESIS
NEUROSCIENCES
2017

Metallothionein family:

THE MULTIPURPOSE PROTEIN

Influence of Metallothionein-1 in the Tg2576 mouse model of Alzheimer's disease

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Overexpression of metallothionein-1 modulates the phenotype of the Tg2576 mouse model of Alzheimer's disease

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Influence of transgenic MT-1 on gliosis, CA1 neuronal loss, and brain metal levels of the Tg2576 mouse model of Alzheimer's disease

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Abbreviations

%EOA Percentage of entries in the open arms
%TOA Prcentage of time in the open arms
OH• Hydroxyl
6-OHDA 6-Hydroxypamine
8-OHdG 8-Hydroxy-2'-deoxyguanosine
aa Aminoacid
ACE Angiotensin-converting enzyme
AD Alzheimer's disease
ADAM A disintegrin and metalloproteinase
AICD AβPP intracellular domain
ALS Amyotrophic lateral sclerosis
AOA Area occupied by astrocytes
AβPP Amyloid-β precursor protein
APPMT1+2KO Amyloid precursor protein positive/metallothionein-1/2 deficient
APPMT3KO Amyloid precursor protein positive / metallothionein-3 deficient
APPWT Amyloid precursor protein positive / metallothionein wild type
ARE Antioxidant response element
AU Arbitrary units
Aβ Amyloid beta
BACE β-site APP cleaving enzyme
BBB Blood-brain barrier
CA Closed arms
CAA Cerebral amyloid angiophaty
CBF Cerebral blood flow
CNS Central nervous system
CQ Clioquinol
CSF Cerebrospinal fluid
CTF C-terminal fragment
CTX Cortex
DAB Diaminobenzidine
EAE Experimental autoimmune encephalomyelitis
ELISA Enzyme-linked immunosorbent assay
ER Endoplasmic reticulum
FAD Familial Alzheimer disease
GC Glucocorticoid
GEE Generalized estimating equations
GFAP Glial fibrillary acidic protein
GIF Growth inhibitory factor
GLZ Generalized linear model
GRE Glucocorticoid response element
GSH Glutathione
HB Hole board
HD Head dipping
HFD Hight fat diet
HIP Hippocampus
ICP-MS Inductively coupled plasma mass spectrometry
IFN Interferon
IHC Immunohistochemistry
IL Interleukin
LTP Long term potentiation
MRE Metal response element
MS Multiple sclerosis
MT Metallothionein
MT1+2KO Deficient for metallothionein-1 and 2
MT3KO Deficient for for metallothionein-3
MTF-1 Metal-regulatory transcription factor 1
MTWT Wild type for metallothioneins
MWM Morris water maze
NFT Neurofibrillary tangle
NSAIDs Nonsteroidal anti-inflammatory drugs
O/N Over night
OF Open field
PCR Polymerase chain reaction
PFA Paraformaldehyde
PFs Proteolytic fragment
PHF Paired helical filaments
PM/EPM Elevated plus maze
PS Presenilin
RAGE Receptor for advanced glycation end products
ROS Reactive oxygen species
RPA Ribonuclease protection assay
RT Room temperature
RT-PCR Real time PCR
sAβPP Soluble amyloid-β precursor protein
SDS Sodium dodecyl sulphate
SEM Standard error of mean
SOD Superoxide dismutase
STAT Signal transducer and activator of transcription
TBARS Thiobarbituric acid reactive substances
TGN Trans golgi network
TNF Tumor necrosis factor
TQ Target quadrant
TTR Transthyretin
USF Upstream stimulator factor
WB Western blot
WT Wild typeBekris et

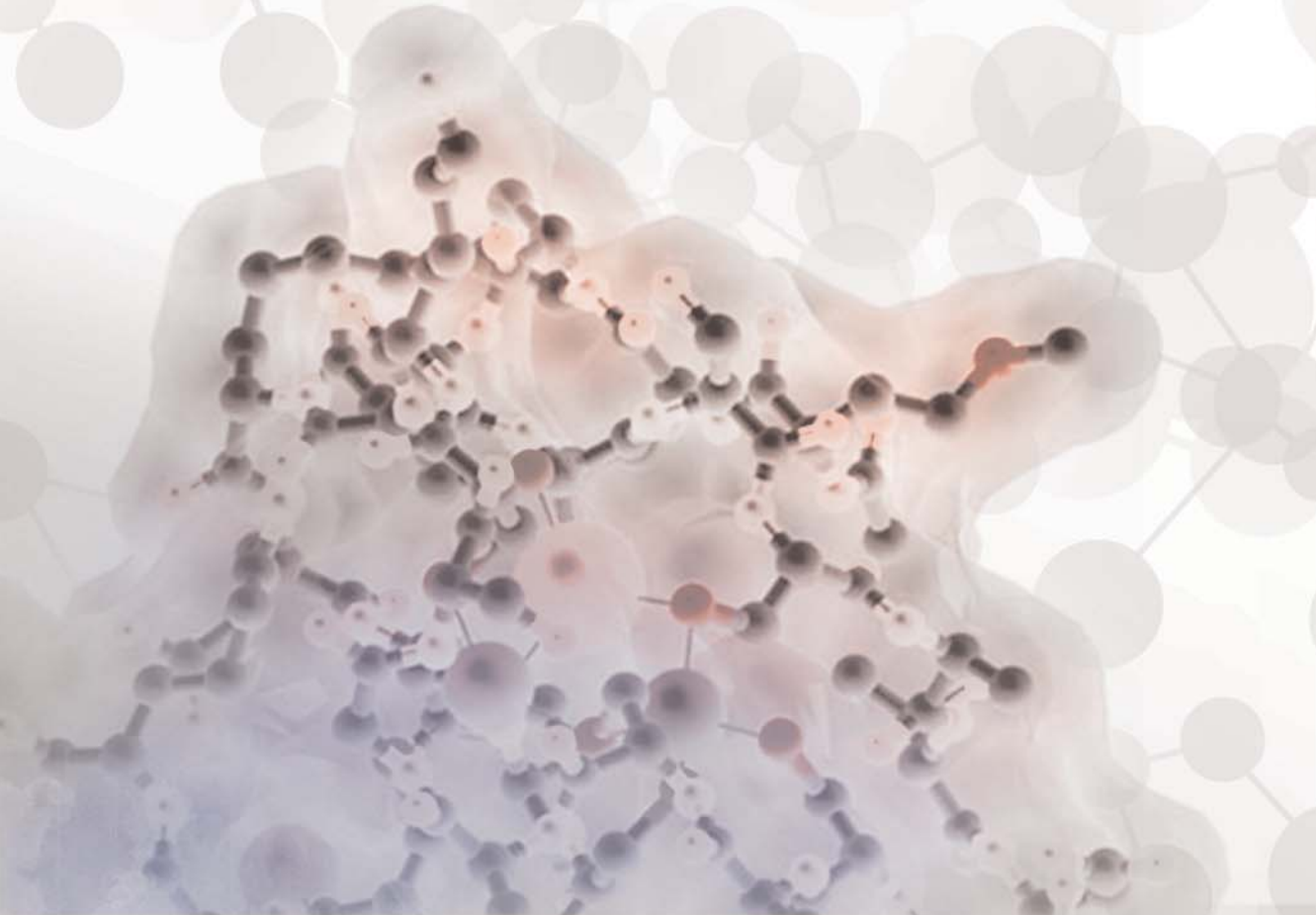
1 Introduction Hypothesis & Objectives

Alzheimer's disease (AD)

The role of inflammation, oxidative stress, metals, tau and NFTs in AD
Animals models of AD

Metallothioneins

General characteristics and structure
MT-1 and MT-2 regulation and functional aspects
MTs and brain disease



Alzheimer's disease

Introduction

According to alz.org®, **dementia** is a *general term for a decline in mental ability severe enough to interfere with daily life*. Alzheimer's disease (AD) is the most common form of dementia, accounting for 50-60% of all cases (Blennow et al. 2006). The incidence of AD increases almost logarithmically with age until an inflection point is reached at the age of 85. With the increasing of geriatric population and the lack of cure or preventive therapies yet available, AD represents an important global economic and societal burden (Tanzi et al. 2004; Blennow et al. 2006; Robinson et al. 2017).

First described by Alois Alzheimer in 1906, AD is clinically defined by a progressive decline of cognitive functions including memory, judgment, orientation, language, abstract reasoning, executive function or decision making as well as for a behavioural, emotional, interpersonal and social decline, leading to dementia and death (Bekris et al. 2010; Bertram et al. 2010). Neuropathologically AD is characterized by the aggregation and deposition of β-Amyloid peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$) in the form of extracellular senile plaques (amyloid plaques) and intracellular deposits of hyperphosphorylated tau protein (neurofibrillary tangles). These two hallmarks are accompanied by profuse microvascular damage, including vascular amyloid deposits, pronounced inflammation and oxidative stress and nerve and synapse loss of the affected brain regions such as the hippocampus and the cortex (Bertram et al. 2010; Ittner and Götz 2011). Transition metals such as Cu or Fe, together with Zn, contribute to oxidative stress and Aβ aggregation and precipitation in the AD brain (Bush et al. 1994; Bush 2003).

Although **age** is the major risk factor for AD, **family history** represents the second-greatest risk factor. Genetically, AD is classified into familial cases with an autosomal dominant inheritance with an early-onset (EOFAD) (≤ 60 years) and sporadic cases with later appearance of the disease (LOAD) (≥ 60 years) (Bertram et al. 2005). The EOFAD cases are caused by mutations in three genes which are related with amyloid cascade hypothesis and affect directly the amyloid production, aggregation and removal (Swerdlow 2007; Bertram et al. 2010; Kaminsky et al. 2010): the *App* gene, on chromosome 21, encodes the Amyloid Precursor protein (AβPP), a type I transmembrane glycoprotein from which Aβ is proteolitically derived, and *Psen1* and *Psen2* genes, on chromosomes 14 and 1 respectively, encode Presenilin-1 and Presenilin-2 proteins that lie at the catalytic centre of the γ-secretase complex involved in the proteolytic processing of AβPP (Swerdlow 2007; Bekris et

al. 2010; Bertram et al. 2010; Kaminsky et al. 2010). All the mutations lead, thus, to abnormal production of Aβ resulting in an overabundance of $A\beta_{42}$ species accumulation in the brain of these patients. EOFAD represents only a small fraction of all AD cases (≤5%) (Ashe K. H. 2010; Bertram et al. 2010).

On the other hand, susceptibility for LOAD has no apparent familial aggregation and is related to an array of common risk alleles across different genes and an interaction with environmental factors. The ε-4 allele of the apoliprotein E gene (APOE) on chromosome 19 has been established as a major genetic risk factor for AD by multiple meta-analysis and Genome-wide association studies (GWAS). APOE is a lipoprotein involved in regulating the metabolism of cholesterol and triglycerides. Three allelic variants of the APOE gene exist in the population (ε2, ε3 and ε4), with different biochemical properties at the protein level. ε4 isoform is overrepresented in subjects with AD compared with the general population, and the inheritance of one or two alleles heightens the likelihood of developing AD at early ages compared with subjects harbouring ε2 and or ε3 alleles. In contrast, the inheritance of the ε2 allele may confer protection against the development of AD and cognitive decline (Robinson and Bishop 2002; Haass and Selkoe 2007) (Dennis et al., 2001, Robinson et al., 2002). APOE-ε4 is neither necessary nor sufficient to cause AD but instead operates as a genetic risk modifier by decreasing the age of onset in a dose-dependent manner. Despite its genetic relation in the disease is well-known and established, its biochemical consequences are not fully understood but encompass Aβ-aggregation/clearance and/or cholesterol homeostasis (Bertram et al. 2005). LOAD represent the majority cases of the AD (90% of cases). There are many **other risk and protective factors** that may affect the progression or development of AD. These factors can be due to a pre-existing conditions or disease or life style choices: obesity, type 2 diabetes mellitus, physical activity and diet, mentally demanding activities, stress, etc. (Robinson et al. 2017).

The 'amyloid cascade hypothesis': from amyloid precursor protein to Aβ peptide

The cloning of the Amyloid-β Precursor Protein (AβPP) and the elucidation of its role in generating the Aβ peptide (Goldgaber et al. 1987) and the isolation and sequencing of the Aβ peptide (Glenner and Wong 1984; Glenner 2012), main constituent of the senile plaques (Masters et al. 1985), represented major advances in the study of AD. Subsequently, the identification of the missense mutations in the AβPP and Presenilin genes (Goldgaber et al. 1987), which affected directly the production of Aβ peptide, in some FAD pedigrees and the fact that individuals with Down's syndrome,

with 3 chromosome 21 and therefore 3 copies of App gene, develop earlier clinical and pathological signs of AD, lead to the elaboration of the Amyloid Cascade Hypothesis (Hardy and Higgins 1992; Hardy and Selkoe 2002).

Amyloid cascade hypothesis postulated that an altered proteolysis of AβPP leads to an overproduction of $A\beta_{42}$ in the interstitial fluid of the brain (**Fig. 1**). $A\beta_{42}$ oligomerizes and deposits into diffuse extracellular plaques providing a focus for the subsequent deposition of $A\beta_{40}$ and other proteins (Robinson and Bishop 2002), leading to a neuronal loss, vascular damage and dementia (Hardy and Higgins 1992). Recent studies focus on small soluble aggregates of Aβ peptide as the primary impetus of the disease progression (De Strooper et al. 2010). However, although the amyloid cascade hypothesis suits the familial forms of the disorder, less consensus has been found among the scientific community in relation with the pseudoesporadic forms of AD (Robinson and Bishop 2002; Zhu et al. 2007; Herrup 2010). Even with new theories and approximations to the onset and developing of the disease, the 'amyloid cascade hypothesis' has dominated the field of AD investigation and the study of the proteolytic generation of Aβ from AβPP is the most prevalent area of research in AD studies (De Strooper et al. 2010).

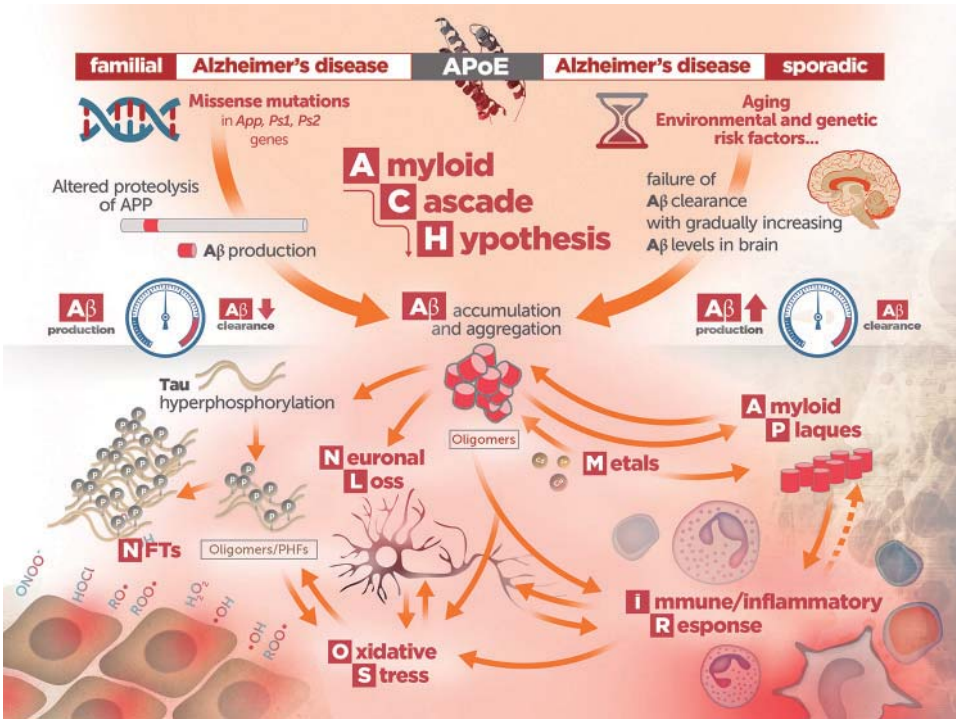


Figure 1. Theoretical scheme of the amyloid cascade hypothesis.

AβPP: family, trafficking, proteolytic processing and putative functions

AβPP protein belongs to a conserved gene family that also includes the mammalian AβPP-like proteins APLP1 and APLP2. AβPP family members are type-1 transmembrane proteins that have a relatively large extracellular domain, and short intracellular domain (Haass et al. 2012). The AβPP gene contains 19 exons, of which exons 7, 8 and 15 can be alternative spliced generating three isoforms: AβPP695, AβPP751 and APP770, the former being highly expressed in neuronal cells and the two latter being ubiquitously expressed in all kind of cells (De Strooper and Annaert 2000; Selkoe 2001).

During its transit from the endoplasmatic reticulum (ER) to the plasma membrane (**Fig. 2**), AβPP is posttranslationally modified in several different ways by N- and O-glycosylation, phosphorylation, and tyrosine sulfation. The majority of APP localizes to the Golgi and TGN; in non-neuronal cells, when AβPP reaches the cell surface, it is rapidly internalized due to the presence of the YENPTY internalization motif near the C-terminus of APP and trafficked through endocytic and recycling compartments back to the cell surface or degraded in the lysosome (Selkoe 2001; Thinakaran and Koo 2008).

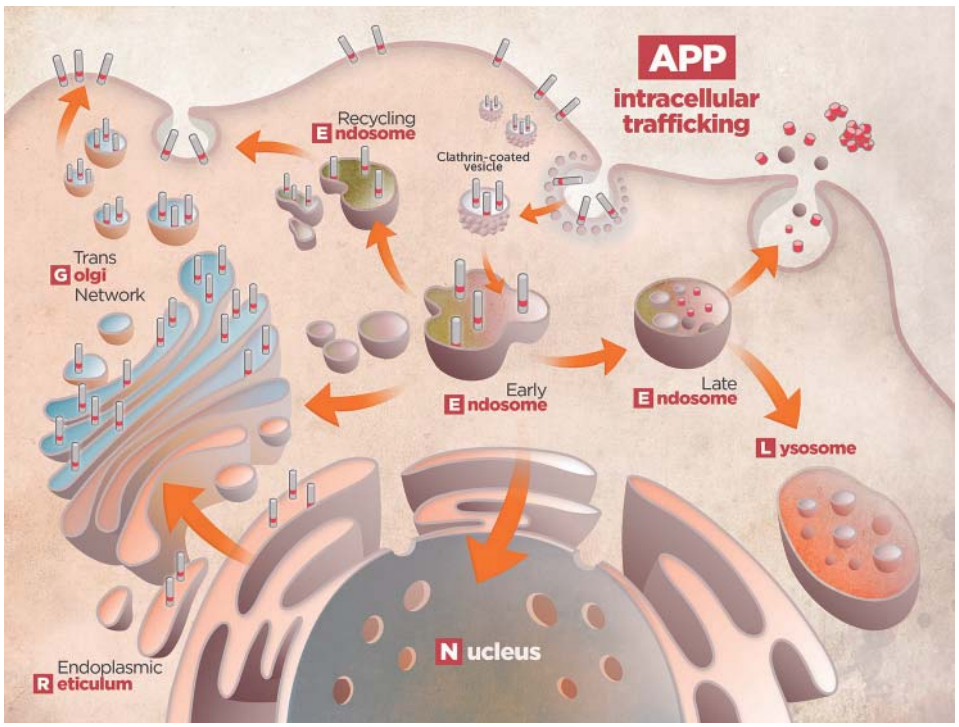


Figure 2. AβPP intracellular trafficking.

Three protease activities, α-, β- and γ-secretase are involved in the proteolytic processing of APP that is divided into two principal pathways (**Fig. 3**): the **non-amyloidogenic pathway**, which is the physiologically most relevant proteolysis of APP and which prevents Aβ generation, and the **amyloidogenic pathway**, which leads to Aβ generation (Haass et al. 2012).

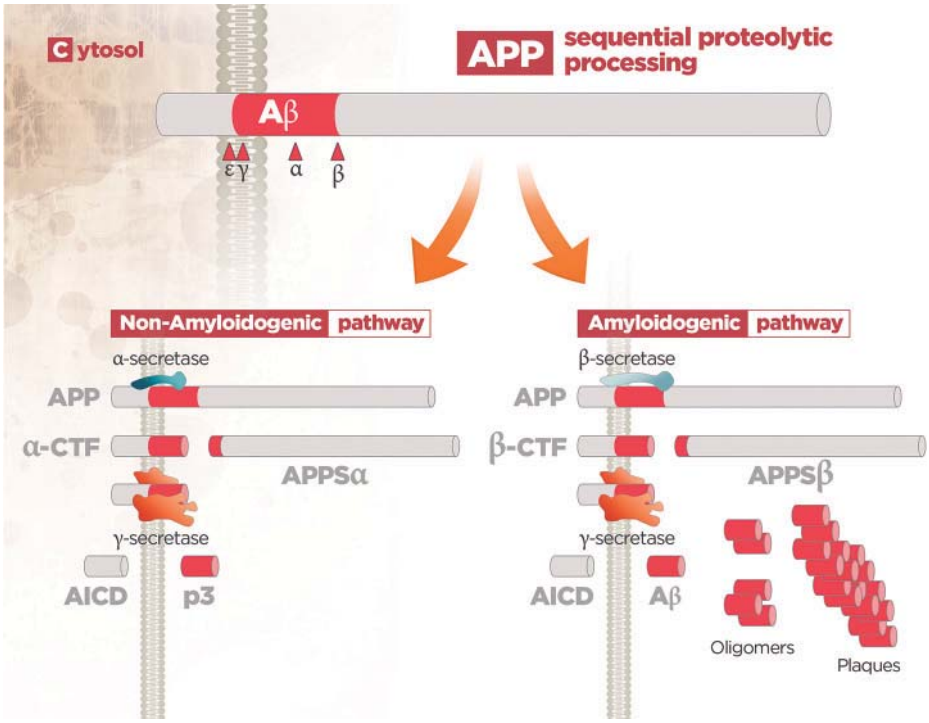


Figure 3. AβPP sequential proteolytic processing.

Anti-amyloidogenic processing occurs mainly in the cell surface where α-secretase complex is present and cleaves the APP within the Aβ sequence (between Lys613-Leu614) resulting in the release into the lumen space of the N-terminal ectodomain, APPsα, and the retention of a C-terminal membrane-bound fragment of 83 aminoacids (C83 or CTFα). This fragment can be further cleaved by γ-secretase giving away a 3 KDa peptide and a cytoplasmic polypeptide, AβPP intracellular Domain (AICD).

The amyloidogenic processing of APP is initiated through cleavage by β-secretase which cuts 16 residues N-terminal to the α-cleavage site, generating a smaller ectodomain, APPsβ, and a transmembrane APP C-terminal fragment of 99 aminoacids (C99 or βCTF), which begins at residue 1 of the Aβ region. CTFβ is then processed as well by γ-secretase, generating Aβ (~4 KDa) and AICD (Jacobsen and Iverfeldt 2009; Haass et al. 2012).

The set of proteases responsible for α-secretase activity are plasma membrane-bound zinc metalloproteinases, and they are all members of the ADAM (a disintegrin and metalloproteinase) family such as ADAM10, ADAM9/MDC-9/Meltrin γ, ADAM17/TACE (TNF-α Converting Enzyme) and BACE2 (aspartyl protease homolog to BACE1).

BACE1 (β-site AβPP cleaving enzyme-1) is the term designed for the enzyme harbouring β-secretase activity. It is predominantly localized in the late Golgi /TGN and endosomes but briefly transits to the cell surface to locations where APP can also be found. The protease is ubiquitously expressed with highest levels in the brain (neuronal cells in particular) and pancreas. The combination of high levels of BACE and APP make the brain the main tissue generating Aβ and help to explain why AD is a brain disease despite APP is ubiquitously expressed.

γ-secretase is an enzyme complex constituted by four essential subunits: presenilin-1 (PS1) or presenilin 2 (PS2), nicastrin, anterior pharyx defective 1 (APH-1) and presinilin enhancer 2 (PEN2). Presenilins are aspartyl proteases that are the major components for proteolytic activity of γ-secretase but the association of the other 3 subunits is required. The processing of APP by γ-secretasse is not restricted to a unique site and Aβ peptides generated length range from 37 to 43 residues. This fact is very relevant for the understanding of the AD pathology because $A\beta_{42}$ species are more prone to aggregation and confer more toxic and pathologic effects to the brain. In neurons, γ-secretase complex activity is present in ER, Golgi, TGN, endosomes and plasma membranes. In non-neuronal cells Aβ mainly is generated in the TGN and endosomes as APP is trafficked through the secretory and recycling pathways. A number of studies also evidence that amyloidogenic processing occurs in cholesterol- and sphingolipid-enriched membrane raft microdomains of intracellular organelles (De Strooper and Annaert 2000; Thinakaran and Koo 2008; Jacobsen and Iverfeldt 2009; De Strooper et al. 2010).

The **biological function of APP** is still unclear (Jacobsen et al., 2009). *In vitro* and *in vivo* studies have yielded strong evidence for roles of APP and its derivates in the developing and adult nervous system, cell adhesion, neuronal survival, neurite outgrowth, synaptogenesis, vesicular transport, neuronal migration, modulation of synaptic plasticity, and insulin and glucose homeostasis (Thinakaran and Koo 2008; Jacobsen and Iverfeldt 2009).

Aβ peptide: aggregation and clearance

Aβ peptide is currently the most widely studied neuroimaging biomarker for the diagnosis and monitoring of the AD (Adlard et al. 2014). In contrast to what was thought initially, Aβ is produced in a constitutive manner and can be detected in both cerebrospinal fluid and plasma in healthy subjects throughout life (Selkoe 2001).

As described above, Aβ peptides are the natural product of the proteolytic processing of APP by β- and γ-secretasse cleavages through the amyloidogenic pathway. In particular γ-secretase is responsible of the generation of different Aβ peptides; depending on the cleavage site three principal forms of Aβ (from 39 to 42 residues) are produced. This fact is particularly relevant because $A\beta_{42}$ peptide tend to oligomerize and form fibrils (that in turn have propensity to form β-pleated sheet structures) respect to the shorter peptides (Di Carlo 2010). The enriched production of the $A\beta_{42}$ peptide due to an AβPP mutation, for example, leads to an increase in absolute levels of this isoform and consequently in an increase of $A\beta_{42}/A\beta_{40}$ ratio. The $A\beta_{42}$ peptide with its C-terminal alanine and isoleucine residues is more prone to aggregation forming stable Aβ oligomers (trimeric or/and tetrameric) at an early time point. Actually, elevated levels of $A\beta_{40}$, which do not lead to plaque formation, may serve to retard the deposition of $A\beta_{42}$ in fibrils or amyloid plaques, acting as an 'anti-aggregation factor' (Haass and Selkoe 2007).

The mechanisms of the Aβ **aggregation** are not fully understood but it seems like the proteins seek to adopt a quaternary structure with a stable conformation. An alternative but stable 'misfolded' state may make Aβ prone to aggregation and mutations associated with FAD may predispose to misfolding. This abnormal aggregation and precipitation could be the cause of amyloidosis in AD (Adlard et al. 2014). It is generally accepted that fibril formation is a multistep process, whose onset is dependent on an initial nucleation step (like seeding a crystallization process) (Di Carlo 2010). However, there is mounting evidence that soluble oligomers are not obligate intermediates for fibril formation and the oligomers and fibrils represent separate and distinct aggregation pathways (Necula et al. 2007).

Amyloid has been demonstrated to be toxic to neuronal cell cultures (Adlard et al. 2014) but, in vivo, amyloid burden correlates weakly with the disease severity, suggesting a lesser role for insoluble Aβ fibrils, while soluble oligomers appear to play the major part in neurotoxicity. These evidences, support the hypothesis that 'soluble oligomers' rather than mature amyloid

fibrils are the more deleterious factors in AD (Di Carlo 2010; Adlard et al. 2014). It is quite likely that different oligomeric species are in dynamic equilibrium with each other and that more than one of these species results neurotoxic (De Strooper et al. 2010). In fact, there is now considerable evidence that decreased hippocampal long term potentiation (LTP) and impaired memory function can be directly attributed to soluble oligomers (Klyubin et al. 2005; Haass et al. 2012).

Taking all this information into account, it seems that the formation of amyloid-β plaques could represent a protective process against toxic oligomeric Aβ (Lee et al. 2005), inhibiting the extracellular spread of Aβ toxicity and/or reducing the amount of soluble oligomeric Aβ (Takahashi et al. 2017).

A variety of β-Amyloid plaques are present in AD brains, and thus, various authors have been trying to identify and classify plaque types which represent sequential maturation of the amyloid deposit (Mrak 2009):

> **Diffuse, non neuritic plaques/pre-amyloid deposits:** early amorphous non-congophilic deposits of Aβ with low abundance of associated microglia and astrocytes and no evidence of associated neuritic damage.
>
> **Diffuse neuritic plaques:** later deposits with some granular condensation of Aβ but without a defined congophilic core. Associated-activated microglia and astrocytes are increased and there is some evidence of neuritic damage in the form of dystrophic neurites.
>
> **Dense core neuritic plaques:** centre core of dense, congophilic amyloid is present and surrounded by many associated activated microglial and astroglial cells as well as associated damage in form of dystrophic neurites.
>
> **Dense core, non-neuritic plaques/burn out plaques:** only a dense congophilic core persists devoid of associated activated microglia, astrocytes or neuritic damage.

Two main hypothesis have been developed, concerning the existence of different plaque type; the first postulate that one type of plaque is converted into another, and therefore, different plaque types represent different stages in the 'life history' of a single type of plaque. And the second theory is that each plaque type evolves independent of the others and so, unique factors are involved in their formation (Dickson and Vickers 2001).

The observation of the diversity of morphological types of β-amyloid deposits in both the preclinical and end-stages AD cases, suggests that the theory of plaque development is unlikely to be the correct one (Dickson

and Vickers 2001). However, quantitative analysis demonstrated that clinical AD was associated with a proportional shift to particular plaque types, principally the fibrillary form, and that the fibrillary plaques showed a higher tendency to be neuritic in end-stages of AD cases.

It has been hypothesized that extracellular Aβ fibrils exert their toxic effects on the surrounding neurons and their processes being more disruptive to synaptic plasticity (Takahashi et al. 2017); numerous studies support that intraneuronal **accumulation** of Aβ within the neurites and synapses leads to their dysfunction and final destruction. After the destruction of numerous neurites and synapses, amorphous remnants may be further shaped as plaques by activated microglia which may contribute to the formation of plaques and other AD pathologies. In conclusion, both extracellular and intracellular Aβ are important in the pathogenesis of AD (Takahashi et al. 2017).

Nevertheless, some authors have proposed that the classification through the immunohistochemically appearance of the plaque is underestimated and does not explain why some plaque types have inflammatory cells associated and others not, why some plaques are abundant in some brain areas and lacking in others, why are dense core plaques similar in shape but diffuse plaque are more heterogeneous, why dense core plaques contain DNA, RNA and cytoplasmic proteins resistant to proteolysis, etc. To answer these questions D'Andrea et al., (D'Andrea and Nagele 2010) proposed that morphologically distinct plaques have different origins and pathogenesis. For example, diffuse plaques (vessel-derived) do not have associated microglia and astroglia and do not contain neuron-derived DNA or cytoplasmic proteins, suggesting that diffuse plaques are not associated with cell death and lysis and do not affect local axons or dendrites surrounding them. This fact explains that the presence of this type of plaques may be insufficient to cause relevant cognitive impairment. By contrast, many factors support neuronal origin for dense core, inflammatory plaques, such as the presence of proteolytically resistant neuronal proteins, the absence of proteolitically sensitive neuronal proteins as MAP2, the presence of centromeric DNA repetitive sequences indicating nuclear degeneration, immunopositive NeuN proteins, the presence of gliosis, the correlation between the size of dense core plaques and the size of local neurons, which can easily explain the inverse association of increasing plaque number and decreasing pyramidal neurons in the cerebral cortex of AD brains. Thus, the neuronal origin of dense core plaques allow us to link the presence of intracellular amyloid in neurons with amyloid plaque formation.

Other than studied mechanisms of aggregation and accumulation of Aβ in AD brains, there is a crucial event that has to be taken into account, the **clearance and removal** of Aβ (**Fig. 4**). An inefficient clearance system may produce an imbalance between production and removal of Aβ, leading to an increased level of Aβ in the brain. While in FAD cases the increased levels of Aβ is due to $A\beta_{42}$ overproduction, deficient $A\beta_{42}$ clearance is observed in the brains of LOAD patients (De Strooper et al. 2010). Many of the mechanisms reviewed below are mediated by glial cells (Ries and Sastre 2016).

The mechanisms of Aβ clearance involve either Aβ removal to the peripheral blood and lymphatic systems, with a series of clearance receptors, including LRP1 and VLDLR and/or degradation within the CNS tissues by proteases and peptidases. Disturbances in the clearance produce an accumulation of Aβ peptides in the blood vessel walls, causing the vascular component or cerebral amyloid angyopathy (CAA) of AD (Tanzi et al. 2004; De Strooper et al. 2010). Low-density lipoprotein receptor-related protein (LRP), located at the BBB, mediates the efflux of Aβ from the brain to the periphery. $A\beta_{42}$, more prone to aggregation, requires an initial binding to the LPR ligand/chaperone APOE and α2M to an effective clearance, while

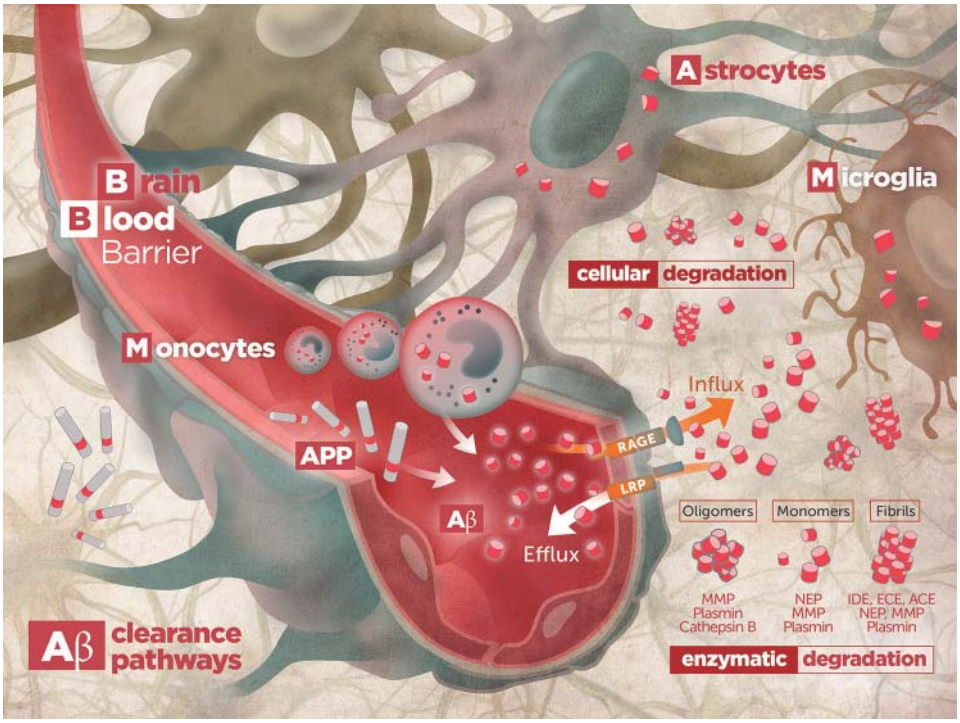


Figure 4. Aβ clearance pathways.

$A\beta_{40}$ and less fibrillogenic species could efflux direct through LRP. Aβ removed is internalized to late endosomes which can be delivered to lysosomes for latter degradation or to be targeted for transcytosis across the BBB into plasma. Mutations in genes encoding α2M, APOE and LRP have then been associated with increased risk for AD (Tanzi et al. 2004). Aβ can also enter from the bloodstream into the brain principally via the RAGE receptor which is a multifunctional receptor in the immunoglobulin superfamily which binds a large variety of different ligands such as Aβ.

On the other hand, the peptidolytic removal of Aβ involves two major endopeptidases: insulin degrading enzyme (IDE) and neprylysin (NEP), although ECE and plasmin have also been implicated. IDE is only able to hydrolyse Aβ monomers and while numerous studies have implicated NEP as a rate-limiting Aβ-degrading enzyme in the brain. The localization of NEP on the plasma membrane with its catalytic site exposed extracellularly makes the peptidase prone to peptide degradation in Aβ-containing diffuse deposits and neuritic plaques. In contrast, IDE is localized majority in the cytosol while only a small fraction resides in the plasma membrane (Tanzi et al. 2004). Lastly another way to clear Aβ from the brain is by the uptake and phagocytosis of Aβ by cells such as microglia, astrocytes and macrophagues.

Inflammation and AD

If we talk about AD we have to talk about neuroinflammation. Already at the very beginning of AD research, Fischer et al., described a local inflammatory reaction around extracellular deposits. At that time the role of microglia in brain was uncertain due the immunochemistry limitations. The discovery of microglia by Pío del Río-Hortega together with the improvement and introduction of monoclonal antibodies brought back the interest in the concept of the role for innate immunity not only in AD but in most neurodegenerative diseases (Eikelenboom et al. 2006; Mrak 2009).

Minor signs of neuroinflammation are found in the normal aging brain, while in AD brains exist a strong activation of inflammatory systems indicating that an increasing variety or qualitatively immunostimulants are present and critical for the pathological progression of AD (Heneka et al. 2015). In the last decades many studies shown a variety of **inflammatory mediators**, including acute phase proteins, cytokines, and chemokines within the proximity of plaques. These inflammatory mediators, including complement factors, were not blood-derived but produced locally by microglia (microgliosis), astrocytes (astrocytosis) and neurons (Eikelenboom et al. 2006). The complement system is found fully activated in AD brains since Aβ it is

a powerful activator and can bin C1q and trigger the classical complement pathway in an antibody-independent fashion. When this occurs, the cascade produces anaphylotoxins which promote further inflammation, opsonising components which mark material for phagocytosis, and the membrane attack complex (MAC) which is directly lytic to cells (Schwab and McGeer 2008; McGeer and McGeer 2010).

In vitro studies indicate that Aβ peptides requires a certain degree of compactation to triggers pro-inflammatory reactions of microglia and astroglia. This information is consistent with the immunohistochemical data that shows no or poor immunostanig for early components in diffuse plaques with low grade fibrillar Aβ peptide (Eikelenboom et al. 2006).

Microglia and astroglia are capable of detecting ab through several sensors like, Toll-like receptors (TLR), RAGE and NOD-like receptors (NLRs). TLR, and in particular TLR4 together with CD14 and MD2 in microglia, leads the activation of signal-dependent transcription factors that drive expression of downstream inflammatory response genes and participate in the phagocytosis of Aβ plaques by microglia. Aβ peptides and Aβ oligomers bind to RAGE (receptor for advanced glycoxidation end-products), and activate glia cells and especially microglia. RAGE also is implicated in the clearance of Aβ and involved in APOE-mediated cellular processing signalling. NLRs represents the third sensing system and when Aβ oligomers and fibrils induce lysosomal damage, NLR is expressed in microglia and induce apoptosis as well as the maturation of pro-inflammatory mediators like IL-1β and IL-18 (Glass et al. 2010). Similar inflammatory reactions take place in disorders involving misfolding of tau and α-synuclein (Schwab and McGeer 2008).

Microglia

Microglia cells (10-20% of glial cells) represent the first line of defense of the brain innate immune system against pathogens. Under pathological situations such as stroke, traumatic injury, tumoral invasion and neurodegenerative disease, microglia become activated and migrate to the injured area playing similar as phagocytic active macrophages. Microglia when activated suffer dramatic morphological changes from a resting ramified phenotype, with long cytoplasmic extensions that are in continuous movement, to motile activated ameboid cells which can be recognized by the expression of ionized calcium binding adapter molecule 1 (Iba1) or cluster of differentiation CD68 markers. The transition among one phenotype to another is promoted by various extracellular cytokines or factors such as lipids or lipopolysaccharides (LPS). Activated microglia is also classified

into inflammatory (M1) and alternative activated (M2) phenotypes that switch continuously between both. M1 is induced by agents like interferon (IFN)-γ, lipopolyssachariде (LPS), and Aβ aggregates and produce and release pro-inflammatory cytokines such as tumor necrotic factor (TNF)-α, IL-6, IL-23, IL-1β, IL-12, nitric oxid (NO), and chemokines. In contrast, M2 is activated by Il-4, IL-10 and IL-13 and express anti-inflammatrory molecules such as IL-10 and transforming growth factor (TGF)-β and extracellular matrix molecules. In AD patients, a mixed of alternative and classical activation is shown (McGeer and McGeer 2010; Bolós et al. 2017) .

Some aspects of microglia function may be beneficial since microglia is able to reduce Aβ accumulation (by phagocytosis), clearance (IDE enzyme is released by microglia and neurons) and degradation. Furthermore, microglia can also secrete several trophic factors with neuroprotective function such as the glia-derived neurotrophic factor (GDNF) (Heneka et al. 2015).

Astrocytes

Astrocytes are the major and most numerous cells of CNS and represent the main element of brain homeostatic system providing metabolic and trophic support to neurons (survival, regeneration and differentiation) by expressing an extensive range of growth factors, control of ion neurotransmitter environment and generation, regulation and maintenance of the brain blood-barrier (BBB) (Schwab and McGeer 2008; Rodriguez et al. 2009).

Under pathologic conditions, astrocytes become activated and suffer hypertrophy, proliferation and progressive thickening of their cellular process and migrate to the injured area. Astrocytes participate in β-amyloid clearance and degradation, thus, we can find a large number of them associated with Aβ deposits which in turn generate chemotactic molecules that mediate astrocyte recruitment (Schwab and McGeer 2008; Rodriguez et al. 2009; McGeer and McGeer 2010). Astrocytes gradually accumulate, by phagocytosis, $A\beta_{1\text{-}42}$ of locally degenerated dendrites and synapses, especially in the molecular layer of the entorhinal cortex of AD brains. Recent evidences suggest that the phagocytosis of Aβ peptides may depend on their APOE status, suggesting that APOE polymorphisms may influence the risk to develop AD, by affecting astroglial ab phagocytosis. In contrast, astrocytes could act as a source of ab because they overexpress BACE1 in response to chronic stress. If astrogliosis have a key role in generation or phagocytosis in amyloidosis is still unknown but the certain is that has an important contribution to inflammation, releasing pro- and anti-inflammatory mediators such as complement factors, complement inhibitors, chemokines, cytokines

or neurotrophic factors, in addition, astrocytes secrete immune modulators that are involved in the regulation of microglia activation and regulation (Heneka et al. 2015). Astrocyte and microglial activation could be an early event in the disease, even before Aβ depositions, but rather to a response to Aβ oligomers or protofibrils. Because cytokines like TNF-α, interleukines IL-1β and IL-6 directly impair neuronal function and supress hippocampal LTP, early focal inflammatory events may contribute to neuronal dysfunction well before neuronal cell death and parenchymal volume reduction become apparent (Heneka et al. 2015).

Neurons

Against what was assumed, neurons are capable of producing inflammatory mediators *per se* and exacerbate local inflammatory reactions, contributing to their own destruction and degeneration of typical AD brains. Alternatively, pro-inflammatory mediators such as TNF-α and low levels of NO may confer neuroprotection instead of destruction in the local inflammatory reactions (Heneka et al. 2015).

In conclusion, **brain inflammation response is a double-edged sword, which has beneficial but also deleterious effects when the inflammation becomes dysregulated and chronically activated** (Eikelenboom et al. 2006). The challenge falls on exploit the beneficial aspects of the neuroinflammation, while neutralizing its harmful sequelae.

Oxidative stress and AD

Oxygen (O_2) is an indispensable molecule for most life forms and participate in the major part of the reactions which take place in the organisms. Oxidative stress reflects an imbalance between the **reactive oxygen species (ROS)**, produced as physiological by-products of normal metabolism (**Fig. 5**), and the capability of the organism to readily detoxify the reactive intermediates or to repair the resulting damage by cell antioxidants mechanisms.

There are several ROS with pernicious effects (superoxide radical anion, $O_2^{-}\bullet$; hydrogen peroxide, H_2O_2; hydroxyl radical. OH•) and Reactive Nitrogen Species (RNS: Nitric Oxide, NO•; peroxynitrite, $ONOO^{-}$). To cope the harmful effects of these reactive species, cells have developed highly elaborated mechanisms of regulation, defence and repair including antioxidant enzymes (Superoxide Dismutase, SOD, Catalase, Glutathione Peroxidase, GSHPx, Gluthatione Reductase, GSHRd) and more adaptative cellular responses (J. et al. 2004).

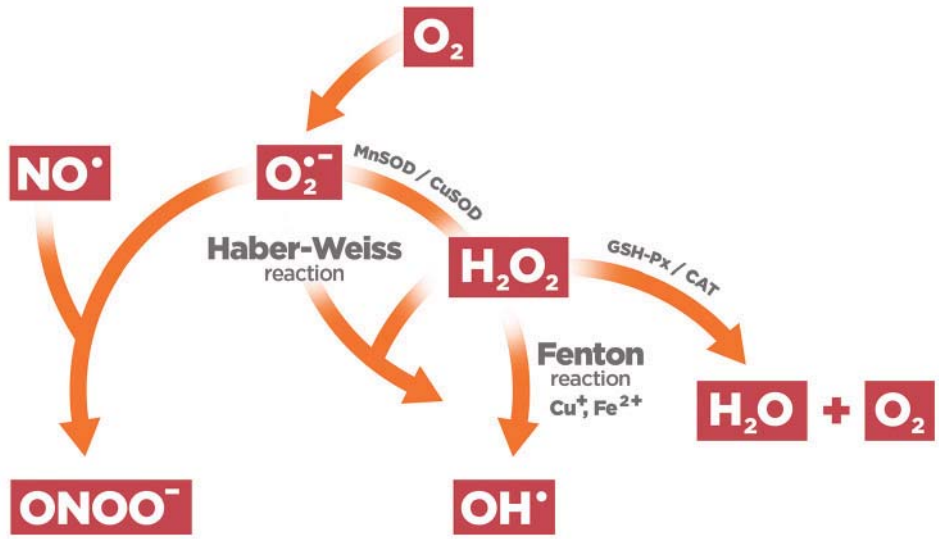


Figure 5. Scheme of ROS and RNS production.

ROS and RNS can induce peroxidation of cell membrane lipids, modifying the biological properties of the membrane such fluidity and inactivating the membrane-bound receptors or enzymes, impairing normal cellular function and, thus, increasing cellular damage and generating new oxidized products. Also, ROS can damage physical structures of proteins, and therefore most of functional processes within cells altering immunogenicity. Moreover, protein damage products can contribute to secondary damage to other biomolecules, such as DNA. DNA bases are vulnerable of hydroxylation, protein carbonylation and nitration (García-Blanco et al. 2017).

These resulting products can be measured in peripheral fluids as an oxidative stress index through oxidative markers such as Malondialdehyde-thiobarbituric acid-reacting substance (MDA/TBARS), 4-Hydroxynonenal (HNE) or Isoprostanes in case of lipid peroxidation, protein carbonyls, nitrotyrosine and Advanced Glycation End Products (AGEs) in protein oxidation and glycol-oxidation and DNA strand breaks and 8-OH-2'-deoxyguanosine (8-OHdG) in DNA oxidation (Praticò and Delanty 2000; Sultana and Butterfield 2010).

The brain is particularly vulnerable to oxidative damage due to its oxygen consumption and high energy requirements. It is rich in peroxidizable fatty acids and in transition metals which may catalyse through the Fenton reaction the formation of •OH. Also, is relatively deficient in antioxidants compared with other organs (Praticò and Delanty 2000; Guglielmotto et al. 2010).

The ability of Aβ peptides to induce protein oxidation and to inhibit the activity of oxidation-sensitive enzymes is consistent with the hypothesis that Aβ can lead to impair cellular function and cell death and consequently to cognitive impairment and AD-like pathology (Sultana and Butterfield 2010). Also, oxidant agents and oxidative products increase intracellular and secreted Aβ by increasing APP synthesis or by increasing γ-secretase activity which in turn increases BACE1 activity and thus Aβ production (Guglielmotto et al. 2010).

Metals and AD

Metals are widely distributed in nature and in biological systems. Biometals such as copper (Cu), zinc (Zn) and iron (Fe) have physiological functions that serve to maintain the homeostasis and the normal function of the cellular processes due to their capability of ionization and bonding properties. Metals, such as aluminium (Al) and lead (Pb), are classified as toxicological metals, with no biological function in the organisms. Moreover, when there is a mis-metabolism of any ion metal which result in levels outside the normal physiological range, we talk of toxicological process that induce biological damage (Adlard 2006).

Thus in AD abnormal distribution and imbalanced homeostasis of transition metals have been shown, particullary Zn, Cu and Fe and specially in increased levels within the plaques. In addition, Cu levels are found to be decreased in bulk tissue of the AD-affected.

Metals have been postulated to be involved in the pathogenesis of AD for several reasons and one is the interaction of Aβ with particular metals (Cu and Zn) that drives Aβ pathogenicity and downstreams AD pathology according to 'The metal hypothesis of AD' (A.I. 2008). Ions such as Cu, Zn and Fe are constitutively found in neocortical areas most predisposed in AD pathology and markedly increased levels within the amyloid plaques in AD brains (Bush 2003).

Aβ has selective high and low affinity Cu and Zn binding sites that mediate its aggregation (and to a lesser extent Fe) via histidine residues located at the N-terminal end if the Aβ sequence. The affinity of Aβ for $Cu^{2}+$ is greatest for $A\beta_{42}$(human)> $A\beta_{40}$(human)> $A\beta_{42}$(mouse)> $A\beta_{40}$(mouse) because of the enhanced ability of the longer peptide to form a $Cu^{2}+$-coordinating oligomer and correlate well with the increased redox activity and the toxicity (Maynard et al. 2002; Bush 2003). However, the affinity of variant Aβ

species e.g. $A\beta_{40}$ and $A\beta_{42}$ for $Zn^{2}+$ is equal (Bush 2003). At physiological pH, $Zn^{2}+$ is the most powerful inducer of Aβ aggregation and binds to Aβ to form insoluble aggregates, while $Cu^{2}+$ binding is competitive inducing a soluble conformation (Maynard et al. 2002). In acidic conditions, which occurs in aged brain and in response to inflammation, $Cu^{2}+$ displaces $Zn^{2}+$ from Aβ. Presynaptic Zn released in the extracellular space leads to β-amyloid formation in mutant APP transgenic mice. Mice deficient in Zn transporter (ZnT3) and thus deficient in synaptic Zn significantly inhibited β-amyloid pathology and congophilic angiopathy (CAA). Zn release during transmission might explain gender effect of AD since female mice exhibited age-dependent hyperactivity of the ZnT3 transporter associated with increased amyloid deposition, which was abolished in Tg2576/*ZnT3*$^{-}$/$^{-}$mice. This suggests that when Aβ is precipitated by synaptic Zn, it co-precipitates with $Cu^{2}+$ and $Fe^{3}+$, a possibility supported by the observation of selective $Cu^{2}+$ and $Zn^{2}+$ binding sites in Aβ (Bush 2003). Thus, the balance between metals such as $Zn^{2}+$ and $Cu^{2}+$ and the maintenance of neutral pH could be important factors to prevent Aβ aggregation and amyloid formation (Duce and Bush 2010).

If we focus on Aβ degradation, metals may also be implicated in this process since the two main AB degrading enzymes (IDE and NEP) are Zn metallopeptidases. Besides, ApoE isoforms prevent copper-mediated aggregation of Aβ in a manner that correlates with the risk for AD, and the precipitation of Aβ by Zn and Cu is reversible with the chelation, in contrast with the fibrilization, which is reversible (A.I. 2008).

Apart from assembling Aβ and binding ion metals, Ab reduces $Cu^{2}+$ and $Fe^{3}+$, producing H_2O_2 by double electron transfer to O_2, which in presence of reduced metals and in the absence of sufficient detoxifying enzymes (tipically of AD), gives rise via the Fenton reaction, to the generation of the toxic •OH radical and the subsequent oxidative stress. Such oxidative damage typifies AD neuropathology and precedes Aβ deposition (Bush 2003) . The redox activity (metal reduction and H_2O_2 and •OH formation) is greatest for $A\beta_{42}$ than for $A\beta_{40}$ (Adlard 2006, Huang 1999).

On the other hand, metals regulate APP synthesis and APP in turn participates in their homeostatic regulation. Moreover, metals may also influence APP processing as secreatases (which are implied in processing of APP) interact with different metal spices. Particulary, BACE1 posseses a Cu –binding sites and γ-secrestase has been reported to be inhibited by low levels of $ZN^{2}+$ (Bush 2008).

Altogether, evidence of the **potentially pleiotropic character of metals and the significance of its homeostatic regulation in the pathophysiology of AD, at the same time that raises new therapeutic targets** to prevent the onset and progression of the disease (Duce 2010).

Tau and neurofibrillary tangles (NFT)

NFT, the other neuropathological hallmark of AD, are a major intracellular microscopic lesions located especially in large pyramidal neurons of Ammon's horn of the hippocampus and the cerebral neocortex, though are also present in other regions. The main component of tangles is the Microtubule Associated Protein (MAP) family tau (τ) which is a normal axonal protein that, physiologically, binds to microtubules through its microtubule-binding domains, promoting microtubule assembly and stability (Blennow et al. 2006). Tau is present in all nucleated cells and relatively abundant in neurons, predominantly found in axons and in less proportion in dendrites (Castellani et al. 2011). The biological function of Tau is regulated by several kinases (GSK-3β and CDK5) and phosphatases (PP-1 and PP-2). An imbalance between these kinases and phosphatases results in an abnormal phosphorylation of 38 or more serines and/or threonine amino acids on tau (Biran et al. 2009).

In AD like in other taupathies such as Pick's disease, progressive supranuclear palsy, corticobasal degeneration and others, exist a hyperphosphorylation of tau that starts intracellularly and leads to sequestration of normal tau and other microtubule-associated proteins. This loss of tau function compromise axonal transport and contribute to the synaptic degeneration observed in AD (Blennow et al. 2006; Castellani et al. 2011). Hyperphosphorilated tau also becomes prone to aggregation into insoluble fibrils in tangles, compromising the neuronal function (Blennow et al. 2006).

The aggregation is a multi-step process initiated by a rate-limiting nucleation step, which is followed by the progressive addition of tau proteins in an elongation process. Firstly there is a redistribution of microtubule-bound tau to the cytoplasmic pool of tau by various factors: phosphorylation, mutations, proteolysis, etc. Cytoplasmic tau is highly soluble, and additional factors, including proteolysis, are necessary to convert this tau into nucleating forms of tau, which induce pretangles, paired helical filaments (PHF) and neurofibrillary tangles (De Strooper et al. 2010)

The current idea is that like Aβ cascade model, the intermediate aggregates of abnormal tau molecules are cytotoxic and impair cognition. However,

NFT and the intermediates tau aggregates exist within the cytoplasm of viable neurons (Castellani et al. 2011).

Tau in tangles becomes ubiquitinated for non-lysosomal degradation, but this process is inefficient, and tangles may finally lead the neurons to death (Blennow et al. 2006). Albeit experimental results indicate that Aβ accumulations precedes and drives tau aggregation, it has been demonstrated recently that tau is implicated in Aβ toxicity since reduction of endogenous tau levels prevented behavioural deficits in transgenic mice expressing APP, without altering their high ab levels, suggesting that tau reduction could block ab-induced neuronal dysfunction (Roberson 2007).

Biomarkers, therapies and future perspectives in AD

In these last decades, AD research have been trying to highlight the aetiology and pathogenesis of the disease with the hope to find successful therapies targeting different disease events.

Multifactorial disease are very common in the elderly population, and treatment of these disease usually requires a combination of different approaches (De Strooper et al. 2010), in fact, as many as all the pathways which are implicated in the pathology: Aβ aggregation and plaque development, hyperphosphorilation and aggregation of tau protein and formation of tangles, inflammation, oxidative stress and the consequent loss of synaptic integrity and progressive neurodegeneration (Hampel et al. 2010).

One of the problems in the research of the therapy is the lack of reliable **biomarkers**. A biomarker is defined as an indicator of normal or/and pathological processes or pharmacological responses to a therapeutic intervention (Giacomelli et al. 2017). Biomarkers should serve as surrogate end points for clinical outcomes, increasing objectivity and efficiency in regulatory decision-making. Also, could serve in diagnostic of pre-symptomatic identification of patients with AD and to aid treatment decisions and individualized care. Finally, could work as screening tool for disease prevention programmes. Studies in transgenic mouse models talk about the importance of intervention in early stages of the disease to be more effectiveness than when there is severe plaque pathology and neurodegeneration (Hampel et al. 2010).

Imaging techniques at CNS such as MRI, fMRI, MRS or PET are the most reliable methods for detection of AB deposits and NFT of tau, as well as,

ELISA and WESTERN-BLOT are used in to detect peripheral (CFS) biomarkers (Giacomelli et al. 2017).

The current AD **therapies** are only symptomatic and consist in the administration of acetylcholinesterase inhibitors (AChEIs) which increase AChE concentrations in the synaptic cleft and enhance cholinergic transmission, and the administration of a non-competitive N-methyl-D-aspartate (NMDA)-receptor antagonist memantine, which protects neurons against glutamate-mediated excitotoxicity and inhibits tau hyperphosforilation and aggregation biran . Often both drugs are used together in spite of the beneficial but modest effects on cognitive and behavioural tests and functional outcomes. Also, the majority of AD patients are treated with antipsychotics or antidepressants to manage neuropsychiatric and behavioural symptoms. These current therapies provide only temporary symptomatic relief but do not inhibit or reverse the disease mechanisms. There are more than 50 compounds in different stages of clinical investigation (www.alzforum.org): statins, peroxisome proliferator-activated receptor-γ agonists, non-steroidal anti-inflammatory drugs (NSAIDs), neurotrophic molecules, metabolic or nutritional drinks and other molecules in pre-clinical stage of development.

Mainly the **pharmacological agents** being developed target the principal cerebral proteins implicated in the AD (**Fig. 6**): tau and Aβ. AD pharmacotherapies targeting tau consist of modulators of tau phosphatases and kinases, and tau aggregation inhibitors namely TAIs. Pharmacotherapies targeting Aβ, modulates Aβ production through inhibitors or modulators of the secretases, inhibit Aβ aggregation and passive or active immunization (Biran et al. 2009).

Pharmacotherapies targeting metal ions have been developed in the last years since is known that metal homeostasis is essential for the maintenance and well-being of physiological functions and that this homeostasis appear dysregulated in neurodegenerative disease. Two therapeutic strategies are the use of antioxidants, which neutralize free or incorrectly bound metals and decrease the generation of ROS and other radicals, and the use of metal chelators which bind metal ions and convert them in inert forms. Metal-complexes are emerging as a potential therapy which deliver metals to cellular compartments which are metal-deficient (using metal complex of pyrrolidine dithiocarbamate $–M^{2}$+-PDCT-) or prevent the harmful binding of Cu to AB, using platinum 1.10 phenanthroline derivates (L-$PtCl_2$). Metal-protein attenuating compounds (MPACs) bind metals with weak reversible affinity and compete with endogenous ligands for metal ions main-

taining the physiological metal levels in specific cellular compartments. Clioquinol (CQ and PBT-1) and PBT-2 are examples of MPACs which have demonstrated beneficial effects in the treatment of AD and other neurodegenerative disease. Now in a phaseI and phase II of clinical trials (respectively), these therapies highlight the potential role of the metals in modifying AD progress (Roberson and Mucke 2006; A.I. 2008; Biran et al. 2009).

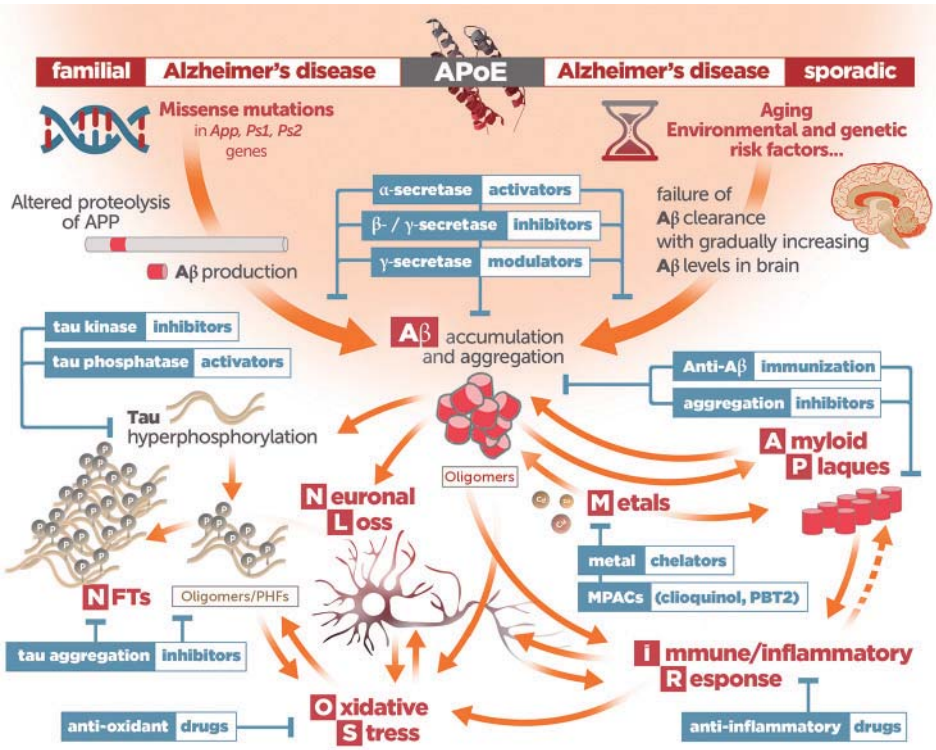


Figure 6. Current therapeutic approaches for the AD treatment.

Animal models of AD

Genetically engineered animals exhibiting age-dependent behavioural, functional and histopathological features of Alzheimer's disease, represent one of the most powerful tools to study the disease *in vivo*. Despite some models have been generated in different species, indisputably, mice are the specie used *par excellence* in this field. Regardless of the species chosen, modelling requires the disease to be associated with a genetic mutation or at least for hypothesis to exist regarding the likely pathophysiology of the

disorder that can be modelled by a genetic modification. AD may in many ways be regarded as the perfect disease for modelling in transgenic animals (Elder et al. 2010).

The majority of the transgenic mouse models of AD are based on the **overexpression of transgenes containing EOFAD mutations**: on *App* and *Psen* genes. Tg lines carrying missense mutations in *App* commonly display a progressive Aβ deposition between 6-9 months, Cerebral Amyloid Angiopathy (CAA), astrocytosis, microgliosis, cytokine production, oxidative stress and cognitive impairment in an aged related manner. In these models no NFT and neuronal and synaptic loss is associated (Janus 2000; Bilkei-Gorzo 2014).

Transgenic lines carrying *Psen* gene mutations show increased brain levels of $A\beta_{42}$ with no effect in $A\beta_{40}$ levels and neither plaque pathology nor NFT and neuronal loss. Despite the lacking of plaques, these models exhibit increased lipid and protein peroxidation, impaired hippocampal neurogenesis in adult brain and cognitive and behavioural abnormalities. Double transgenic mice carrying both *App* and *Psen* mutations have an early onset of the AD phenotype and show a robust age-dependent Aβ pathology, probably owing to the increase in $A\beta_{42}$ production, together with cognitive impairment and inflammation among other features, compared to single transgenic APP lines (Thomas Wisniewskia 2010). However, bigenic lines do not show some aspects of the disease such as neuronal loss and tau deposition (Puzzo et al. 2015).

Among all the APP-based Tg mice, **Tg2576** is the most widely used and characterized AD model. This model overexpress the 695-amino acid isoform of human APP containing a Lys^{670}→Asn, Met^{671}→Leu mutation (APP 695, K670N/M671L), which was found in a large Swedish family with early-onset AD, under the control of the hamster prion protein (Prp) promoter (Hsiao et al. 1996). Brain levels of Aβ begin increasing at 6-7 months age and amyloid deposits star developing between 9 to 12 months, when plaques become evident (Hsiao et al. 1996; Kawarabayashi et al. 2001; Thomas Wisniewskia 2010). This model also exhibit a marked congophilic angiopathy and the deposition of a large amount of AB_{40} caused presumably by some combination of species, strain, promoter, expression level, and mutated transgene (Kawarabayashi 2001).

It is generally accepted that Tg2576 mice display an age-dependent alterations in cognitive performances e.g increased locomotor activity and decreased anxiety in open field or the plus maze tests (Lalonde et al. 2003; Ognibene et al. 2005; Gil-Bea et al. 2007), and impaired learning and spatial

memory in Morris Water Maze (Westerman et al. 2002). However, there are some variations among the results of different laboratories (King and Arendash 2002; Deacon et al. 2008; Reed et al. 2010). Other AD features such as inflammation (microgliosis and astrogliosis) (Frautschy et al. 1998) or oxidative stress (Smith et al. 1998) are also present in this aged tg mouse line, but no neuronal loss is found.

In addition to AβPP models, Tg mice expressing mutated human tau have been created in order to reproduce the developing of NFT which are absent in App/Psen based models. Models of tau pathology exhibit NFT (although its distribution differs from that found in AD), neuronal death and behavioural deficits (McGowan et al. 2006; Thomas Wisniewskia 2010).

Triple transgenic mice (3xTg), combining App, Psen and Tau mutations are developed to obtain more complete AD mouse mode. This triple Tg mice develop Ab plaques previously to NFT pathology with a temporal and spatial pattern similar to that observed in AD, inflammation, synaptic dysfunction and cognitive decline (Oddo et al. 2003; Giménez-Llort et al. 2007).

Metallothioneins

General characteristics

Metallothionein (MT), was first isolated as a cadmium and zinc protein in horse kidney by Margoshes and Vallee 60 years ago. Some years later, Kägi et al. (1984), named them referring to its high content of metals and cysteine residues.

MTs have been defined through years as a non-enzymatic, polypeptidic superfamily which is expressed in all eukaryotes, including plants and other phylogenetic groups, and in some procaryotes (Kagi et al. 1983; Ghoshal and Jacob 2001). Typically, MTs are low molecular predominantly cytoplasmic proteins (<7000 Da) with high metal content, specially Zn and Cu or Cd, and highly conserved 18-23 cysteine residues and no aromatic amino acids or histidine (Coyle et al. 2002). The characteristic feature of all MTs is the distribution of cysteinyl residues such as Cys-Xaa-Cys and Cys-Cys (where Xaa stands for a residue different from Cys) (Hamer 1986; Kägi 1987; Binz P-A and Kägi J 1999; Romero-Isart and Vasák 2002).

Based on its sequence similarities and phylogenetic relationship, they have been classified into 15 families. Mammalians MTs, which belong to the first family, are subdivided in 4 distinct isoforms, MT-1 through MT-4. In hu-

mans, the MT genes are clustered in the q13 region of chromosome 16 (Palmiter et al. 1992; West et al. 2008) and consist of seven functional MT-I genes (*MT-1A, -B, -E, -F, -G, -H* and *-X*) and a single gene encoding each of the other MT subfamilies, namely MT-II (the *MT-2A* gene), MT-III and MT-IV. Mice have simpler MT gene structure with only one functional gene for each isoform (MT-I through MT-IV), located in chromosome 8 (Palmiter et al. 1992; West et al. 2008). All human and murine *Mt* genes consist of three exons that are highly homologous among isoforms that in mice are separated by two introns highly conserved but divergent among isoforms (Ghoshal and Jacob 2001). Heterogeneity of different isoforms results from postranslational acetylation and/or variations in metal composition (metalloforms). Isoforms may be distributed in different ratios in individual tissues and have different rates of degradation (Coyle et al. 2002). Mt-1 and Mt-2 are widely expressed in all the tissues (especially Mt-2A), being liver, kidney, intestine and pancreas the organs with higher levels of these proteins. The expression of Mt-3 and Mt-4 genes is restricted to SNC and the stratified squamous epithelia, respectively (Uchida et al. 1991; Palmiter et al. 1992; Quaife et al. 1994).

Structure

Much what we know about the biological actions of MTs has arisen from comparative analysis of their chemical and structural features (Vasák and Hasler 2000a).

All CNS MTs are single polypeptide chain (**Fig. 7**) of 61 to 68 amino acids, 20 of which are highly conserved cysteine residues which are able to bind metals through thiolate bonds established between the thiol groups (SH-) and the metal ion (Hamer 1986). Each protein binds 7 divalent metal ions (e.g. Zn(II)) and up to 12 monovalent copper ions, divided into two metal-thiolate clusters. These clusters are localized in two independent but interacting globular domains which are connected with a flexible hinge region of a conserved Lys-Lys (lysine) sequence in the middle of the polypeptide chain. The C-terminal α domain (residues 33-61) is encoded by exon 3 and incorporates four divalent or six monovalent metal atoms through 11 Cys residues. The N-terminal β domain (residues 1-29) is encoded by exons 1 and 2 and coordinate only three divalent or six monovalent metal atoms through 9 Cys residues. The α domain is more stable and less flexible than β domain. The metal ions in both clusters are tetahedrally coordinated by both bridging and terminal thiolate ligands (Hidalgo et al. 2001).

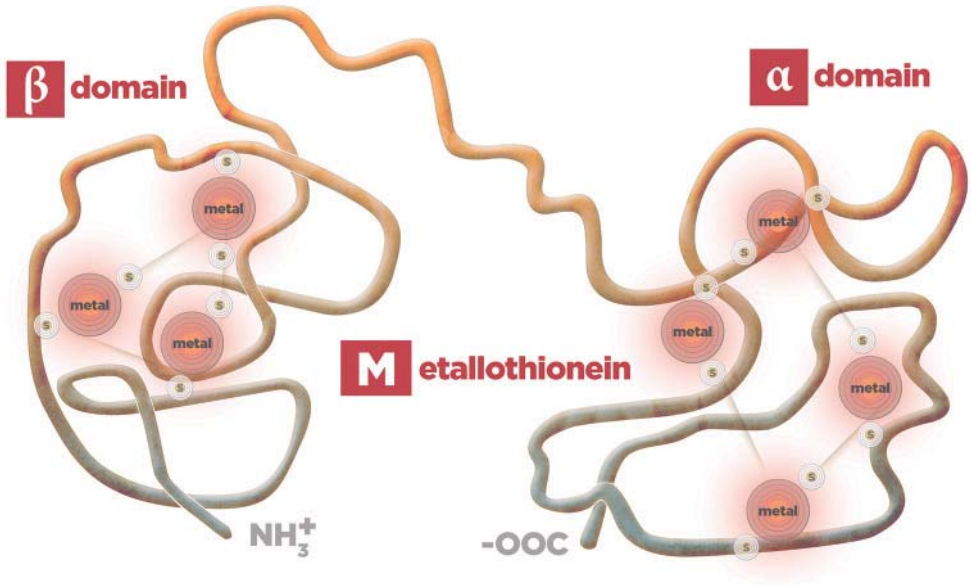


Figure 7. MT structure.

MT-1 and MT-2 (61-62 aa) isoforms are structurally similar but they differ in their total charge due to the differences in certain amino acids other than Cys (). Physiologically, they usually bind 7 divalent metal ions (Zn(II), Cd(II)) and up to 12 monovalent copper. However, MTs can bind other metal ions such as Cd^2 and Hg^2 *in vivo* or Pb^2+ and Ag+, among others, *in vitro*.

In comparison, MT-3 (68 aa) has two inserts: a single Thr in the N-terminal region and an acidic hexapeptide in the C-terminal region. In addition, it contains a conserved motif Cys(6)-Pro-Cys-Pro(9) which is absent in the other isoforms and the possible responsible of the growth inhibitory activity in neuronal assays that characterizes MT-3 isoform, also named GIF (growth inhibitory factor) for this reason (Ghoshal and Jacob 2001; Hidalgo et al. 2001). In contrast with MT-1 and MT-2, native MT-3 contains both Zn^2+ and CU+ ions (Cu_4, Zn_3MT-3), organized in Cu_4- and Zn_3-thiolate clusters. The fact that in neuronal assays GIF, but not MT-1 and MT-2 isoforms, exhibited biological activity indicates a distinct function and distinct inducibility and regulation of this protein (Uchida et al. 1991; Erickson et al. 1994; Vasák and Hasler 2000b).

MT-1 and MT-2 regulation and functional aspects

The highly conserved structure of MT isoforms among higher eukaryotes suggests that these proteins perform important biological functions although the primary function has not yet been elucidated (Ghoshal and Jacob 2001). The certain is that MTs are implicated in biochemical reactions due to its unusual structure and chemical features: high content of Cys and the content of metals (Valle 1995, Ghoshal and Jacob 2001).

*Mts e*xpression is regulated at transcriptional level (**Fig. 8**), where *cis*-acting DNA elements (located in the promoter region of the MT genes) respond to *trans* elements or transcription factors, modulating the gene expression. TATA-box and elements such GC-box (binding site for Sp1) are essential for basal transcription of the gene, regulated by repressors as well. Besides, other *cis* elements like MREs or USF/ARE and *trans* elements as MTF-1, Sp1 and USF/ARE are involved both in basal and induced expression of *Mt* genes (Haq et al. 2003).

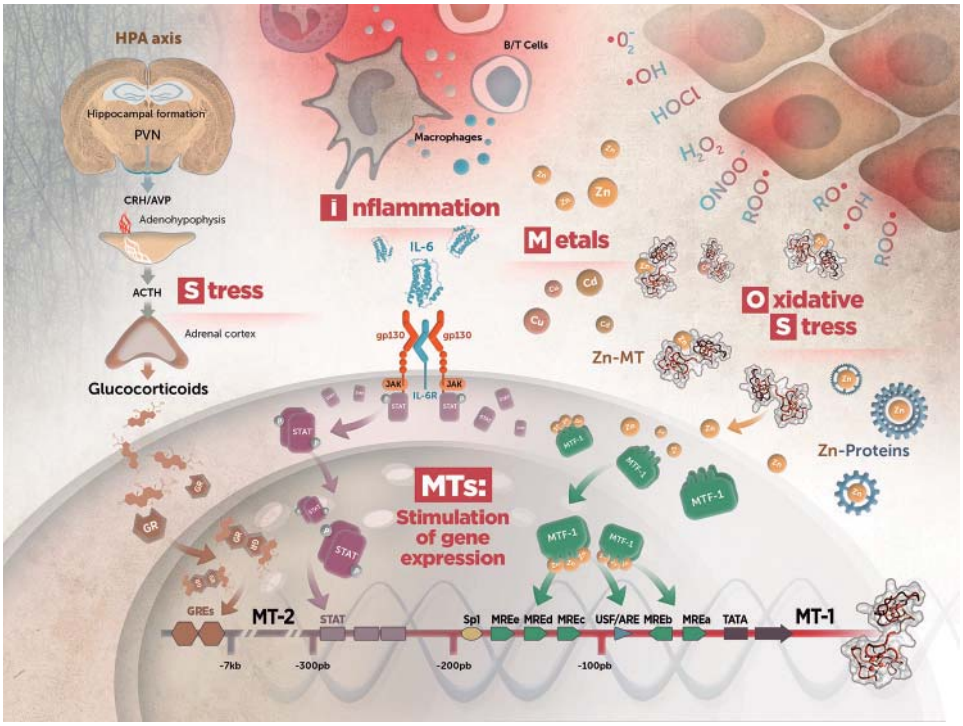


Figure 8. MT-1/2 promoters and regulation.

MT-1 and MT-2 isoforms are both basally expressed and highly inducible. Events inducing MT-1 and MT-2 generally do not enhance MT-3 and MT-4 expression (Haq et al. 2003). **MT-1 and MT-2 genes in higher species are rapidly induced by a variety of stimuli** including metals, hormones, cytokines, oxidants, stress, etc.

Metals

Through to the ability to bind various heavy metals such as Cd, Hg, Cu and Ag, MTs were mainly considered as mediators of cellular detoxification (Ghoshal and Jacob 2001). *In vivo* and *in vitro* studies evidenced that exposure (either by diet or environment) to heavy metals result in an elevation of MT-1 and MT-2 synthesis especially in the liver. Similar exposure to metals in MT-1 and MT-2 knockout mice leads to metal toxicity, while MT-1 and MT-2 overexpressing mice are relatively protected to toxicity (Coyle et al. 2002).

Nevertheless, MTs binds biologically to metals such as Zn^{2}+ and Cu+, and thus they have an important key role in metal homeostasis. They also act as a reservoir for these biometals, and facilitate the reversible transfer of these ions to cellular macromolecules which needs zinc for their biological activity. MTs may act as a zinc donor (MT) or a zinc acceptor (apothionein) modifying the activation or inhibition of transcription factors. This reversible activation of transcription factors by MT is specific for zinc-finger proteins (Ghoshal and Jacob 2001).

Changes in dietary zinc contents affected mice lacking MT-1 and MT-2, particularly during the development. In contrast, MT overexpressors showed relatively resistant to these conditions (cites que fa ghoshal). The role in copper homeostasis was studied in a mouse model for Menekes disease that has cpper deficiency due to mutations in a copper effluxing ATPase, *ATP7A* (Kelly and Palmiter 1996). Deletion of MT-I and MT-II genes in these mice was lethal (Ghoshal and Jacob 2001).

MTs metal induced synthesis is mediated by Metal Response Elements (MREs), which are cis-acting DNA sequences with a conserved consensus sequence (TGCRCNC), located in the promoter region of all mammalian *Mt* genes. The MREs are binding sites for several transcription factors involved in the basal expression of Mt genes. Among these factors, the Metal transcription Factor-1 (MTF-1), a zinc-sensitive trans-acting factor composed of six zinc fingers, is essential for the constitutive and induced expression of Mt-1 and Mt-2 genes (Ghoshal and Jacob 2001).

Hormones and stress

MT synthesis in mammals respond to a number of stress stimuli including psychological stress (restraint, immobilization), especially in liver, but also in heart and brain (Hidalgo et al. 1990, 1994). The attempts to characterize the putative factors involved in the control of stress, evidenced that glucocorticoids (GC), and catecholamines might mediate the response to stress. Moreover, other hormones like glucagon, glutathione, endogenous opioids and cytokines such as IL-6, have also been involved in the regulation of the MT. Nevertheless, studies in rat (but not in mice) indicated that neither glucocorticoids, nor catecholamines nor endogenous opioids seems to mediate the effect of stress on liver MT-1 and MT-2 synthesis. This data suggests that other factors than endocrine system could participate in the stress response. Glucocorticoids Response Elements (GREs) are responsible for Mt expression in response to GC through the binding of the activated receptor of this hormone to GRE.

Oxidative agents

Oxidative stress-inducing agents which generate reactive oxygen spices (ROS) chemically react with MTs and induce rapidly *MT* genes at transcriptional level (Ghoshal and Jacob 2001). Free radicals can be generated by UV or X-radiation, as a result of reactions catalysed by metals, produced by neutrophils and macrophages during inflammation and as products of the mitochondrial respiratory chain (Ruttkay-Nedecky et al. 2013). Free radicals are part of many cellular signalling systems and are beneficial in the immune response to infection, but at high concentrations they may be important mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acids, playing a key role in the development of disease, such as cancer, arteriosclerosis, arthritis and neurodegenerative diseases.

Through the years the hypothesis that MT functions as antioxidant against reactive oxygen and nitrogen species have been confirmed in different organisms. *In vitro* studies, using a cell-free system, have demonstrated the ability of MT as a free radical scavenger such as free superoxide ($O_2^{-}\bullet$) and hydroxyl (OH•) radicals. All 20 cysteine sulfur atoms are involved in the radical quenching process, and the rate constant for the reaction of hydroxyl radical with MT is about 340-fold higher than that with GSH, which is considered of fundamental importance in cell antioxidant defence (Thornalley and Vašák 1985; Vasák and Hasler 2000a; Yu et al. 2011; Ruttkay-Nedecky et al. 2013). Experimental studies with transgenic mice robustly suggest an antioxidant role of MTs, being KO mice more sensible to damage caused by pro-oxidants

and mice overexpressing MTs or pre-treated with MT inducers (e.g metals like Cd) be relatively protected from oxidative stress damage. However, some studies report no differences in oxidative stress levels of MT-1&2 null cells/mice compared with control mice (Vidal et al. 2008; Hyldahl et al. 2010) suggesting the existence of compensatory mechanisms for the absence of these MTs (Vidal et al. 2008), despite the levels of GSH, or CuZn-SOD, catalase or GSH-peroxidase activity remain unchanged in MT1&2 KO embryonic cells (Lazo et al. 1995). MTs could protect cells from oxidative stress not only by acting as free radical scavenger, but also through metal binding/release dynamics. For instance, beneficial effects of MTs can drive from release upon Cys oxidation or from the complexation of metal cations able to generate hydroxyl radicals through Fenton reaction in presence of H_2O_2 (Viarengo 2000 and Hidalgo 2009).

The *cis* regulatory elements that respond to free radicals generators are the Antioxidant Response Elements (ARE). The ARE overlap with USF (Upstream Stimulator Factor) elements and have been described to induce *MT* in response to H_2O_2 but no to tBHQ (tert-butylhydroquinone) suggesting that specific signal transduction cascades may mediate response to different forms of oxidative stress (Andrews 2000; Ghoshal and Jacob 2001). Also, MREs which respond to metals, may n in the cell, that would act as a secondary messenger activating the DNA-binding activity of MTF-1. MTF-1, under certain circumstances is able to interact with AREs (Andrews 2000).

Inflammation and cytokines

Significant upregulation of MT-1/2 in the brain occur by factors such as endotoxin (Durnam et al. 1984), stress (Hidalgo et al. 1991), glutamate analogues and seizures (Dalton et al. 1995), traumatic lesions (Carrasco et al. 1999; Ding et al. 2002), ischemia (Campagne et al. 2000) and neurodegenerative diseases (Carrasco et al. 2006; Hidalgo et al. 2006). In all these models, cytokines and/or oxidative stress are likely to be involved (Hidalgo 2004). Inflammatory acute phase cytokines such as IL-1, IL-6, TNF-α, IFN-α and IFN-γ are essential mediators of the immune system and might be relevant in MT-1/2 regulation *in vivo* (Hidalgo et al. 2009). Signal Transducer and Activator of Transcripion (STAT) binding sites mediate the increased *Mt* expression in response to some inflammatory factors through STAT transcription factors (Lee et al. 1999).

The exogenous administration of IL-1α/β, IL-6, TNF-α and IFN-γ was found to increase the liver MT-1/2 levels and studies with transgenic mice expressing either IL-6, TNF-α, IL-3 or IFN-α under the control of GFAP gene pro-

moter, causing a targeted expression of the astrocyte, also shown a dramatic upregulation of these MTs and a marked gliosis and neuronal damage (Hidalgo 2004). Studies with transgenic mice deficient for MT-1/2 showed a delayed recovery after injury displaying an enhanced inflammatory response including increased recruitment of macrophagues, lymphocytes and enhanced secretion of pro-inflammatory factors, compared with normal mice. *Mt1* overesspressing mice showed increased synthesis of anti-inflammatory cytokines, growth factors and neurotrophins facilitating the recovery (Fu et al. 1998; van Lookeren Campagne et al. 1999; Penkowa et al. 2002, 2005; Kang et al. 2003). Overall, all the studies are compatible with a role of MT-1/2 decreasing oxidative stress, inflammation and apoptosis in the CNS (and in other tissues)(Lazo et al. 1995; Kondo et al. 1997; Fu et al. 1998)

MT has been observed to interact with the plasma membrane of immune cells (Youn et al. 1995; Borghesi et al. 1996). Extracellular MT decreases both cytotoxic T lymphocyte induction (with an important role in protective immunity) and cytolytic activity against allogenic target cells, suggesting an important role of MT in the immune function. Indeed, MT injection, demonstrated that MT can modulate in vivo humoral immune response to T-dependent antigens by decreasing the immune response (Borghesi and Lynes 1996; Hidalgo et al. 2009). In line with this results, genetic inability to express MT-1/-2 in MT knockout mice leads to elevated humoral response to T-dependent antigen challenge (Crowthers et al. 2000).

Extracellular roles

In addition to important intracellular roles described above, MTs have also interesting extracellular roles. MT-1/2 can be detected in the extracellular fluids *in vivo*, such as serum, bile and urine (Bremner I., Mehra RK. 1987), and is actively secreted by adipocytes (Trayhurn et al. 2000; Trayhurn and Beattie 2001). Moreover, *in vitro* studies reported that extracellular MT-1/2 proteins are internalised by kidney tubule cells through endocytic receptor megalin, responsible of the 30-60% of the uptake by these cells. The specific region of MT molecule responsible or megalin binding resides in the α domain of the protein (Klassen et al. 2004). *In vitro* assays indicated that MT-1/2, unlike inhibitory neuronal survival activity of MT-3 isoform, promote neurite outgrowth of cortical (Chung et al. 2003) and dopaminergic neurons (Køhler et al. 2003), and also promote axon regeneration of injured neurons following cortical injury to the rat brain (Chung et al. 2003). Exogenously administratered MT-1/2 in mice exerted the same functions described for the endogenous proteins supporting a role of extracellular MT (Penkowa et al. 2002; Jiang et al. 2005; Morellini et al. 2008; Helal and Helal 2009; Kiliç and

Kutlu 2010). In summary, extracellular MTs have important role in **regulating cellular function**, including **neuronal differentiation and survival** and **immune response** (Hidalgo j., Chung R., Penkowa M. 2009).

Whereas MT apparently are not essential for life, as evidenced by normal reproductive capacity and long-term survival of mice lacking functional MT genes, there is consistent evidences for a survival advantage of MT in situations of stress, including exposure to oxyradicals and toxic metals, inflammation, infection and low Zn nutrition. Concluding that the relevance of this pleiotropic protein depends very much on specific evolved requirements of the particular organisms, from more primitive life forms to mammals (Coyle et al. 2002). Moreover, a number of reports of *in vitro* and *in vivo* assays have demonstrated that enhanced expression of MT in cells induces anti-apoptotic effects, unlike lacking of MTs in null mice which showed increased susceptibility to apoptotic cell death (excluding the altered Zn levels as the cause of the enhanced apoptotic state) (Vasák and Hasler 2000a).

It is important to highlight that apoptosis is one of the major cellular responses to carcinogen exposure and may play an important role in tumour formation (Deng et al. 1998). Expression of MT has been demonstrated in various types of human tumours (Cherian 1994) and recent studies hypothesised that MT-1 serve as a tumour suppressor (Fu et al. 2017). In addition, an inappropriate apoptosis may precipitate many diseases including AD, PD, autoimmune disorders, cardiovascular damage and more (Kondo et al. 1997). NF-κB which is linked to anti-apoptotic or pro-apoptotic role depending of the situation, is activated by variety stimuli that are those that also induce the synthesis of mammalian MTs: TNF-α, IL-1, hypoxia and reactive oxygen species. A specific interaction between the p50 subunit of NF-κB and MT has been concluded to be required to stabilize DNA binding of NF-κB, suggesting a potential role for NF-κB in mediating the anti-apoptotic effects of MT (Abdel-mageed and Agrawal 1998; Vasák and Hasler 2000b).

Metallothioneins in the central nervous system (CNS)

Distribution

In normal adult brain MT-1 and MT-2 isoforms levels are relatively low and the main cell type expressing these MTs are subsets of astrocytes (particularly in protoplasmic astrocytes, in humans) in the gray matter and to a lesser extent in the white matter. However, we can find these isoforms in other

tissues such as endothelial cells of blood vessels, meningeal cells of the pia matter, ependymal cells and epithelial cells of the choroid plexus. Neurons in the brain and spinal cord have been reported to express these isoforms also, however, these results are inconsistent and in any case, the expression levels are considerably lower than that observed in astrocytes (Hidalgo et al. 1994; Kiningham and Xuguang 1995; van Lookeren Campagne et al. 1999). In spinal cord, MT-1/2 are found especially in gray matter astrocytes. In addition, oligodendrocytes have been reported to express MT-1/2 during mammalian development, especially in cerebral cortex, but not in adult brain (Holloway, et al. 1997). Finally, microglia cells in basal conditions are devoid of MT but in response to injury, the synthesis of MT-1/-2 isoforms are up-regulated, indicating that reactive but not resting microglia is available to synthesize MT-1/2 *in vivo* and *in vitro* (Vela et al. 1997; Agullo et al. 1998).

MTs are mainly localized in the cytoplasm, although they have also been found in the cell nucleus. Even though MTs lack conventional secretion sequences, MT-1 and MT-2 have been detected, *in vitro* and *in vivo*, within the extracellular milieu of the injured brains (Chung et al. 2008). Adding MT-1/2 to injured neurons in culture, in absence of immune system cells, lead to an increase of regenerative sprouting, suggesting that there is a robust and generic neuronal response to extracellular MT-1/2 (Chung et al. 2003). The hypothesized mechanism proposed for extracellular presence of MTs is that astrocytes respond to neuronal trauma by up-regulating MT-1/2 synthesis and release them to the extracellular environment (Chung and West 2004). Extracellular MTs would then be internalized by neurons mainly via megalin receptor and exert their neurodegenerative effect (Chung et al. 2003, 2008).

Functional roles

Equivalent to peripheral tissues, brain MT-1 and MT-2 synthesis is induced by metals, glucocorticoids, catecholamines, cytokines, endotoxin, psychogenic stress, oxidative stress and inflammation (Hidalgo et al. 1990, 1991, 1994; Gasull et al. 1994; Belloso et al.1996; Hernández et al. 1997; Vela et al. 1997; Hernández and Hidalgo 1998; Acarin et al. 1999). Hence, although the primary function is still not well identified, MT-1/2 in the SNC have similar roles to those described in the periphery: metal homeostasis and detoxification, antioxidants, anti-inflammatory, etc. Supporting these roles, many *in vivo* models of brain injuries (Carrasco et al. 1999; Chung et al. 2008), seizures (Dalton et al. 1995) or ischemia (van Lookeren Campagne et al. 1999) evidenced increased levels of MT-1/2. Mice overexpressing MT-1 showed lower infarcts and better functional recovery after mild focal cerebral ischemia and

reperfusion (van Lookeren Campagne et al. 1999; Penkowa et al. 2005) and the contrary was observed in MT-1/2 null mice (Penkowa et al. 1999; Hidalgo et al. 2001; Giralt et al. 2002). Despite there are many different models of brain injury, MT-1/2 exerted similar effects in all cases, decreasing oxidative stress, inflammation and apoptosis, in other words offering neuroprotection. MTs levels have been reported to be altered in many neurodegenerative diseases as well as in a number of brain disease animal models, suggesting a role for MTs in brain disorders (see the following section for further discussion).

Metallothioneins and brain disease

Metallothioneins have been described to be affected in many neurodegenerative diseases, including AD, which will be discussed in detail in the next section, as well as in a number of brain disease animal models, suggesting a role of MTs in brain disorders.

Aging is considered one of the major risk factors for neurodegenerative disorders. Aged brains increase their susceptibility to environmental factors like hormonal changes, infections and immunological disorders leading the brain to a variety of typically pathological events. Factors such as high levels of free radicals or neuroinflammatory phenomena are important and altered in neurodegenerative diseases and MTs participate actively in those processes (Hidalgo et al. 2001)

Amyotrophic lateral sclerosis (ALS) is a lethal motor neuron disease causing selective and progressive motor neuron degeneration in the cortex, brainstem and spinal cord. Patients with ALS exhibit elevated levels of MT-1/-2 in astrocytes within the spinal cord (Sillevis Smitt et al. 1992; Blaauwgeers et al. 1996). Transgenic mice for this model express SOD-1 bearing a mutation that leads to muscular wasting and neurodegeneration, displays increased MT-1/2 synthesis in astrocytes within the white and gray matter of the spinal cord. Elevated MT-1/2 levels (both mRNA and protein) are apparent prior to onset of motor deficits and continue as neurodegeneration progress indicating that MTs are participating in the evolution of the disease (Gong and Elliott 2000). Investigations performed with double transgenic mice (SOD-1*MT-1/2 KO), showed that MT-1/2 deficiency produced faster onset and accelerated progression of the disease and faster mortality, suggesting a neuroprotective role of the MTs (Puttaparthi et al. 2002).

Multiple sclerosis (MS) is a chronic demyelinating disease of the CNS. Experimental autoimmune encephalomyelitis (EAE) is an animal model for MS, with clinical signs and CNS lesions similar to those observed in MS (Martin

et al. 1992). Although the actual pathogenic mechanisms of EAE/MS are not fully understood, the implication of ROS in the ongoing inflammation and CNS damage might be the cause while MTs may protect cells from oxidative stress and may reduce inflammation (Espejo and Martínez-Cáceres 2005). Levels of MT-1/2 are found elevated in astrocytes and activated monocytes/macrophagues, corresponding to the level of the expression with the severity of the disease. Interestingly, MT-1/2 levels were mildly greater in inactive lesions than active lesion, suggesting that MT are involved in the remission periods of the disease (Penkowa et al. 2003). When EAE was induced in MT-1/2 KO mice, the severity of the disease was significantly greater including increased inflammation and neuronal apoptosis (Penkowa et al. 2001).

The dopaminergic neurons death that occurs in the **Parkinson's disease** is accompanied by an inflammatory reaction mediated by activated microglia and enhanced oxidative and nitrative stress. Although no changes were observed in MT-1/2 levels in PD patients (Mirza et al. 1999), studies with transgenic mice indicated that MTs also have a role in PD (Ebadi et al. 2005).

Besides neurodegenerative diseases, there are several studies showing MT-1/2 induction in animal models of **inflammation**, **stress**, **excitotoxicity**, **traumatic brain injury**, **ischemia** and **gliodegeneration**.

Metallothioneins in AD

As mentioned above, AD is one of the disease which the synthesis of MTs is found to be altered. Particulary, mRNA and protein levels of MT-1/2 isoforms are consistently reported to be increased in AD brains (Duguid et al. 1989; Zambenedetti et al. 1998; Hidalgo et al. 2006) even at early 'preclinical' AD stages preceding inflammation (Adlard et al. 1998).

Mouse models of AD, including Tg2576 mice, showed a prominent upregulation of MT-1/2 in the vicinity of the amyloid plaques, consistent with inflammatory context (gliosis, inflammation, oxidative stress, metals accumulation, principally Zn) normally found in them (Hidalgo et al. 2006). Moreover, immunofluorescence stainings demonstrated that astrocytes and microglia and macrophages surrounding the deposits expressed MT-1/2 (Carrasco et al. 2006).

Recent *in vitro* studies demonstrate that Zn_7MT-2A is capable to decrease Aβ neurotoxicity of cultured cortical neurons presumably due to a metal swap between Zn_7MT-2A and Cu(II)-Aβ (Chung et al. 2010) preventing the toxicity from Cu mediated aggregation of $A\beta_{40}$ and $A\beta_{42}$. As well as, recent studies (Siddiq et al. 2015) have demonstrated an inhibitory effect of MT-1/2 iso-

forms on the *in vitro* activity of one of the α-secretase (ADAM17/TACE) of the non-amyloidogenic pathway, favouring the amyloidogenic pathway and increasing the levels of Aβ peptides. Interestingly, MT-3 isoform has been reported to increase the activity of another α-secretase such as ADAM10, in the mouse neuroblastoma Neuro2A (Park et al. 2014) Swedish APP cells, suggestive of MT isoform-specific roles.

A pilot study in our laboratory demonstrated that aged Tg2576 mice chronically injected by Zn_7MT-2A reverted the behavioural phenotype of Tg2576 (i.e. deceased anxiety and increased activity), but did not affect the cortical amyloid burden or the GFAP response in the cortex and tended to increase in the hippocampus. In addition, soluble Aβ content (monomers of $A\beta_{40}$ and $A\beta_{42}$) evaluated by western blotting increased in the hippocampus and the rest of the brain but not in the cortex (Manso et al. 2011). For the first time, we demonstrated *in vivo* that exogenous administration of MTs might modulate the behavioural phenotype and amyloid pathology of Tg2576 mice.

More evidences of the putative role of MT family in AD, *in vivo,* were collected in our laboratory when we obtained a double transgenic mice presenting on the one hand AD pathology and on the other hand lacking MT-1/2 and MT-3. The lack of MT-1/2 tended to reverse the effect of hAβPP on activity and anxiety in a gender and age depended manner (significant in young males). In the Morris water maze, the absence of MT-1/2 reverted the inability of AβPP+ females to switch from a spatial strategy to the stimulus-response strategy needed in the visible platform test, suggesting that MT-1/2 absence could be delaying or preventing some of the hAβPP-induced changes in behaviour. In older mice, the deficiency of MT-1/2 diminished the amyloid burden and microglia with no significant effect in the astrogliosi in cortex and hippocampus (Manso et al. 2012a). Moreover, MT-3 deficiency resulted disadvantageous in the development of Tg2576 phenotype, especially in females since hAβPP-induced lethality while amyloid plaque and gliosis were partially prevented when MT-3 was absent (Manso et al. 2012b).

From the point of view of our research group, the study of double transgenic mice, presenting AD pathology in addition to deficiency or overexpression of either MT-1/2 or MT-3 isoforms could it is crucial to provide important insight into the role of these proteins in AD since there are many deficits in our understanding of how MTs may function.

A part from the expected actions related with the anti-inflammatory, anti-oxidant and metal binding properties, MTs interact with several proteins that could directly or indirectly be involved in AD at different levels. Trans-

thyretin (TTR), although is not the only protein that binds Aβ, is the major Aβ sequestering protein in human CSF, preventing the aggregation and amyloid formation in AD brains (Schwarzman et al. 1994; Choi et al. 2007). Screening human liver cDNA library, identified MT2 as a putative TTR-associated protein, which diminished TTR-Aβ binding (Gon??alves et al. 2008; Martinho et al. 2010). Other studies suggest that MT-2A could be implicated in the mechanism through which HIPK (homeodomain-interacting protein Kinase 2) alters the conformational state of p53, leading to an impaired and dysfunctional response to stressors in AD (Puca et al. 2009; Lanni et al. 2010).

Hypothesis and objectives

Due to the putative role of the metallothionein family, we hypothesize that they could **modulate the phenotype of the Tg2576 mouse model of Alzheimer's disease**.

To this purpose, we proposed the following objectives:

To obtain Tg2576 mice with *Mt1* overexpression and proper controls for the analysis.

To validate the model of the transgenic *Mt1*overexpression.

To study if *Mt1* overexpression can modify the phenotype (physiological, behavioural and neuropathological) of the Tg2576 mouse model of AD.

2

Overexpression of metallothionein-1 modulates the phenotype of the Tg2576 mouse model of Alzheimer's disease

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Journal of Alzheimer's Disease 51 (2016) 81–95
DOI 10.3233/JAD-151025

Alzheimer's disease (AD) is the most commonly diagnosed dementia, where signs of neuroinflammation and oxidative stress are prominent. In this study we intend to further characterize the roles of the antioxidant, anti-inflammatory, and heavy metal binding protein, metallothionein-1 (MT-1), by crossing *Mt1* overexpressing mice with a well-known mouse model of AD, Tg2576 mice, which express the human amyloid-β protein precursor (hAβPP) with the Swedish K670N/M671L mutations. *Mt1* overexpression increased overall perinatal survival, but did not affect significantly hAβPP-induced mortality and weight loss in adult mice. Amyloid plaque burden in ~14-month-old mice was increased by *Mt1* overexpression in the hippocampus but not the cortex. Despite full length hAβPP levels and amyloid plaques being increased by *Mt1* overexpression in the hippocampus of both sexes, oligomeric and monomeric forms of Aβ, which may contribute more to toxicity, were decreased in the hippocampus of females and increased in males. Several behavioral traits such as exploration, anxiety, and learning were altered in Tg2576 mice to various degrees depending on the age and the sex. *Mt1* overexpression ameliorated the effects of hAβPP on exploration in young females, and potentiated those on anxiety in old males, and seemed to improve the rate of spatial learning (Morris water maze) and the learning elicited by a classical conditioning procedure (eye-blink test). These results clearly suggest that MT-1 may be involved in AD pathogenesis.

Introduction

Alzheimer's disease (AD) is the most commonly diagnosed dementia. Clinically, it is defined by a progressive loss of cognitive functions. Neuropathologically, AD is characterized by the presence of extracellular deposits of the amyloid-β (Aβ) peptide (senile/amyloid plaques) and intracellular deposits of hyperphosphorylated tau protein (neurofibrillary tangles). In addition, it is common that amyloid plaques are accompanied by neuroinflammation, oxidative stress, and neuronal death in brain areas such as the hippocampus and the cortex [1, 2]. Oxidative stress and Aβ are closely related since Aβ induces oxidative stress *in vitro* and *in vivo*, which, in turn, increases the production of Aβ [3]. Transition metals such as Cu or Fe, together with Zn, are also an important source of oxidative stress and Aβ aggregation and precipitation in the AD brain [4, 5]. Metallothioneins (MTs) are low molecular weight (6-7 kDa), cysteine rich proteins with high metal content that are subdivided into four subfamilies (MT-1- MT-4) in mammals. Although the primary biological role of MTs remains unknown, there is mounting evidence to suggest that MTs confer a survival advantage under stress or tissue injury [6]. As could be expected, MT-1/2 are upregulated in several human neurodegenerative diseases including AD, whereas the results for MT-3 are less consistent [6, 7]. The expression of the different MT isoforms has been studied in several AD mouse models, including Tg2576 mice, and while MT-3 is little affected, MT-1/2 are clearly upregulated in cells surrounding the amyloid plaques [8, 9]. Recent *in vitro* studies show that MT-2 reduces Aβ induced changes in ionic homeostasis and the subsequent neurotoxicity in cultured rat cortical neurons by a metal swap between Zn_7MT-2 and Cu-Aβ [10]. Besides direct inhibition of Aβ neurotoxicity, it has also been demonstrated an indirect role of MTs by suppressing Aβ-dependent microglial activation [9]. *In vivo* studies are scarce. In a preliminary study where we administered MT-2 to old Tg2576 mice, it was suggested that MTs might modulate the neuropathology of this AD model [11]. Moreover, results obtained in transgenic mice crossing the Tg2576 mice with *Mt1&2* KO mice further demonstrated a complex role of MTs in this widely used model [12]. Still, there are many deficits in our understanding of how MTs may function. Therefore, in order to assess the robustness of these proteins affecting AD-related neuropathology in the present study we analyzed if the overexpression of MT-1 results in a reversal of the phenotype observed with MT-1/2 deficiency.

Materials and methods

Animals

The parental strains used in this study were TgMT mice, which carry 56 copies of a minimally mutated *Mt1* (*Mt1**) gene [13] (B6.Cg-Tg(Mt1)-174Bri/J; The Jackson Laboratory), C57BL/6JOlaHsd as a wild-type (WT) strain (Harlan), and the AD mouse model Tg2576 which expresses the human $A\beta PP_{695}$ harboring the Swedish K670N/M671L mutations under the control of the hamster prion protein promoter [14] (B6;SJL-Tg(APPSWE)2576Kha; Taconic Europe A/S; Ry, Denmark).

To produce the desired double transgenic mice and controls, we proceeded as follows. Two large, separate experiments were carried out. In the first one, hemizygous Tg2576 mice were crossed with WT and with homozygous TgMT mice, which produced the four genotypes of interest: WT (hAβPP–/–) and APPWT (hAβPP+/–); and TgMT (hAβPP–/–/TgMT+/–) and APPTgMT (hAβPP+/–/TgMT1+/–), respectively. To further assure genetic homogeneity, in the second experiment a different crossing strategy was used. Tg2576 mice were first crossed with TgMT mice, and from the resultant offspring (hAβPP–/–/TgMT+/– and hAβPP+/–/TgMT+/–) the hAβPP+ mice were selected and crossed with WT mice to obtain the four genotypes to be studied, being littermates in this case. In this second experiment, body weight and mortality were monitored regularly from weaning until sacrifice at ~6 and ~14 months of age. We will present the results of Experiment 2 unless otherwise stated because of genetic homogeneity. Genotype was determined by PCR as described [12] for hAβPP transgene and as recommended by Jackson lab for TgMT.

Mice were housed with free access to food and water in a 12-h dark-light cycle under constant temperature. Animals were killed by decapitation and the brain quickly removed on ice. The right hemisphere was dissected into cortex and hippocampus, frozen with liquid nitrogen, and stored at –80ºC. The left hemisphere was immersed in 4% paraformaldehyde (PFA), stored in 70% ethanol at 4ºC, and processed for paraffin-embedding and subsequent histology. All experimental procedures were approved by the Ethics Committee in Human and Animal experimentation from the Autonomous University of Barcelona.hemisphere was immersed in 4% paraformaldehyde (PFA), stored in 70% ethanol at 4ºC, and processed for paraffin-embedding and subsequent histology. All experimental procedures were approved by the Ethics Committee in Human and Animal experimentation from the Autonomous University of Barcelona.

Behavioral characterization

Young and old mice were tested in several paradigms to characterize their behavioral phenotype.

Hole-board, elevated plus maze, rotarod and sensorimotor tests

Exploratory activity and anxiety were evaluated with the hole-board and the elevated plus maze tests as described elsewhere [11, 15]. The hole board apparatus is a white wooden box (36 × 36 × 15 cm) with four holes (3 cm diameter) equally spaced in the floor, which in addition is divided into 16 areas. The mice were allowed to freely explore the maze for 5 min, and the number of areas crossed and rearings (activity), and the number of head dips and the time head-dipping were measured. The elevated plus maze consists of two open arms and two closed arms forming a square cross with a 5-cm square center piece, elevated 40 cm above the floor. The mice were allowed to freely explore the maze for 5 min, and the time and number of entries in the open arms and closed arms were measured. Sensorimotor reflexes and coordination were assessed with a battery of tasks including a cylindrical rod and a coat hanger, as reported previously [16, 17]. Briefly, in the case of the round rod, a horizontal cylindrical rod (1 cm wide/diameter × 50 cm long and 40 cm from floor) was used. The mice were placed in the center of the rod for 20 s and the latency to fall was measured. Each animal was tested twice with 10 s inter-trial period.

Morris water maze

Spatial memory and learning were assessed with the Morris water maze (MWM) as described elsewhere [12, 16] but introducing small changes in the protocol. Briefly, black and white cues were placed on the curtain that surrounded the pool to help mice to locate the platform. For the hidden platform test, animals were trained for 4 consecutive days (4 trials per day) and probe trial tests were done the days 4, 5, and 7 to assess retention. For the reversal test, animals were challenged to relearn a new platform location and they were trained for 3 consecutive days (4 trials per day) and probe trial tests were done the days 9 and 10 to assess retention. For the visible platform test mice were trained for 1 day (4 trials per day).

Instrumental conditioning in the Skinner box

A total of 39 animals were selected for the oper-ant conditioning task. Training and testing took place in standard Skinner box modules (n = 3) me-

asuring 12.5 × 13.5 × 18.5 cm (MED Associates, St. Albans, VT, USA). The operant chambers were housed within a sound-attenuating chamber (90 × 55 × 60 cm), which were constantly illuminated (19 W lamp) and exposed to a 45 dB whitenoise (Cibertec, S.A., Madrid, Spain). Each Skinner box was equipped with a food dispenser from which pellets (Noyes formula P; 20 mg; Sandown Scientific, Hampton, UK) could be delivered by pressing a lever. Prior to the task, mice were handled daily for 7 days and food-deprived to 80% of their free-feeding weight. Conditioning took place for 20 min during five successive days, in which mice were trained to press the lever to receive pellets from the food tray using a fixed-ratio (1:1) schedule. Animals were maintained on this 1:1 schedule for 5 days of training [18]. Cumulative records of lever pressing and pellet rewards were stored online on a computer connected to the Skinner boxes, and results were processed for statistical analysis using the SPSS program. The percentage of lever presses for a maximum of 20 lever presses/session (100%) was computed for the four groups of animals.

Classical eyeblink conditioning test

The surgical preparation for classical eyeblink conditioning [19, 20], was as follows. Under deep anesthesia (Ketamine, 35 mg/kg and Xylazine, 2 mg/kg, i.p.), animals were implanted with four electrodes in the upper eyelid of the left eye. Electrodes were made of Teflon-insulated, annealed stainless steel wire (50 μm in diameter, A-M Systems, Carlsborg, WA, USA). One pair of electrodes was aimed toward the supraorbital nerve, and served for the application of electrical stimuli. The second pair of electrodes was implanted in the ipsilateral *orbicularis oculi* muscle, and served for recording its electromyographic (EMG) activity. The four electrodes were connected to a 4-pin socket (RS-Amidata, Madrid, Spain), which was fixed with dental cement to the cranial bone. After surgery, and before the beginning of the experiment, animals were kept for 5–7 days in independent cages, with free access to food and water, for a proper recovery. They were also maintained in individual cages for the rest of the experiment.

The classical conditioning procedures were as follows. For EMG recordings, animals were placed in individual (15 cm × 5 cm × 10 cm) methacrylate cages, and the wires plugged into their implanted sockets were connected with the stimulating/recording system. A trace conditioning paradigm was carried out. For this, animals were presented with a tone (2400 Hz, 70 dB, 20 ms) as a conditioned stimulus (CS), followed 250 ms from its start by an electrical stimulation (500 μs, 3 × Threshold) of the supraorbital nerve

as an unconditioned stimulus (US). Paired CS-US presentations were separated at random by 30 ± 5 s. For habituation and extinction sessions, only the CS was presented, also at intervals of 30 ± 5 s. During 19 days, a total of 4 habituation, 10 conditioning, and 5 extinction sessions (of 60 trials each) were presented to each animal.

The EMG activity of the *orbicularis oculi* muscle was recorded using differential amplifiers within a bandwidth of 1 Hz to 10 kHz (Grass Technologies, West Warwick, RI, USA). Data were stored directly on a computer through an analog/digital converter (CED 1401 Plus, Cambridge, England), at a sampling frequency of 11 kHz and an amplitude resolution of 12 bits. Data were analyzed off-line for quantification of conditioned responses (CRs) with the help of the Signal Average Program (Cambridge Instruments, Cambridge, England). As a criterion, we considered a CR those EMG responses, recorded during the CS-US period, that presented the following characteristics: i) the EMG activity lasted >10 ms; ii) the EMG was not preceded by any spontaneous activity in the 200 ms preceding CS presentation; iii) the EMG activity was initiated >50 ms after CS onset; and iv) the integrated EMG activity was at least 2.5 times larger than the activity recorded 200 ms before CS presentation [21].

Collected data were quantified, through a purpose-designed Excel worksheet, as the percentage of CRs per session—i.e., the proportion of stimulations within a session of 60 trials that generated an EMG activity satisfying the above-mentioned criteria [18].

In situ hybridization for Mt1 and Mt3 mRNA

In experiment 1, determination of *Mt1* and *Mt3* mRNA levels in the brain was carried out by *in situ* hybridization (and using macro– and microautoradiography) as described [8, 22]. Briefly, serial sagittal sections (20 μm in thickness) were obtained and mounted on slides coated with poly-L-lysine. *Mt1* and *Mt3* cDNAs were labeled with (^{35}S) a-UTP using a SP6/T7 transcription kit (Boehringer Mannheim, Mannheim, Germany). *In situ* hybridization was performed using procedures described by Yuguchi et al. [23] with some modifications: The sections were incubated with 0.1 N HCl instead of proteinase K, and we used RNAse at 10 μg/ml instead of 1 μg/ml to digest the free probe. The concentration of probe used was 1×10^6 dpm/90 μl/slide. Autoradiography was performed exposing the autoradiographic film to the slides, all sections simultaneously prepared and exposed to the same film. *Mt1* or *Mt3* mRNA levels were determined in 3 sections per brain area and animal, by measuring the optical densities and the num-

ber of pixels in defined areas. The *Mt1* and *Mt3* mRNA values shown are expressed in arbitrary units (number of pixels x optic density).

Microautoradiography

After macroautoradiography was performed, the slides were coated with Hypercoat LM-1 emulsion (Amersham) following the instructions of the manufacturer. The slides were exposed at 4ºC into a light-tight box, and then they were developed in D-19 (Kodak).

Protein extraction and western blotting and enzyme-linked immunosorbent assay (ELISA)

Cortical and hippocampal tissues were homogenized by sonication in 50 mM Tris-HCl (pH 7.6), 0.01% NP-40, 150 mM NaCl, 2 mM EDTA, 3% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% deoxycholate and protease inhibitor cocktail (Sigma-Aldrich). Total homogenate samples were stored at –80ºC. Protein concentration was estimated with the BCA protein assay according to manufacturer's instructions (Pierce, Thermo Fisher Scientific Inc; Rockford, USA).

Western blot for hAβPP, Aβ, and other AβPP-derived proteolytic fragments such as CTF-β (6E10–$A\beta_{1-16}$– 1:2000, Signet, Dedham, MA, U.S.A; WO2 – $A\beta_{5-8}$– 1:50, in-house antibody) was carried out as previously described [12]. Membranes were developed with ECL reagent (Amersham, GE Healthcare, Buckinghamshire, UK) and images were captured and quantified using the Bio-Rad laboratories (Hercules, CA, U.S.A) software *QuantityOne ChemiDoc*.

Determination of $A\beta_{1-40}$ and $A\beta_{1-42}$ was done using a sandwich ELISA commercial kit from Invitrogene as described [12].

Immunohistochemistry

Fixed brains were paraffin-embedded and cut sagitally in 8 μm-thick sections for assessing the amyloid plaque load (primary antibody: 4G8–$A\beta_{17-24}$ – 1:5000, Signet; secondary antibody: Anti-mouse IgGbiotin conjugate 1:400, Sigma) as described [12]. Six slides per animal were studied.

Statistical analysis

Data was analyzed using the Statistical Package for Social Sciences (SPSS) version 17.0. Males and females were analyzed separately. Survival was analyzed using Kaplan-Meier survival test, using genotype as a factor with

four levels (WT, TgMT, APPWT, APPTgMT). The rest of the data was analyzed using either Generalized Linear Model (GLZ) (i.e., latency in freezing behavior, etc.) or Generalized Estimated Equations (GEE) for repeated measures (i.e., body weight or acquisition in the MWM). In both cases, hAβPP and TgMT were used as factors with two levels each: hAβPP+ and hAβPP– for hAβPP and TgMT– and TgMT+ for TgMT. In addition, in GEE analysis "time" was used as within-subject factor. In parameters such as amyloidosis, which is not present in hAβPP– genotypes, only hAβPP+ genotypes were analyzed, using TgMT as grouping factor. Statistical significance was defined as $p \leq 0.05$. The number of mice used will be stated in the text or in the figure as needed. Because of the high mortality of the hAβPP+ mice the number of mice per group varies widely. Results shown are mean ± SEM.

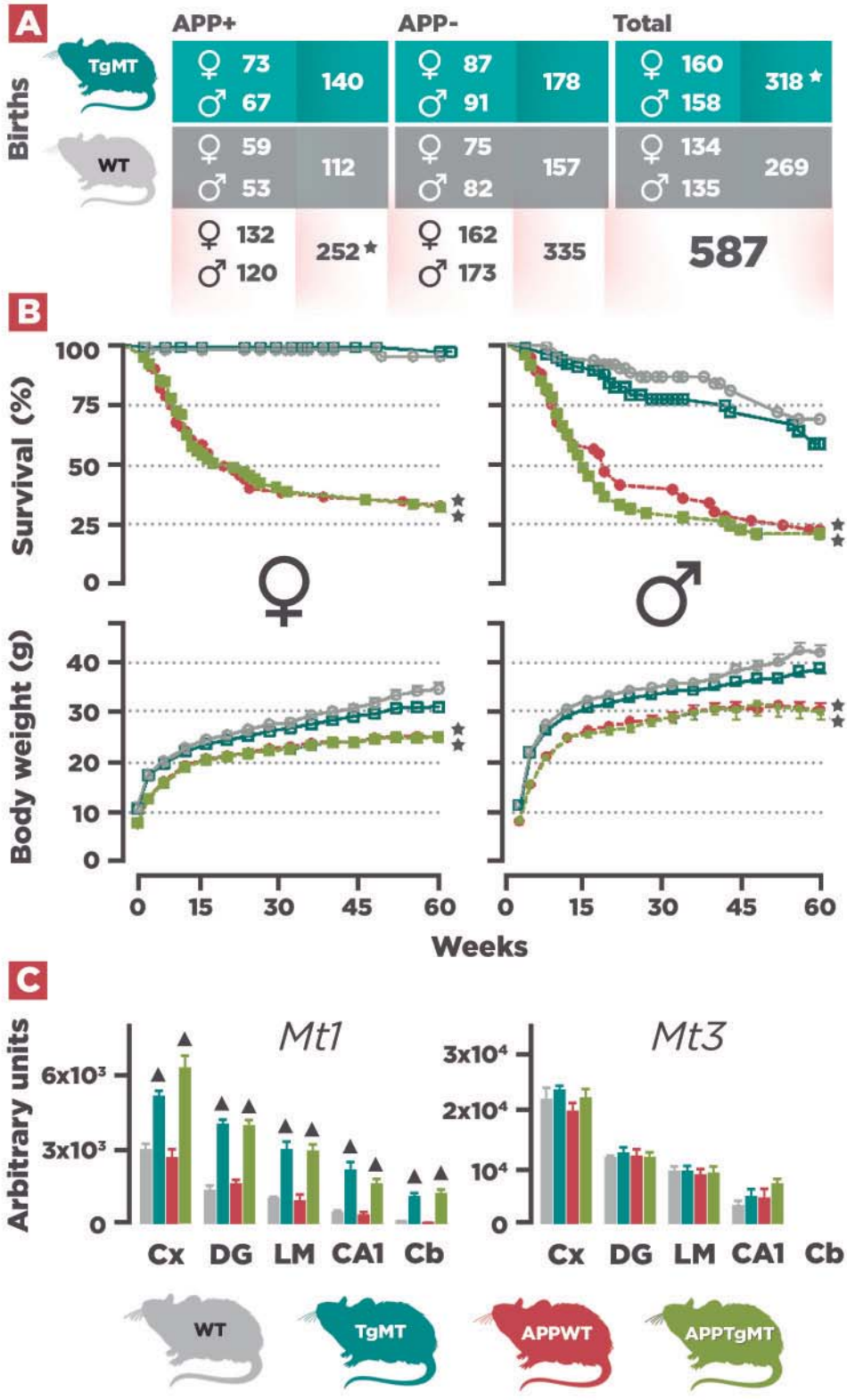
Results

Mt1 mRNA but not Mt3 levels are increased in TgMT mice

To validate our transgenic overexpressing model we measured *Mt1* and *Mt3* **mRNA levels** by *in situ* hybridization (**Fig. 1C**; experiment 1; n = 3–12). As expected [13, 24], *Mt1* mRNA levels were significantly increased in all brain areas studied in TgMT mice. *Mt1* mRNA levels, as measured by macroautoradiography, were not significantly increased by hAβPP expression. By microautoradiography, a ~2.6 fold increase in the *Mt1* signal was observed in plaque areas compared to areas without plaques in APPWT mice; a similar increase was observed in APPTgMT mice (data not shown). This is consistent with a previous study which demonstrated that the *Mt1* signal is only increased in areas surrounding the amyloid plaques [8]. Also in line with that study, *Mt3* mRNA levels were not significantly altered by either hAβPP or *Mt1*expression.

Figure 1. Effect of *Mt1* overexpression on survival and body weight.

(A) ***Analysis of the offspring genotyped at weaning***. A significant effect of hAβPP decreasing survival at this early age was observed ($\chi^2 = 11.736$, $p < 0.001$). In contrast, *Mt1* overexpression increased survival ($\chi^2 = 4.090$, $p < 0.05$). **(B)** ***Survival and body weight of female*** (left) and male (right) mice were followed up from weaning to sacrifice at 60 weeks of age. **(C)** ***As expected, in situ hybridization analysis*** showed increased *Mt1* (but not *Mt3*) mRNA levels in TgMT mice throughout the brain (Cx, DG, LM, CA1, Cb: Cortex, dentate gyrus, lacunosum moleculare, *Cornu Ammonis* area 1, and cerebellum); there were no differences between hAβPP+ and hAβPP– mice. ★ and ▲ p at least ≤0.05 versus hAβPPAPP– and TgMT- mice, respectively.


A
Births
APP+
APP-
Total
TgMT
♀ 73
♂ 67
140
♀ 87
♂ 91
178
♀ 160
♂ 158
318 ★
WT
♀ 59
♂ 53
112
♀ 75
♂ 82
157
♀ 134
♂ 135
269
♀ 132
♂ 120
252 ★
♀ 162
♂ 173
335
587
B
Survival (%)
100
75
50
25
0
♀
♂
Body weight (g)
40
30
20
10
0
0 15 30 45 60
Weeks
C
Arbitrary units
Mt1
6x10³
3x10³
0
Mt3
3x10⁴
2x10⁴
10⁴
0
Cx DG LM CA1 Cb
WT
TgMT
APPWT
APPTgMT

Mt1 overexpression rescues perinatal mortality and tends to the opposite in adulthood

Analysis of the Mendelian distribution of the mouse litters at weaning (n = 53–91) revealed the existence of **early mortality** associated with the pres- ence of the hAβPP transgene since hAβPP+ mice were born and/or survived to weaning at a less-than expected ratio (observed: 252, expected: 294, χ^2 = 11.736, $p < 0.001$ with one degree of freedom (**Fig. 1A**). On the other hand, Mt1 overexpression also had an impact on perinatal mortality since TgMT+ mice were born and/or survived to weaning at a more-than expected ratio (observed: 318, expected: 269, χ^2 = 4.090, $p < 0.05$ with one degree of freedom) (**Fig. 1A**).

As expected and in line with perinatal mortality, the **expression of hAβPP** dramatically increased mortality in both male and female mice after weaning, starting at early ages and reaching up to ~75% mortality by 60 weeks of age (**Fig. 1B**, top). In contrast to the significant prosurvival role identified at weaning, in adult mice Mt1 overexpression, if anything, tended to potentiate mortality in male mice.

In agreement with the survival data, the analysis of **body weight** gain (n = 53–91 at weaning) evidenced a clear detrimental effect of hAβPP transgene, with the body weight of APP+ mice lower than that of controls throughout the experiment (**Fig. 1B**, bottom). Mt1 overexpression did not significantly affect body weight, and furthermore, the interaction between factors was not significant. Nevertheless, should the comparison be carried out only in hAβPP-mice, the TgMT mice tended to show a decreased body weight gain compared to WT mice (p = 0.05 in males, and 0.053 in females).

Mt1 overexpression increases amyloid plaque load

The **amyloid plaque load** of old mice was evaluated in ~14 month-old mice by immunohistochemistry (**Fig. 2A**). The results indicate that Aβ staining intensity tended to be increased in APPTgMT versus APPWT mice in both sexes. Since the immunostaining for each sex was carried out in separate batches, the values were transformed to percentage of the mean value of APPWT mice of each sex and a combined statistical analysis using Mt1 overexpression and sex as main factors was carried out. With these normalized data, Mt1 overexpression significantly increased the plaque load in the hippocampus in bothsexes, whereas in the cortex no significant effects were observed (**Fig. 2B**; n = 11–18).

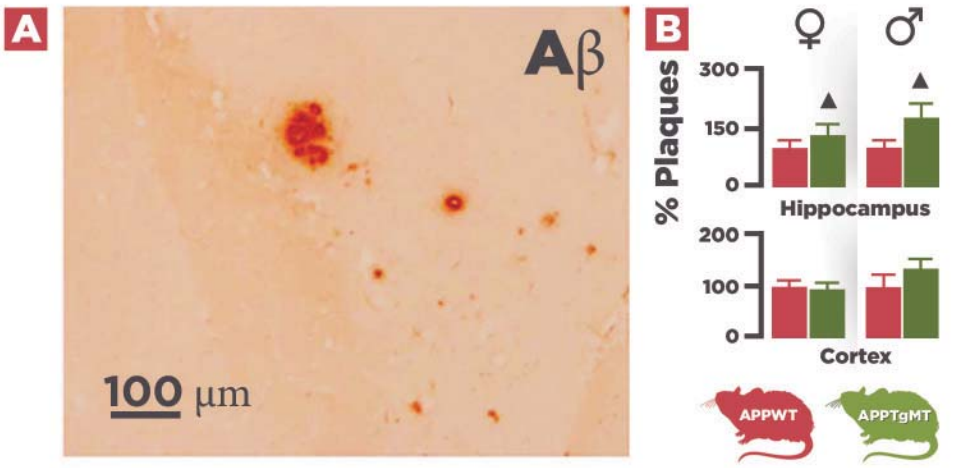


Figure 2. Effect of *Mt1* overexpression on amyloid load.

(A) ***Representative immunostaining for Aβ*** (amyloid plaques). **(B)** ***Quantification of Aβ immunostaining*** in the hippocampus and cortex of ~14 month-old mice. *Mt1* overexpression significantly increased amyloid load in the hippocampus of both sexes. ▲ *p* at least ≤0.05 versus APPWT mice.

Mt1 overexpression influences the amyloid cascade

hAβPP and its proteolytic fragments were studied in ~14-month-old mice by western blot in total hippocampal and cortical homogenates, allowing the assessment of the levels of human full length AβPP (hAβPP), Aβ peptides, C-terminal fragment-β (CTF-β), and the putative Aβ trimer (**Fig. 3A**). hAβPP levels were significantly increased in APPTgMT mice compared to APPWT mice in the hippocampus and cortex of both sexes (**Fig. 3B**; n = 10). In the hippocampus there was a clear sex-dependent difference in the proteolytic fragments observed, since inAPPTgMT males the CTF-β and the monomeric and trimeric forms of Aβ were significantly increased, whereas in females the opposite tendencies were observed for Aβ forms (significant only for the trimeric form) (**Fig. 3B**, left). In contrast, in the cortex Mt1 overexpression increased CTF-β levels in both sexes whereas the monomeric and oligomeric forms were not different between genotypes (**Fig. 3B**, right). Further analysis by ELISA revealed similar results in the hippocampus. Thus, $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels tended to decrease in APPTgMT females and to increase in APPTgMT males, albeit the only significant effect was observed for $A\beta_{1\text{-}40}$ levels in females (**Fig. 3C**; n = 9-10). At early ages (~6 month-old mice), only the hAβPP and the CTF-β bands were detectable. Only in the hippocampus of males were there significant differences between genotypes, with the APPTgMT animals having increased hAβPP levels (data not shown).

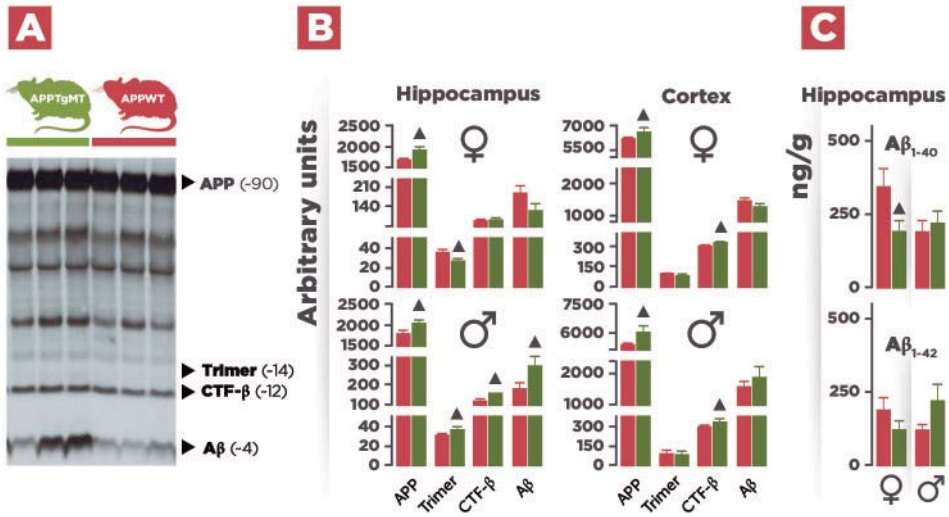


Figure 3. Effect of *Mt1* overexpression on amyloid cascade.

(A) ***Full length human AβPP*** and its proteolytic fragments were analyzed in ~14-month-old mice by western blot. **(B)** ***Quantification of hAβPP*** (~90 kDa), c-terminal fragment (CTF)- β (~12 kDa) and the monomeric (~4 kDa) and oligomeric (trimer (~14kDa)) forms of Aβ in the hippocampus and cortex. **(C)** ***ELISA analysis of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels*** in the hippocampus. ▲ *p* at least ≤0.05 versus APPWT mice.

Mt1 overexpression influences the behavioral phenotype of Tg2576 mice

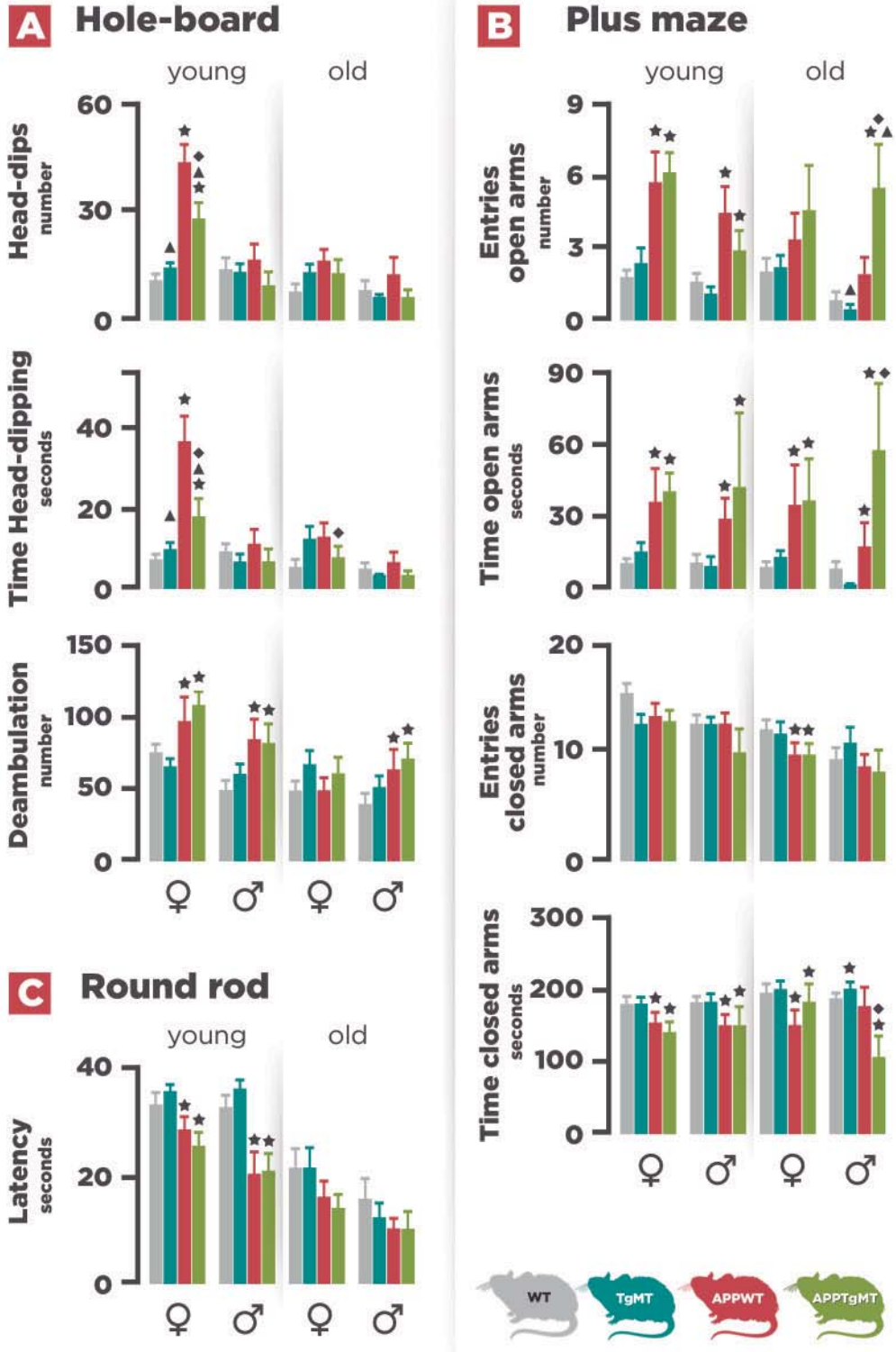
Male and female mice ~5-6 (young) and ~13-14 (old) months of age were characterized behaviorally. **Exploratory behavior** (number head-dips and time of head-dipping) and **activity** (deambulations and rearings) were assessed using the hole-board paradigm (**Fig. 4A;** young and old females, n = 15–33 and 13–16; young and old males: 9–15 and 11–14). hAβPP expression increased exploratory behavior in young female mice only, a behavioral change partially reversed by Mt1 overexpression. Deambulation (horizontal activity) but not rearings (vertical activity, data not shown) was also increased in young hAβPP+ mice of both sexes, and only in males in the case of old mice. No significant effects of Mt1 overexpression were observed. **Anxiety** was evaluated with the elevated plus maze (**Fig. 4B**; young and old females, n = 15–33 and 11- 12; young and old males: 9–15 and 7–12). Young hAβPP+ male and female mice were significantly less anxious than their littermate controls, as evidenced by the increased number of entries and time spent in the open arms of the maze and the decreased time spent in the closed arms. The results in old hAβPP+ mice were similar. Regarding Mt1 overex- pression, it did not alter anxiety in young mice, but potentiated the hAβPP-induced decrease in anxiety present in old male mice.

Motor coordination and strength was evaluated with a battery of tests. To assess balance, mice were placed on a round rod (1 cm in diameter) and the latency to fall was measured. As depicted in **Fig. 4C** (young and old females, *n* = 18–33 and 14–19; young and old males: 8–15 and 11–14), the latency to fall was significantly decreased in hAβPP+ mice of both sexes and ages, with the effect of hAβPP more prominent in young than in old animals (likely because the latter were already deteriorated per se). Similar results were obtained in the coat hanger test (which tests forepaw grip capacity and strength, data not shown). Mt1 overexpression did not modify this phenotype.

Mt1 overexpression influences learning in Tg2576 female mice

Spatial memory and learning capacity were studied with the MWM in young female mice (**Fig. 5A**; *n* = 4–8). When challenged to locate a submerged platform using external cues as reference, hAβPP+ mice showed impaired spatial learning since they learned more slowly how to find the platform compared to controls (**Fig. 5A** top). Mt1 overexpression rescued this deficit. However, no significant differences between genotypes were found in the retention during the probe trial test (**Fig. 5A** bottom) or when animals were challenged to learn a new location of the platform or to find a visible platform (data not shown). In the instrumental (operant) conditioning (Skinner box), young female mice learned to get food pellets by pressing a lever. As depicted in **Fig. 5B** (*n* = 9-10), that shows the mean percentage of the maximum number of lever presses, all genotypes increased the percentages of lever presses across the sessions. Notably, by the end of the test (5th session), the APPTgMT females showed the highest percentage of lever presses, reaching 93.5 ± 5.9% of the criterion, followed by the WT mice with 76.1 ± 12.4% and the TgMT and APPWT groups which reached 69.5 ± 12.7% and 69.5 ± 12.7%, respectively. If only APPWT and APPTgMT groups are considered, then a significant Mt1 overexpression x day interaction is obtained ($p < 0.05$) since the APPTgMT mice performed better the last two days of conditioning. However, when analyzed all together no significant differences were achieved.

Learning was also studied in ~18-month-old female mice with the eyeblink test (**Fig. 5C**; Experiment 1. *n* = 2–4). During the habituation sessions, the percentage of putative CRs diminished slowly but steadily. Also as expected, during the conditioning sessions the percentages of CRs were significantly increased. However, hAβPP expression dramatically impaired this learning (~56 versus ~15% of CRs in WT and APPWT mice, respectively, in the 10th conditioning session).


A
Hole-board
young
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B
Plus maze
young
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Entries
open arms
number
9
6
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Entries
closed arms
number
20
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Time closed arms
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200
100
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C
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WT
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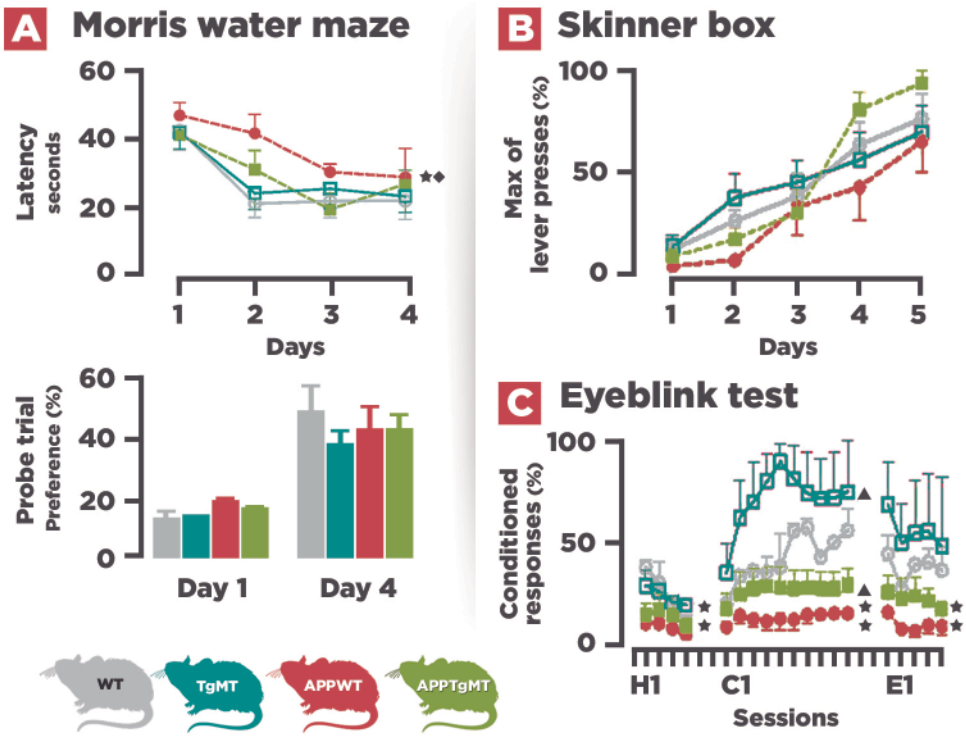

Figure 5. Effect of *Mt1* overexpression on learning.

(A) ***Spatial learning*** ~6-month-old females were assessed in the Morris water maze. In the hidden platform phase hAβPP+ mice showed a somewhat impaired learning and memory since they learned more slowly the position of the submerged platform (top). However, the preference for the target quadrant was unaltered (bottom). *Mt1* overexpression rescued the hAβPP-induced deficit. **(B)** ***Operant conditioning*** of ~6-month-old females in the Skinner box. Learning was similar in the four genotypes, but *Mt1* overexpression increased learning in hAβPP+ mice the last days. **(C)** ***Conditioned responses*** of ~18-month-old females in the classical eyeblink conditioning test. Learning was impaired in hAβPP+ mice, whereas *Mt1* overexpression improved learning in both hAβPP+ and hAβPP– mice. ★, ▲, and ◆ *p* at least ≤0.05 versus hAβPP–, TgMT–, and interaction between AβPP and TgMT, respectively.

Figure 4. Effect of *Mt1* overexpression on the behavioral phenotype of Tg2576 mice.

(A) ***Exploratory behavior (number of head-dips and time of head-dipping) and activity*** in the Hole-board of young (~5, 6-month-old) and old (~13, 14-month-old) male and female mice. Exploration and activity were increased in young hAβPP+ female mice, an effect partially rescued by *Mt1* overexpression in the former case. **(B)** ***Anxiety was assessed in the elevated plus maze.*** Anxiety was decreased in hAβPP+ mice, an effect potentiated by *Mt1* overexpression in old male mice. **(C)** ***The latency to fall from a round rod*** was significantly decreased in hAβPP+ mice. ★, ▲, and ◆ *p* at least ≤0.05 versus AβPP–, TgMT–, and interaction between hAβPP and TgMT, respectively.

Overall, Mt1 overexpression significantly increased the CRs (~75 versus ~29% of CRs in TgMT and APPTgMT mice, respectively). During the extinction sessions the differences between hAβPP+ and hAβPP- groups were still observed, but the effect of Mt1 overexpression was no longer statistically significant ($p = 0.112$).

Discussion

Metallothioneins are key proteins involved in several physiological and pathological processes such as inflammation, oxidative stress, and metal homeostasis, among others. All these processes have been suggested to have a central role in AD pathology [25]. *In vitro*, literature further supports a potential involvement of the MT family in the pathogenesis of AD; however, it is imperative that *in vivo* models are developed to be able to unravel the role of MTs in AD. By using knockout models we demonstrated that MTs do indeed modulate some physiological, behavioral, and biochemical aspects of a widely used AD transgenic mouse model of AD (Tg2576, expressing mutated human AβPP) [12, 16]. To further characterize the role of MTs *in vivo*, we have produced double transgenic mice, namely Tg2576 mice overexpressing the MT-1 isoform.

Survival was monitored in these animals from weaning until sacrifice and as expected, the presence of the hAβPP transgene dramatically reduced perinatal and adult survival (~25% survival by 60 weeks) [26–31] in both sexes. The mechanisms underlying such mortality are poorly known, but one possibility is that of increased oxidative stress. MTs are considered potent antioxidant proteins, and thus could be envisaged as protective proteins regarding hAβPP- induced mortality. Indeed, at weaning *Mt1* overexpression dramatically increased perinatal survival, supporting a pro-survival role of this MT isoform at early ages. However, rather than rescuing from hAβPP-induced mortality, Mt1 overexpression increased perinatal survival in both hAβPP+ and hAβPP– mice. In our previous study using *Mt1&2* KO mice, we did not find a significant overall effect of MT-1/2 deficiency [12], but the results in fact suggested a prosurvival role in hAβPP– mice, in line with the present results. Older data with a different model (GFAP-IL6 mice) also suggested a role of MT-1/2 during development [32]. It has long been known that the MT-1/2 isoforms are highly expressed in visceral yolk sac, placenta, and fetal tissues, and that these proteins provide a reproductive advantage during adverse conditions such as maternal zinc deficiency [33–36]. However, the opposite was observed in AβPP+ mice, i.e., a detrimen-

tal role of MT-1/2 [12]. In the present study such trend was not observed. Whether this is due to a specific role of MT-2 versus MT-1 [37], genetic background or to the need of increasing the number of mice studied will need further studies. Interestingly, hAβPP-induced perinatal mortality was not observed when crossing with Mt3 KO mice [16], which suggests that the number of alleles of these MT isoforms (by the nature of the crossings carried out, the littermate WT mice are not homozygous but heterozygous) may be relevant factor.

hAβPP-induced mortality continued following weaning, reaching dramatic levels (~70–80%) by the time the experiment was terminated (>60 weeks). Mortality was higher in males than in females, in accordance with previous studies despite very different genetic backgrounds [12, 16]. Sex is therefore a major factor in this regard. On the other hand, overall mortality in the present study was higher, which is likely due to the genetic background. The Tg2576 mice obtained commercially are in B6xSJL mixed background, and we have crossed them twice with TgMT mice, which are in B6. Increasing the percentage of C57Bl/6J alleles has been shown to be detrimental for life expectancy in the Tg2576 model, whereas a 129s6 background, as used in our previous reports, would be beneficial for survival [26–31]. *Mt1* overexpression did not affect significantly hAβPP- induced mortality, but a trend for increasing it was observed in male mice, therefore suggesting a detrimental role of MT-1. However, the same trend was observed in hAβPP– mice; thus, it would rather be a general detrimental effect of *Mt1* overexpression. Unfortunately, despite starting the experiment with almost 600 mice, the high mortality reduced severely the number of animals and this may preclude the putative statistical significances. In our previous study with *Mt1&2* KO mice a detrimental role of MT-1/2 isoforms was clearly established, but only in hAβPP+ female mice [12]; interestingly enough, the results were strikingly similar when crossing with *Mt3* KO mice [16]. Why the effect of MTs may depend on sex and situation (hAβPP+ versus hAβPP–) is unclear. It is important to emphasize that in all these cases the four groups directly compared were littermates. In addition, other evidence indicates that the effects of MTs on survival highly depend on the context [38–42]. A very recent study demonstrates a prosurvival role of MT-1/2 in very old mice [43]; thus, aging is an additional factor to consider.

Monitoring of body weight gain evidenced that, as expected [12, 26–31], hAβPP+ mice from both sexes showed a clear reduction in body weight gain compared to WT animals since weaning. *Mt1* overexpression, however, did not affect this phenotype. In contrast, *Mt1* overexpression decreased body

weight in the AβPP- mice from both sexes, which is consistent with some studies carried out with *Mt1&2* KO mice which showed increased body weight, particularly when fed a high-fat diet [44–46]. However, the role of MTs in the control of body weight is again obscured by results in other studies, showing either no effect of MT-1/2 deficiency [12] or a decreasing effect [32] on body weight. Again, the number of alleles and/or the genetic background are likely relevant. Clearly, much remains to be understood.

In Tg2576 mice, insoluble Aβ and amyloid deposits have been described to first appear at 6 and 9–10 months of age, respectively [47]. Therefore, to further characterize our double transgenic model, we assessed the plaque load in ~14-month-old mice by immunohistochemistry and the amyloid cascade in ~6- and ~14-month-old mice by western blot. As expected, amyloid plaques were seen throughout the hippocampus and the cortex. *Mt1* overexpression slightly but significantly increased the amyloid plaque load in the hippocampus (but not the cortex) in both sexes. This is consistent with the trends observed in old Tg2576 mice when Zn_7-MT-2A was injected subcutaneously on a daily basis (5 days a week) for 19 days [11]. Moreover, Tg2576 mice crossed with *Mt1&2* KO mice showed a decreased amyloid load [12]. Altogether, these results strongly suggest that *in vivo* the MT-1/2 isoforms favor the formation of amyloid plaques as detected by immunohistochemistry, being this effect more consistent in the hippocampus than in the cortex. There may be a number of reasons underlying this effect of MT-1/2 on amyloid burden: changes in the expression of hAβPP, in its processing, in the type of metal interacting with Aβ, and in the activity of glial cells, to name some.

We assessed the expression of hAβPP and the amyloid cascade by western blot. In old mice, the full-length hAβPP was consistently increased in hippocampus and cortex in APPTgMT mice compared to APPWT mice in both sexes. Moreover, hAβPP levels were also increased by *Mt1* overexpression in the hippocampus of young male (but not female) mice. Increased hAβPP levels is a likely cause contributing to the higher amyloid load observed in APPTgMT mice. As could be expected, upon β-secretase proteolytic processing higher hAβPP levels led to higher CTF-β levels, supporting that assumption. Despite higher hAβPP levels and hAβPP processing in APPT- gMT mice in both cortex and hippocampus, increased amyloid plaque burden was only occurring in the hippocampus, which suggests that further mechanisms are in place. To complicate things further, in our previous study with *Mt1&2* KO mice we also observed higher hAβPP levels in the hippocampus (but not the cortex) of old mice, although in female mice

only; and no differences in young mice [12]. Thus, while the present results are more consistent, we do not know the reasons underlying these contradictory results.
In the amyloid cascade, once CTF-β has been produced by β-secretase, γ-secretase will produce Aβ peptides which can then precipitate and eventually form plaques, but oligomers can also be formed, representing separate and distinct aggregation path- ways [48–50]. In the cortex, *Mt1* overexpression did not influence SDS-soluble Aβ monomeric and trimeric peptides. This is consistent with the phenotype observed in APP*Mt1&2* KO mice, except for a small increase in the trimeric form in males [12]. In contrast, in the hippocampus *Mt1* over-expression had opposed effects on Aβ peptides, increasing them in males and decreasing them in females. Importantly, this is again consistent with the phenotype observed in APP*Mt1&2* KO mice [12]. A very recent report [51] has demonstrated an inhibitory effect of MT-1/2 on the *in vitro* activity of one of the putative α-secretases (ADAM17/TACE). Presumably, if the non-amyloidogenic pathway is inhibited by MT-1/2 it would favor the amyloidogenic one. Should this occur in our experimental set-up *in vivo*, with a sex-dependent regulation of TACE and/or other α-secretases, it would be a good explanation. Interestingly, MT-3 has been reported to increase the activity of ADAM10, another α-secretase, in the mouse neuroblastoma Neuro2A Swedish AβPP cells [52], suggestive of MT isoform-specific roles.

MTs could participate directly in the formation of the plaques. A great insight has been obtained *in vitro* that indicates that these proteins may influence the formation of the fibrillary type Aβ aggregates [53] and inhibit the copper-mediated Aβ aggregation and toxicity [54, 55]. Whether or not this is relevant *in vivo* remains to be established.

The Tg2576 behavioral phenotype has been thoroughly characterized over the years, and while not very robust, mice bearing the hAβPP transgene usually show a less anxious/more exploratory phenotype together with impaired learning and memory, having age, sex, and genetic background considerable importance [14, 56–60]. In line with our previous reports [12, 16], and despite the different genetic background, young hAβPP+ female (but not male) mice did more head-dipping behavior in the hole board, indicative of a greater exploratory activity. This behavioral change mostly disappeared in old mice, which is in contrast to our previous studies, which showed increased exploration in old hAβPP+ mice in both sexes, highlighting again the relevance of the genetic background for behavior [57]. *Mt1* overexpression partly rescued this behavior in young female APPTgMT mice, and this

same trend was observed in young males and old animals of both sexes, which is somewhat reminiscent of the results observed following the administration of Zn_7MT-2A [11]. However, MT-1/2 deficiency had no significant effect [12]. hAβPP expression also decreased anxiety as revealed by the increased visits to and/or time spent in the open arms of the plus maze, and this happened in both young and old mice in both sexes. Although hAβPP increased ambulation (as observed in the hole-board), this decreased anxiety is not simply the consequence of altered ambulatory rates since the entries in the closed arms were not increased but rather the opposite. *Mt1* overexpression did not rescue hAβPP-induced decreased anxiety in young mice, and in fact exacerbated it in old males. Since the administration of Zn_7MT-2A [11] and MT-1/2 deficiency had no clear effects on anxiety [12], we must conclude that the putative effects of these MT isoforms are not critical in this regard.

We also looked into the putative role of MT-1 on hAβPP-induced learning alterations. In the MWM, APPWT young female mice had a somewhat impaired spatial learning process in that they learned to find the submerged platform in a more slowly fashion than WT mice. *Mt1* overexpression rescued this phenotype, suggesting that the MT-1/2 isoforms could be involved in the regulation of this type of learning. MT-1/2 deficiency did not exacerbate the phenotype of Tg2576 mice [12], which in principle does not support that claim. However, when the *Mt1&2* KO mice (in 129Sv background) were backcrossed with C57Bl/6J mice, they had baseline impairments in spatial learning in the MWM [61], which are in line with the present results obtained in Tg2576 mice crossed twice with B6 mice. Still, the ensuing probe trial was normal, as was the learning of a new location of the platform or finding the visible platform, indicating that overall the spatial learning was essentially normal regardless of the genotype. Nevertheless, in another paradigm measuring spatial learning, the radial-arm maze, *Mt1&2* KO mice (in 129Sv background) were reported to perform worse than WT mice [62]. Learning was also assessed in an operant conditioning test (Skin- ner) and in a classical conditioning test (eye-blink). In the first case, the experiment was carried out in young females, and the results indicated that the four genotypes responded similarly (with the APPTgMT mice nevertheless performing somewhat better than APPWT mice the last two days of conditioning). In older female mice (~18-month-old compared to ~6 in the other tests), however, learning in the classical conditioning eye-blink test was severely impaired in AβPP+ mice, which is consistent with previous studies [63]. *Mt1* overexpression partly rescued this phenotype,

and moreover, TgMT in fact performed better than WT mice, indicating that this is a general beneficial effect of MT-1 rather than something related specifically to the Tg2576 phenotype. Collectively, the data suggest that the MT-1/2 isoforms may be exerting beneficial effects on cognitive functions depending on the specific trait being investigated, age, and situation (i.e., hAβPP- versus hAβPP+).

In summary, the present study, together with that carried out in *Mt1&2* KO mice [12], strongly suggests that MTs are able to modulate *in vivo* key features of AD, such as the amyloid cascade and amyloid plaque burden, at least in the hippocampus. They also influence some of the behavioral changes observed in Tg2576 mice even before plaques are formed. While much remains to be understood, these results provide some insight for AD and will hopefully encourage further studies aimed to deal with this devastating disease.

Acknowledgments

The authors are grateful to grants SAF2011-23272 and SAF2014-56546-R, and to Fundació La Marató de TV3 20142210 to J.H., and BFU2014-56692-R to J.M.D. Y.M. acknowledges her Ph.D. (AP2005-0588) and post-doc (FPDI-2013-17228) fellowships. PAA is supported by an ARC Future Fellowship. In addition, the Florey Institute of Neuroscience and Mental Health acknowledge the strong support from the Victorian Government and in particular the funding from the Operational Infrastructure Support Grant.

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3

Influence of transgenic metallothionein-1 on gliosis, CA1 neuronal loss, and brain metal levels of the Tg2576 mouse model of Alzheimer's disease

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International Journal of Molecular Sciences 18 (2017) 251
DOI 10.3390/ijms18020251

The mouse model of Alzheimer's disease (AD) Tg2576 mice (APP), has provided valuable information, such as the role of the metallothionein (MT) family in their behavioral and amyloidosis phenotypes. In this study, we further characterize the role of MT-1 by crossing *Mt1*-overexpressing mice with Tg2576 mice (APPTgMT). In 14-month-old mice, MT-1(/2) protein levels were dramatically increased by *Mt1* overexpression throughout the cortex (Cx), which showed a prominent caudal-rostral gradient, and the hippocampus (HC). There was a trend for MT-1(/2) immunostaining to be increased in the areas surrounding the amyloid plaques in control male mice but not in *Mt1*-overexpressing mice. Gliosis was elicited by the amyloid plaques, but the effects of *Mt1* overexpression were modest. However, in hippocampal western blots the microglial marker Iba-1 was increased in old male APPTgMT mice compared to APP-wild type (APPWT) mice, and the opposite was observed in young mice. Hippocampal CA1 neuronal loss was observed in Tg2576 mice, but was unaffected by *Mt1* overexpression. Aging increased Zn and Cu levels differently depending on brain area, sex, and genotype. Thus, the effects of *Mt1* overexpression on the phenotype of Tg2576 mice here studied are modest.

Introduction

Alzheimer's disease (AD) is a devastating disease that causes a progressive loss of cognitive functions. It is characterized by the presence of extracellular deposits of amyloid-β peptides (amyloid plaques), intracellular deposits of hyperphosphorylated tau protein (neurofibrillary tangles), neuroinflammation, and oxidative stress in brain areas such as the hippocampus and the cortex [1,2]. Transition metals such as Cu or Fe, together with Zn, contribute to oxidative stress as well as to the aggregation and precipitation of amyloid-β peptides in the AD brain [3,4].

A number of studies suggest that the metallothionein (MT) family of proteins may be important for the understanding of AD. MT-1/2 isoforms have been shown to be upregulated in AD [5–8], whereas the results for MT-3 are less consistent [9,10]. In accordance with the human disease, MT-1/2 protein levels are increased in areas enriched in amyloid plaques in several AD mouse models, including the Tg2576 mice [8,11]. Ascertaining the putative role(s) of MTs in these mouse models is of great interest. Results obtained in transgenic mice crossing the Tg2576 mice with MT-1/2 deficient (*Mt1&2* KO) mice showed that these MT isoforms are involved in the formation of amyloid plaques, particularly in the hippocampus [12]. This possibility has been reinforced by recent results obtained crossing the Tg2576 mice with *Mt1*-overexpressing (TgMT) mice [13]. Moreover, some behavioral traits were also shown to be influenced by MT-1. Nevertheless, much remains to be understood. Here we expand the results by analyzing MT-1/2 immunohistochemistry, gliosis, neuronal survival, and Zn and Cu levels.

Materials and methods

Animals

The parental strains used in this study were C57BL/6JOlaHsd as a wild-type (WT) strain (Harlan, KY, USA), TgMT mice, which carry 56 copies of a minimally marked *Mt1* (*Mt1**) gene [21] (B6.Cg-Tg(*Mt1*)174Bri/J; The Jackson Laboratory, Bar Harbor, ME, USA), and the AD mouse model Tg2576 which expresses the human APP_{695} harboring the Swedish K670N/M671L mutations under the control of the hamster prion protein promoter [23] (Taconic Europe A/S; Ry, Denmark). These strains were crossed and genotyped as previously described [13] to produce WT, TgMT, APPWT (APP in Figures), and APPTgMT mice. Throughout the manuscript, we may refer the two former groups as APP negative mice, and the two latter

as APP positive mice. Mice were killed at ~6 and ~14 months of age. Mice were housed in groups and given ad libitum access to food and water in a 12-h dark-light cycle under constant temperature (~22 ºC). Animals were killed by decapitation and the brain quickly removed on placed ice. The cortex (Cx) and hippocampus (HC) of the right hemisphere were quickly dissected, frozen with liquid nitrogen, and stored at −80 ºC. The left hemisphere was fixed by immersion in 4% paraformaldehyde and stored in 70% ethanol at 4 ºC until further processing for paraffin-embedding. All experimental procedures were approved by the Ethics Committee in Human and Animal experimentation from the Autonomous University of Barcelona (CEEAH2996, 29 May 2015) and Servei de Biodiversitat i Protecció dels Animals (8837, 15 December 2015).

Immunohistochemistry (IHC) and histochemistry (HC)

Fixed brains were paraffin-embedded and cut sagitally in 8 μm-thick sections for assessing MT-1/2 (primary antibody: anti-MT 1/100, DAKO, M0639, Clone 9; secondary antibody: biotinylated anti-mouse IgG 1:300, SIGMA, St. Louis, MO, USA), astrogliosis (primary antibody: anti-GFAP 1:900, DakoCytomation, Glostrup, Denmark A/S; secondary antibody: biotinylated anti-rabbit IgG 1:300, Vector Laboratories, Inc., Burlingame, CA, USA), and microgliosis (primary antibody: anti-Iba1 1:1500, WAKO, Tokyo, Japan; secondary antibody: biotininylated anti-rabbit IgG (H + L) 1:300, Vector Laboratories, Burlingame, CA, USA) as described [12]. All IHC performed were double-stained with Congo red stain (SIGMA) to identify areas with dense plaques with a congophilic core, in order to assess the MT-1/2 IHC and the activation of astrocytes and microglia surrounding the plaques compared to areas without plaques. In order to quantify hippocampal CA1 neurons, 0.1% of Cresyl Violet (SIGMA) was used to stain Nissl substance. An image of CA1 of the hippocampus was acquired using a bright field microscope (Nikon Eclipse E400, Nikon Corporation, Tokyo, Japan). The images were analyzed using Image J software (1.49 v) [36] and the average of three measures of CA1 thickness was taken. Analyses were performed on two non-consecutive sections per mouse. Stained sections were examined with a bright-field microscope (Nikon Eclipse 90i, Nikon Corporation) and images were acquired from the cortex and the hippocampus using a Nikon digital camera DXM 1200F and Nikon Act-1 v. 2.70 software. The images were analyzed using Image J software. A limited area was determined around the dense plaques stained with Congo red and the ImageJ color deconvolution plugin by Gabriel Landini [37] was used in order to separate the DAB and Congo red colors, obtaining afterwards the

quantity of immunostaining associated to dense plaques, the quantity not associated and the total amount of staining of the brain areas studied (Cx and HC). The quantitation of immunostaining in the cortex was divided in three regions: caudal, medial (~above hippocampus) and frontal. Histological analyses were performed on at least three non-consecutive sections per mouse.

Western blotting

Total homogenates of Cx and HC were obtained by sonication in 50 mM Tris-HCl (pH 7.6), 0.01% NP-40, 150 mM NaCl, 2 mM EDTA, 3% sodium dodecyl sulfate (SDS), 1mM phenylmethylsulfonyl fluoride (PMSF), 1% sodium deoxycholate and protease inhibitor cocktail (Sigma-Aldrich, Madrid, Spain). Protein concentration was measured using the bicinchoninic acid (BCA) protein assay as specified by the manufacturer (Pierce, Thermo Fisher Scientific Inc; Rockford, IL, USA) and samples were stored at −80 ºC until they were used. Western blot for astrocytosis (anti-Glial Fibrillary Acidic Protein –GFAP– 1:40,000, DakoCytomation, Denmark A/S) and microgliosis (anti-ionized calcium binding adaptor molecule 1–Iba-1– 1:3000, Wako Pure Chemical industries, Osaka, Japan) was carried out as previously described [12]. Membranes were developed with ECL reagent (Amersham, GE Healthcare, Buckinghamshire, UK) and exposed to autoradiographic film (Kodak, Rochester, NY, USA); for quantification, images were acquired and quantified using the Bio-Rad laboratories (Hercules, CA, USA) QuantityOne ChemiDoc software (version 4.6.3).

Inductively coupled plasma-mass spectrometry (ICP-MS)

Cortical and hippocampal tissues were prepared as described above. Following digestion of the samples with HNO_3 at 60 ºC, and dilution in 1% HNO_3, determination of Zn and Cu was carried out as described [12].

Statistical analysis

Data was analyzed using the Statistical Package for Social Sciences (SPSS) version 17.0. Males and females were analyzed separately. The data was analyzed using Generalized Linear Model (GLZ) using APP (APP positive vs. APP negative) and *Mt1* overexpression (TgMT positive vs. TgMT negative) as main factors. In the IHC, when the intensity around the plaques was compared with the non-associated intensity, "association to plaques" was used as grouping factor; and when several areas of the cortex were studied, this was an additional factor. In the study of the metal content in the hippocampus "age" was used as a factor (young and old). Statistical significance was defined as $p \leq 0.05$.

Results

MT-1/2 immunostaining is dramatically increased in TgMT mice

Representative MT-1/2 immunostaining in wild-type (WT) and TgMT mice (**Figure 1A**) as well as quantification of this staining in the cortex of the different genotypes (**Figure 1B**) clearly indicates that total MT-1/2 protein levels were dramatically increased throughout the brain in TgMT male and female mice ($p < 0.001$).

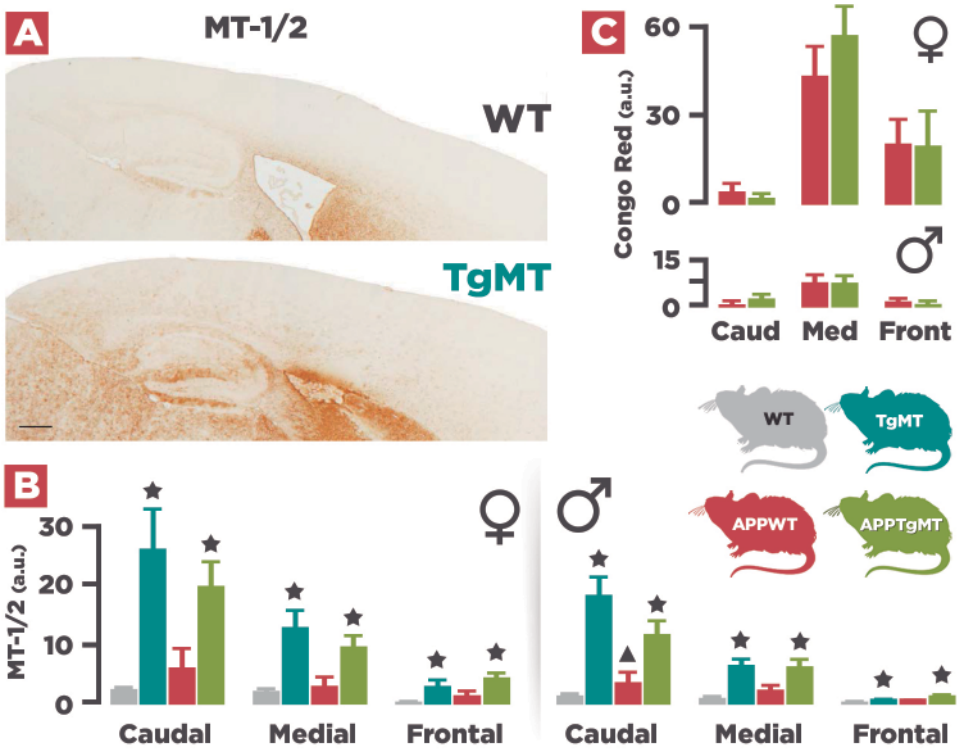

Figure 1. Effect of *Mt1* overexpression on MT-1/2 and Congo Red staining in the cortex.

(A) ***Representative brain MT-1/2 immunostaining*** in type (WT) (**top**) and TgMT (**bottom**) mice. **(B)** ***Quantification of MT-1/2 IHC*** (of the different genotypes in the cortex showed a dramatic increase in *Mt1*-expressing (TgMT and APPTgMT) mice (★ p at least ≤0.05 vs. WT or APP mice, respectively) with a prominent caudal-frontal gradient. As revealed by the significant interaction between APP expression and *Mt1* overexpression (▲ $p < 0.05$ in male caudal region; the rest was not significant), APP expression tended towards an increase in MT-1/2 in WT mice; and the opposite was true in TgMT mice. **(C)** ***The greatest accumulation of dense amyloid plaques*** stained with Congo Red was localized in the medial area in both sexes. Results are mean ± SEM (n = 7–11). Scale bar: 400 µm. a.u., arbitrary units.

The antibody used for MT immunohistochemistry (IHC) recognizes both MT-1 and MT-2 isoforms; the increase in total MT-1/2 levels presumably reflects the expression of the *Mt1* transgene (thus increasing MT-1 protein levels) rather than changes in MT-2 levels. Interestingly, we noticed a prominent gradient in MT-1/2 IHC, with the highest staining in the caudal cortex and the lowest in the frontal cortex (**Figure 1B**; $p < 0.001$). Such a gradient was present regardless of amyloid precursor protein (APP) expression, which produced a major accumulation of Congo Red positive dense amyloid plaques localized in the medial part of the brain but less so in the caudal and frontal cortex (**Figure 1C**; $p < 0.001$). MT-1/2 immunostaining showed a trend for increased levels in APPWT mice (compared to WT mice), whereas the opposite was observed in APPTgMT mice (compared to TgMT mice) (**Figure 1B**).

In the **cortex** of male mice this resulted in a significant interaction between APP expression and *Mt1* overexpression ($p < 0.05$), and between APP expression, *Mt1* overexpression, and area of the cortex ($p < 0.05$).

In the **hippocampus** (**Figure 2A,B**), MT-1/2 immunostaining was also increased in TgMT and APPTgMT mice in both sexes relative to their respective controls ($p < 0.001$). As in the cortex, in the hippocampus of male mice the interaction between these two factors (APP and *Mt1* overexpression) was significant ($p < 0.05$), since there was an increase of MT-1/2 protein levels in APPWT mice compared to WT mice but not in APPTgMT mice compared to TgMT mice (**Figure 2B**).

Double staining with Congo Red and MT-1/2 (**Figure 2A**) allowed comparisons of MT-1/2 protein levels in areas surrounding the dense amyloid plaques (**Figure 2A** right, arrow) to areas without plaques. As might be expected, there was a trend for increased MT-1/2 immunostaining near amyloid plaques, but this was significant ($p < 0.05$) only for male mice (**Figure 2C**). There were no significant differences in this regard in the cortex (data not shown).

Thus, the effect of transgenic *Mt1* expression on MT-1/2 immunostaining is much more evident than changes in its association with amyloid plaques.

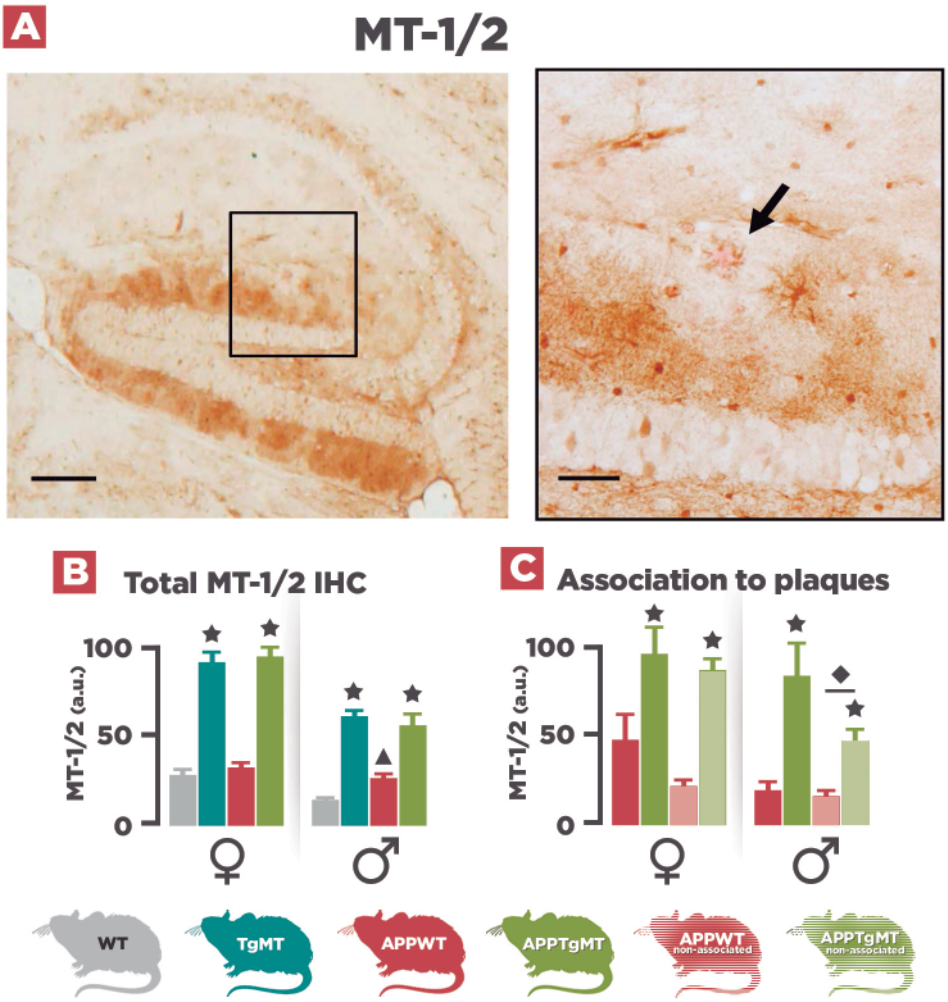

Figure 2. Effect of *Mt1* overexpression on MT-1/2 staining in the hippocampus.

(A) ***Representative immunostaining*** for MT-1/2 counterstained with Congo Red in the hippocampus of APPTgMT mice (**left**); scale bar: 200 µm. A higher magnification of the black lined square area is shown at the right to better demonstrate plaques stained with Congo Red dye (**arrow**); scale bar: 50 µm. **(B)** ***Quantification of total MT-1/2 immunohistochemistry (IHC)*** produced similar results to the cortex, with dramatic increases in TgMT and APPTgMT mice (★ $p < 0.001$ vs. WT or APP mice, respectively). An opposing trend of APP expression was again seen between WT and TgMT male mice (▲ $p < 0.05$ interaction). **(C)** ***Comparison of MT-1/2 levels*** associated with plaques to those not associated with plaques indicated an increased immunostaining in the vicinity of the amyloid plaques only in male mice (◆ $p < 0.05$ vs. staining associated to plaques). Results are mean ± SEM (n = 7–11). a.u., arbitrary units..

Mt1 overexpression has only minor effects on the gliosis elicited by Aamyloid plaques

As expected, amyloid plaques elicited a dramatic gliosis in the hippocampus (**Figure 3**) and cortex (not shown). In contrast to MT-1/2 immunostaining, both GFAP (Glial Fibrillary Acidic Protein) (**astrocytes**; **Figure 3A,B**) and Iba-1 (Ionized calcium binding adaptor molecule 1) (**microglia**; **Figure 3C,D**) immunostainings were significantly ($p < 0.001$) increased in the area surrounding Congo Red-positive plaques compared to areas without plaques. *Mt1* overexpression did not appear to influence these immunostainings for astrogliosis and microgliosis (**Figure 3B,D**, respectively). Thus, the presence or

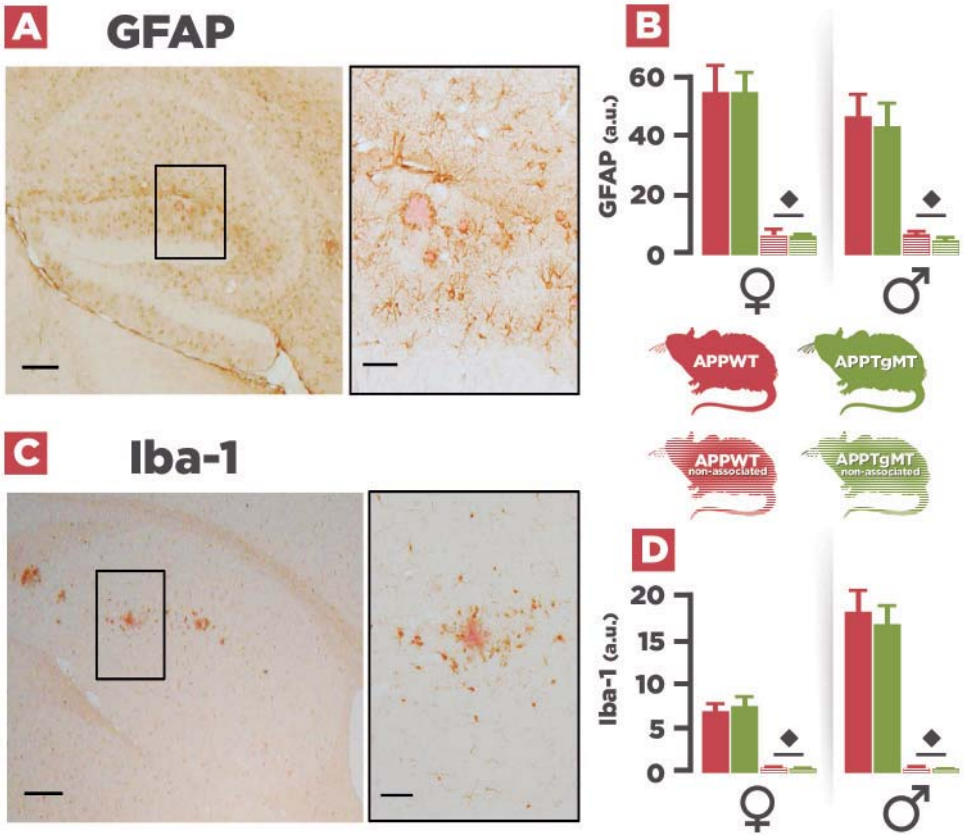

Figure 3. Effect of *Mt1* overexpression on gliosis in the hippocampus.

(A, C) ***Representative immunostaining*** for GFAP (astrocytes) and Iba-1 (microglia), respectively, counterstained with Congo Red, in the hippocampus of APPTgMT mice (**left**); scale bar: 200 µm. On the right, a higher magnification of the black lined square area from left panel shows astroglia and microglia surrounding dense plaques; scale bar: 50 µm. **(B, D)** ***Quantification of GFAP and Iba-1 IHC*** indicated a dramatic increase in the vicinity of the plaques. Results are mean ± SEM (n = 11–18); ◆ $p < 0.001$ vs. plaque-associated staining. a.u., arbitrary units.

absence of amyloid plaques appeared to influence gliosis in the hippocampus more than the expression of *Mt1*, at least in the tissue sections analyzed.

In contrast, results obtained by western blot using the whole hippocampus of one hemisphere did show small, but in some cases significant, effects of *Mt1* overexpression (**Figure 4A,B**). Thus, in the hippocampus of male mice a significant ($p < 0.05$) increase of Iba-1 levels was observed in old mice; the same trend was observed in female mice. In contrast, hippocampal Iba-1 levels were decreased by *Mt1* overexpression in young male mice (**Figure 4B**; $p < 0.05$)).

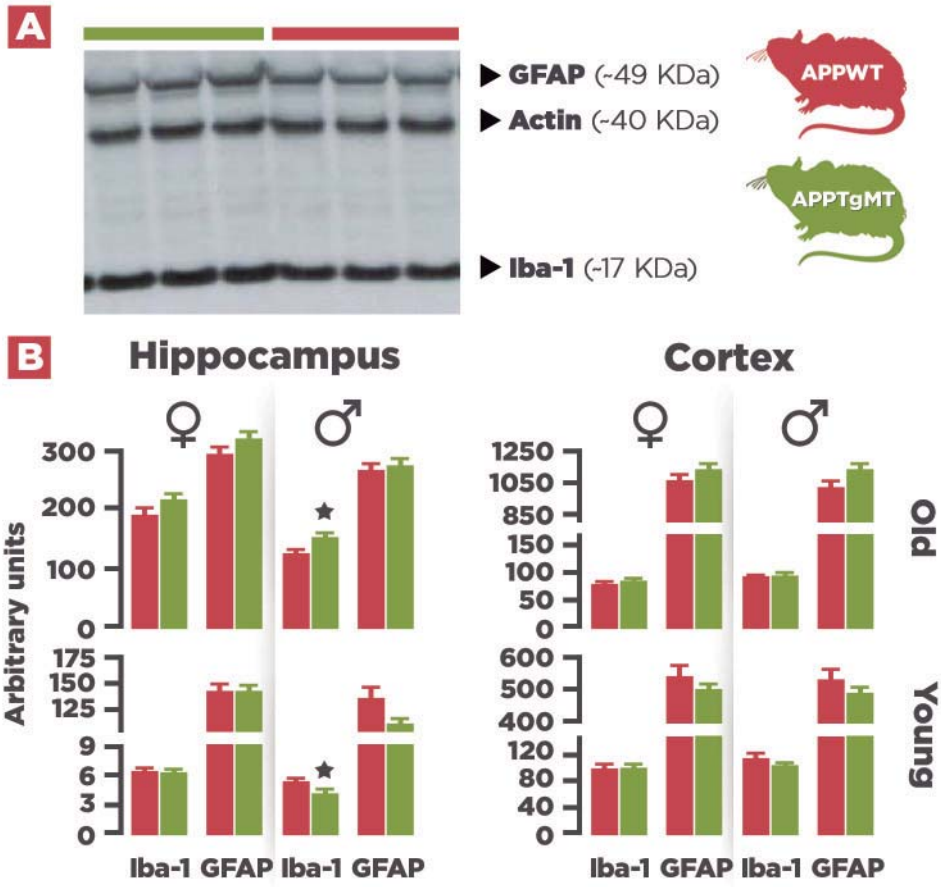

Figure 4. Effect of *Mt1* overexpression on hippocampal gliosis as measured by western blot (WB). Total hippocampal and cortex homogenates were assayed by WB to further characterize gliosis.

(A) ***Representative band pattern of the WB*** (in an autoradiographic film) of old male hippocampus using antibodies for GFAP, Iba-1, and Actin. **(B)** ***Quantification of hippocampal GFAP and Iba-1 levels*** in young and old APPWT and APPTgMT mice. Iba-1 levels were increased by *Mt1* overexpression in old male mice but decreased in young males; the latter also showed decreased GFAP levels. Data are mean ± SEM ($n = 10–11$). ★ p at least ≤0.05 vs. APPWT mice. a.u., arbitrary units.

GFAP levels followed a similar pattern. In contrast to the hippocampus, in the cortex, *Mt1* overexpression did not significantly influence gliosis at any age as evaluated by western blot (**Figure 4B**).

Mt1 Overexpression does not affect hippocampal CA1 neuronal loss

A clear neuronal loss was observed in the CA1 hippocampal area of Tg2576 male and female mice, with a clear thinning of the pyramidal layer as revealed by Nissl staining. This was not influenced significantly by *Mt1* overexpression (**Figure 5**).

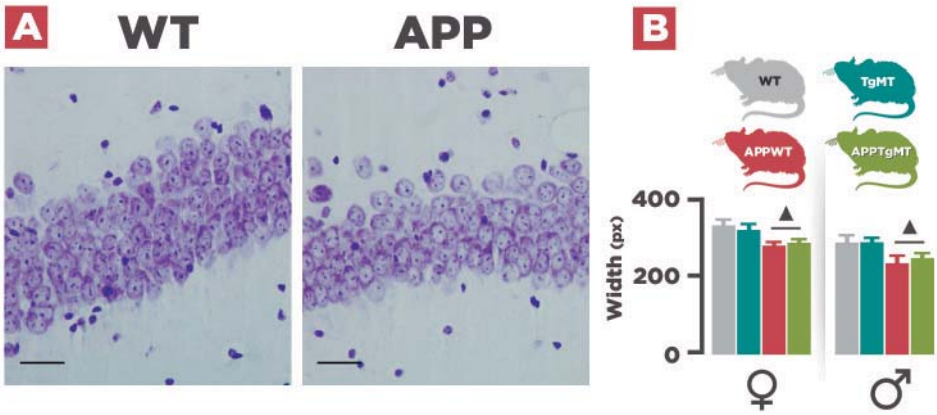

Figure 5. Effect of *Mt1* overexpression on hippocampal CA1 neurons.

(A) ***Representative histochemistry of Nissl body staining*** of neurons in hippocampal CA1 of WT and APPWT mice. Scale bar: 20 µm. **(B)** ***Quantification of the thickness of the CA1 layer*** indicated a significant decrease in APPWT and APPTgMT mice in both sexes, whereas no significant effects of *Mt1* overexpression were observed. Results are mean ± SEM (n = 11–18); ▲ $p < 0.01$ vs. APP negative mice.

Mt1 Overexpression Has only Minor Effects on Zinc and Copper Levels

Total hippocampal and cortical homogenates from young and old mice were used to assess zinc and copper content by ICP-MS (**Figure 6**). In the hippocampus, copper levels were increased by aging ($p < 0.001$), a trend favored by APP expression and in overexpression decreased the effect of APP expression on copper levels of young female had a significant decreasing effect on zinc levels (p at least <0.05) in the hippocampus, but *Mt1* overexpression

did not show a significant effect in this regard. In the cortex, both zinc and copper levels were moderately increased by aging (p at least <0.005); this trend was opposed by APP expression (significantly in male mice), in sharp contrast with the cortex. *Mt1* overexpression tended to increase cortex zinc levels, but variability precluded statistical significance.

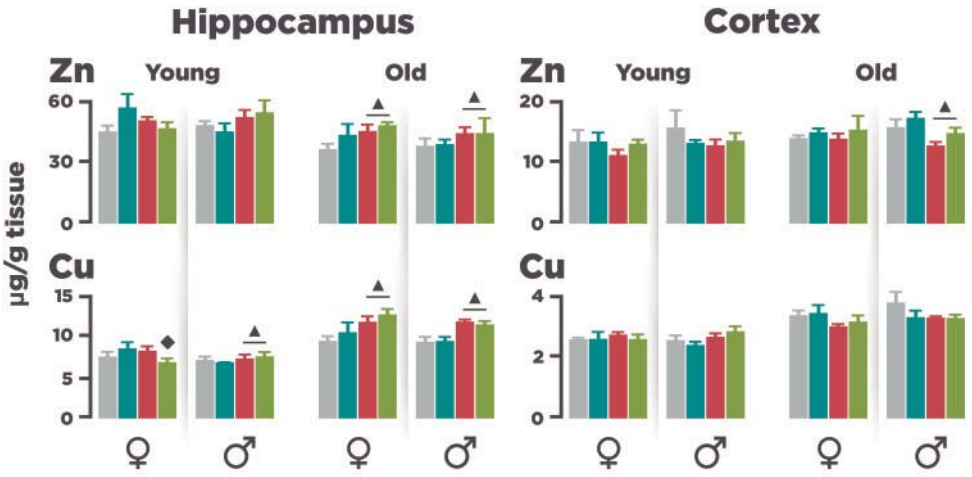

Figure 6. Effect of *Mt1* overexpression on Zn and Cu levels.

Total hippocampal (**top**) and cortical (**bottom**) homogenates from young (~6 months) and old (~14 months) mice were analyzed by ICP-MS. In the hippocampus, copper and zinc levels were increased and decreased by aging, respectively; both metals were increased in the cortex. APP and *Mt1* expression showed different effects depending on the metal and brain area. Results are mean± SEM (n = 7–11); ▲ p at least _0.05 vs. APP negative mice. ◆ $p < 0.05$ interaction between APP and TgMT.

Discussion

We previously showed that *Mt1* overexpression influenced the Tg2576 mice phenotype in a number of ways, including the formation of amyloid plaques and some behavioral traits [13]. It is important to emphasize that the effects caused by *Mt1* overexpression are consistent with those observed in Tg2576/*Mt1&2* KO mice [12]. For instance, *Mt1* overexpression slightly but significantly increased the amyloid load in the hippocampus, whereas trend was observed in *Mt1&2* KO mice. However, the mechanisms underlying MTs on amyloid plaques and other physiological/pathological variables remain to be fully determined. We herewith expand those results by analyzing gliosis, neuronal survival, and accumulation of essential metals in the critical brain areas for AD, cortex, and hippocampus.

Since amyloid plaques are mostly produced in the cortex and the hippocampus, we focused our analysis in these brain areas. We previously showed that *Mt1* (but not *Mt3*) mRNA levels measured by in situ hybridization were clearly increased in TgMT mice [13,14]. Since the antibody used recognizes both MT-1 and MT-2 isoforms, it was expected that MT-1/2 immunostaining would also be clearly increased, and indeed that was the case in both male and female mice. Interestingly, a caudal-frontal gradient in MT-1/2 IHC was observed in the cortex. It is likely that such a gradient could be related to the prominent expression of MT-1/2 in astrocytes [15–17], since these cells tend to show that type of gradient when expressing GFAP [18,19].

The caudal-frontal gradient in the MT-1/2 IHC was present regardless of the APP expression, although certainly some interesting trends could be observed. Thus, when comparing WT and APPWT mice, MT-1/2 immunostaining tended to increase in the latter, presumably because of the amyloid plaques and the associated neuroinflammation. This is in accordance with previous results with in situ hybridization, which indicated that *Mt1* mRNA levels were increased in cells surrounding the amyloid plaques [13,20]. The fact that the Tg2576 mouse model is an AD model with a relative paucity of amyloid plaques, added to the high basal expression of MT-1/2, makes it difficult to see prominent increases of these proteins, which nevertheless we observed, albeit only significantly in male mice. In contrast, when comparing TgMT and APPTgMT mice, MT-1/2 immunostaining tended to decrease in the latter. The very same pattern was observed in the hippocampus. The reason for these opposing trends remains to be established. They might be related to differences between the regulation of the normal (endogenous) MT-1/2 genes and that of the minimally marked *Mt1* (transgenic) gene. According to Palmiter et al. [21], the minimally marked *Mt1* gene, while being expressed about 50% less on a per gene basis, shows a normal tissue distribution, and responds normally to factors such as heavy metals, dexamethasone, and lipopolysaccharide (LPS), which in principle strongly suggests that the transgene is regulated in a similar fashion to the endogenous *Mt1* gene. Therefore, MT-1/2 immunostaining should also be increased by the amyloid plaques in the APPTgMT mice, but this did not occur. Thus, other reasons may be involved, perhaps specifically related to the amyloid plaques (rather than a general phenomenon such as stress) which deserves further attention.

Other putative mechanisms set in motion by *Mt1* overexpression could be related to altered gliosis and neuroinflammation and/or a modulation in normal metal ion homeostasis. MT-1/2 proteins have been shown to

affect gliosis in a number of ways [14,16,17,22]. Astrocytes surrounding the amyloid plaques in old mice were more reactive as revealed by prominent GFAP immunostaining compared to areas without plaques, but, in accordance with our previous study using *Mt1&2* KO mice [12], *Mt1* overexpression did not significantly affect GFAP levels in either the hippocampus or cortex. Similarly, microgliosis, assessed with Iba-1 immunostaining, was not altered significantly by *Mt1* overexpression in the tissue sections of cortex and hippocampus that were analyzed. In contrast, results obtained by western blot using the whole hippocampus (and thus more representative compared to sampled sections) of one hemisphere of old male mice showed an increase of Iba-1 levels; the same trend was observed in female mice. This is consistent with the western blot results found in *Mt1&2* KO mice [12]. It is likely that this effect of *Mt1* overexpression is related to the increased amyloid plaque burden these mice show in the hippocampus [13]; in accordance, *Mt1&2* KO mice show decreased amyloid burden [12]. Thus, through an unknown mechanism, MT-1/2 seem to control the amyloid plaque deposition, which, in turn, drives microglial reactivity.

The situation is different at five months of age, since no amyloid plaques are yet present [23,24]. In contrast to the results found in old mice, *Mt1* overexpression significantly decreased both microgliosis and astrogliosis in the hippocampus; the same trend was present in the cortex. Remarkably, this was occurring again only in male mice. Moreover, the results are generally consistent with those found in *Mt1&2* KO mice [12]. An inhibitory effect of MT-1/2 on microglia has also been suggested in other studies [25–27]. Altogether, the present results suggest that while MT-1 may have a direct inhibitory role controlling microglia, it is overridden by an indirect stimulatory role in the case of APP positive mice because of its effects on the formation of amyloid plaques. In old APP positive (APPWT and APPTgMT) mice, we readily observed neuronal loss of hippocampal CA1 neurons, a known hallmark of mice carrying the "Swedish mutation" [28]; interestingly, this is also observed in AD patients [29]. In this context, it was somewhat surprising that *Mt1* overexpression did not significantly influence neuronal survival [30–32]. Whether or not this is related to different neuronal susceptibilities, to MT-1 levels, or to the specific experimental model causing neurodegeneration remains to be established.

On the other hand, MTs are Zn, Cu-binding proteins [9], metals which have been reported to participate in amyloid-β peptide aggregation [33] and in ROS production [34]. As expected [12], aging, APP and *Mt1* overexpression affected metal content in a modest way. Yet, these were remarkable effects. Aging had different effects on Zn and Cu accumulation in the cortex and

the hippocampus. Thus, in the cortex aging increased Zn (slightly) and Cu levels (more robustly), and this effect of aging was partially blunted in APP positive mice, which is consistent with previous studies [12,35]. In contrast, in the hippocampus, aging increased Cu levels but decreased Zn levels, and both Zn and Cu levels were increased by APP expression. These results highlight the importance of measuring metals in specific areas of the brain rather than bulk brains. Interestingly, increased copper and iron levels with aging have been proposed as a mechanism to explain the age-dependent onset of amyloid neuropathology in the same mice (Tg2576) [35], more so considering hippocampal neuropathology, where APP positive mice had an even higher accumulation of Cu with aging. *Mt1* overexpression only caused minor effects on metal levels, and thus they are unlikely to underlie the phenotype of APPTgMT mice in comparison to APPWT mice (see [12] for further discussion). It should be noted that we have measured total Zn and Cu levels, and therefore we cannot rule out specific effects of MT-1 on free metal ion levels and/or bound metal levels.

In summary, the present study evidences that while MT-1/2 are able to modulate the formation of amyloid plaques and some behavioral traits [12,13], MT-1 shows modest effects on glial activation, neuronal survival, and heavy metal accumulation.

Acknowledgments

The authors are grateful for grants SAF2011-23272, SAF2014-56546-R and to Fundació La Marató de TV3 20142210 to Juan Hidalgo. Yasmina Manso acknowledges her Ph.D. (AP2005-0588) and post-doc (FPDI-2013-17228) fellowships. Olaya Fernandez-Gayol, Paula Sanchis, and Anna Escrig acknowledge their Ph.D. fellowship (FPU2012-00365, BES-2015-071959, and Fundació La Marató de TV3 20142210, respectively).

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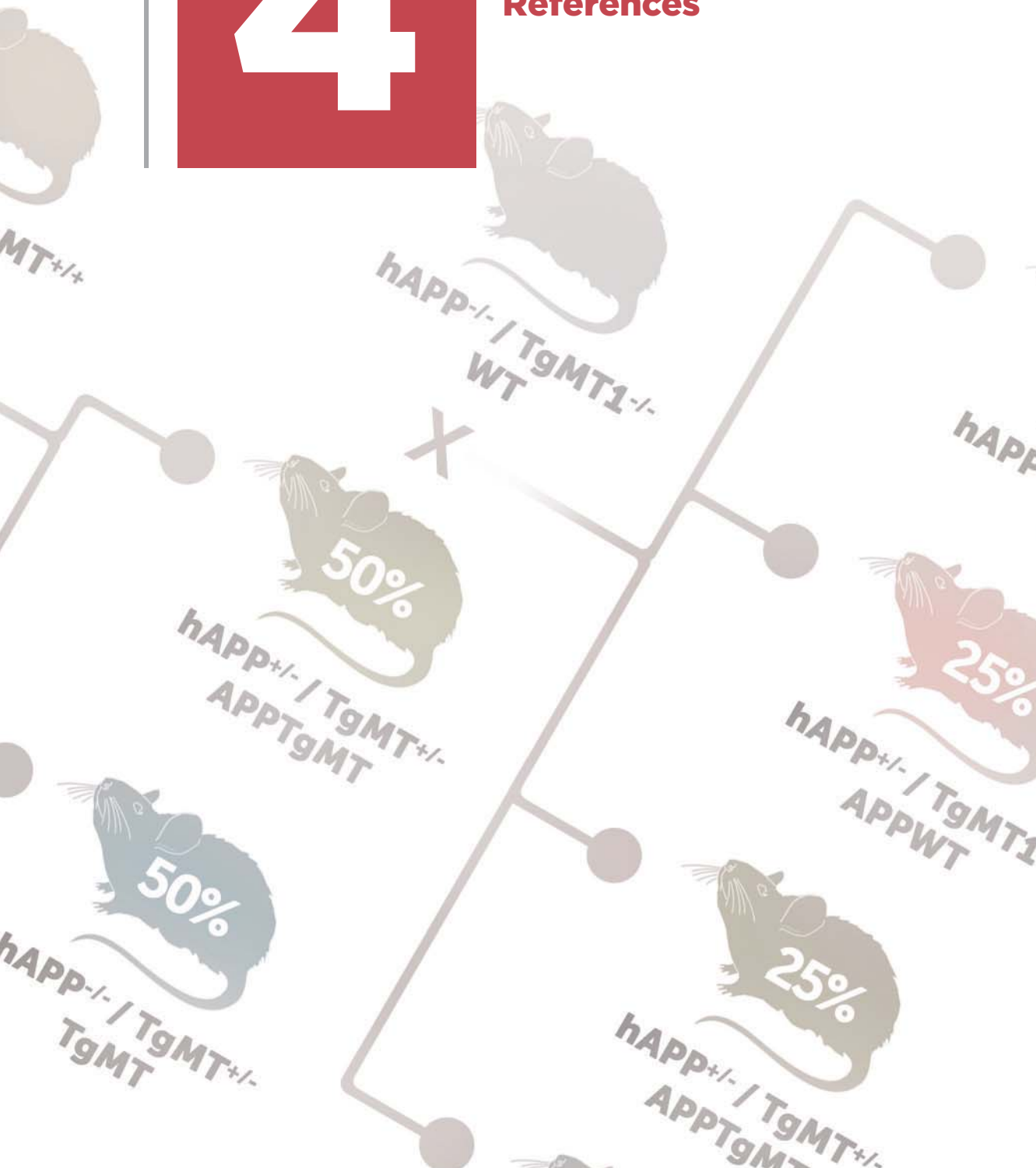
4

Discussion
Conclusions
References



Alzheimer's disease (AD) is the most commonly diagnosed dementia and it is estimated to achieves 81 million of cases in 2040 due to increased life expectancy (Ferri et al. 2005). This represent a major public health problem and economic burden (Blennow et al. 2006).

The most known **hallmarks of AD** such as extracellular Aβ deposits and intracellular neurofibrillary tangles, are accompanied by oxidative stress and inflammation (Kim JH, 2012,Glass et al, 2010, Marchesi et al, 2011).

Metallothionein family confers survival advantage in brain damage and neurodegenerative diseases. The phenotypes of transgenic mouse models of AD, such as Tg2576, have been reported to be modulated by antioxidant proteins (Iadecola et al. 1999) and pharmacotherapies based in metal chelators (Schäfer et al. 2007), despite some conflicting results.

These evidences lead our group to investigate *in vivo* whether the MT family would be capable to modulate the phenotype of Tg2576 mice. **In this thesis, I have studied the role of Metallothionein 1 by crossing Tg2576 mice with mice carrying 56 copies of the minimally mutated *Mt1*** (Palmiter et al. 1993). Several aspects were evaluated in order to characterise the phenotype.

Concerning survival of our mouse model, the results of the present study indicate an altered distribution of the Mendelian inheritance in the four genotypes studied: WT and APPWT; TgMT and APPTgMT. Mice carrying the hAβPP transgene were born and/or survived at weaning in less proportion than APP^- mice. In addition, they showed a dramatic increased mortality after weaning, and at the end of the experiment, at 60 weeks, males and females had ~75-80% of mortality rate in line with survival rates obtained in our previous studies in APP*Mt1&2* KO and consistent with studies of transgenic mice expressing hAβPP transgene with (Carlson et al. 1997; Moechars et al. 1999; Bayer et al. 2003; Leissring et al. 2003; Phinney et al. 2003; El Khoury et al. 2007; Schäfer et al. 2007; Freude et al. 2009) or without AD-related mutations (Carlson et al. 1997; Moechars et al. 1999; Borg and Chereul 2008), or double Tg mice combining hAβPP expression with mutations in other AD-related genes (Etcheberrigaray et al. 2004; Halford and Russell 2009), which have been widely reported to present premature mortality and reduced life span compared to control mice. Interestingly, mice lacking individual or combination of AβPP family member's genes (APP, APLP1 or APLP2) also showed alterations in the Mendelian inheritance and revealed postnatally mortality within the first weeks of birth. These studies have heightened the importance of these proteins for embryogenic and postnatal development, normal growth,

strength/balance and fertility, and evidenced that basal expression of either APP or APLP2 is sufficient to compensate for the loss of the other protein. (Zheng et al. 1995; Von Koch et al. 1997; Heber et al. 2000).

Various parameters have been identified to influence premature death and clinical abnormalities in Tg mice expressing AβPP. Firstly, the levels of APP expression are closely related with death rates being higher levels of expression of the transgene more deleterious. Moreover, the primary structure of AβPP transgene (different amino acid sequence, for instance) might alter the phenotype in different age-dependant penetrance of neophobia and death. Finally, the influence of the host on the Tg phenotype have been demonstrated to play an important role in premature death (Hsiao et al. 1995; Carlson et al. 1997). An increasing percentage of alleles from a determinate background can result in either beneficial (SJL/J and 129S6) or detrimental (C57Bl/6J and FVB/N) effects for life expectancy (Carlson et al. 1997; Krezowski et al. 2004). Some authors postulate that different survival might reflect the host response to APP or its derivate rather than genetic differences in APP processing (Carlson et al. 1997). However, the existence of long-term survivors in some Tg lines overexpressing APP implies that other, as yet unknown factors besides the factors described above, are influencing life span of Tg mice (Hsiao et al. 1995).

Moreover, increased levels of mutated AβPP transgene, and alterations on its processing, might lead to higher levels of the Aβ peptide. Studies in mice expressing Aβ intracellularly, under neuronal-specific promoter, demonstrated that Aβ *per se* is enough to produce important changes within the brains of transgenic mice, starting with neurodegeneration and apoptosis, succeeded by the activation of events such as astrogliosis and ultimately ending with spongiosis. Finally, accompanying the extensive cell death appear other clinical features such as seizures and premature death (LaFerla et al. 1995). Thus, factors such as increased Aβ clearance have been shown to reduce premature death in APP mice with transgenic synthesis of Aβ degrading enzymes (IDE or NEP) (Leissring et al. 2003), or by deletion of Ccr2, a chemokine receptor whose deficiency impairs microglia recruitment favouring an earlier Aβ accumulation particularly in and around blood vessels, which might develops cerebral amyloid angiopathy, a putative risk factor for cerebral haemorrhage and ischemic brain infarction that may lead Tg2576 mice to premature death (J 2001; El Khoury et al. 2007).

In our case, survival was monitored from weaning (21-25 days after birth), so we do not know how much of altered Mendelian inheritance proportion is due to intrauterine mortality or to early postnatal mortality after weaning.

Nevertheless, since AβPP is implicated in differentiation and morphogenesis of the developing nervous system in embryonic stages (Salbaum and Ruddle 1994) and the mutant transgenes of AβPP have been reported to influence the phenotype (Hsiao et al. 1995), we could consider that AβPP is affecting fetal development and therefore, intrauterine mortality is a probable phenomenon occurring in AβPP+ mice. In adults, accumulation of AβPP and Aβ peptides had a detrimental effect in survival.

Fortunately, the transgenic overexpression of *Mt1* had a beneficial impact in perinatal survival since TgMT+ mice were born and/or survived to weaning more than expected ratio but did not show this effect in adult survival, when the overexpression of *Mt1* did not have any effect in female survival and even increased the mortality in AβPP+ and AβPP$^-$ males.

It is well known that MT-1/2 have a role in early stages of embryonic development since gene expression of these isoforms is encountered in many tissues of reproductive tract such as deciduum, placenta, visceral yolk sac and the fetus (Andrews et al. 1991; Andrews and Geiser 1999) I (Webb M, 1987: MT in regeneration, reproduction and development). Studies of the effects of dietary zinc deficiency during pregnancy indicate that mice overexpressing *Mt1* accumulate more zinc in maternal organs providing a local reservoir of zinc to the embryo and thus making it more resistant to the teratogenic effects of zinc deficiency (Dalton 1996, Andrews, 1999), in contrast to mice lacking MT-1/2. These studies propose that one of the physiological functions of MTs is to protect the embryo from stress of zinc deficiency during pregnancy, providing reproductive advantages (Andrews and Geiser 1999). Also, studies of embryonic toxicity to metals such as Zn and Cd, suggest that embryotoxicity is dependent on the stage of the development of the embryo and the metal type exposure. The metal ion responsiveness of the MT genes during preimplantation embryogenesis correlate positively with the resistance to Zn toxicity but inversely with resistance of the embryo to the embryotoxic effects of Cd. Thus, Zn and Cd toxicities involves different mechanisms in the preimplantation mouse embryos possibly because Zn is an essential metal whereas Cd has no known biological role. These studies open an array of possibilities for examining the effects of a variety of other agents such as cytokines, glucocorticoids, X-irradiation that can upregulate MT gene expression affecting embryonic development (Andrews et al. 1991). In this point we might speculate with the idea that premature changes in the preimplantational stages are occurring in AβPP+ mice due to the existence of the transgene and the overexpression of *Mt1* may confer survival advantage to the embryo and its development.

However, we did not see this effect in adult mice when the overexpression of *Mt1* in general had a detrimental effect in both AβPP+ and AβPP$^-$ male mice, contrary to what we expected of the survival role of MTs (Egli et al. 2006; Yang et al. 2006; Zeitoun-Ghandour et al. 2011) and in line with our previous results, that showed a rescue of the hAβPP associated mortality in deficiency of MT1&2 isoforms (Manso et al. 2012a).

Studies in transgenic mice combining APP and overexpressing superoxide dismutase-1 (SOD1), reduced Aβ peptides species (40 and 42) protecting against vascular dysfunction and premature death (Iadecola et al. 1999). In contrast, FGF-2, the most abundant member in the SNC of the family of fibroblast growth factors, which is involved in neurogenesis, axonal growth, differentiation in development, among other functions, exhibited premature lethal effects in a double transgenic mice overexpressing AβPP and FGF-2 (Carlson et al. 1997; Zechel et al. 2010). Thus, we have two cases in which molecules with the same role in survival, after brain injury, react in different way within the same situation.

We cannot obviate the different survival pattern among males and females in AβPP$^-$ mice. The former showed a rate mortality of 20% while only a few deaths were registered in female mice in 60 weeks (WT and TgMT), suggesting that life expectancy is influenced by gender-dependant factors. Whereas sex differences in lifespan in mice vary among strains, laboratories, and environmental factors (Rae and Brown 2015), the presence of female hormones such as estrogens have a beneficial effects in lifespan in ALS mice model (Choi et al. 2008). Despite we find this effect in AβPP$^-$, consistent with the literature, we do not find the same trait in AβPP+ mice, suggesting that the effect of overexpression of AβPP/ Aβ is more powerful than the pro-survival effect in WT females.

In line with survival, the presence of hAPP transgene also had a detrimental effect on body weight; AβPP+ mice show significantly lower weight than AβPP$^-$ mice. The overexpression of *Mt1* did not affect this phenotype, however, it decreased body weight in both genders of WT mice.

Body weight is a parameter widely used in animal research. Weight abnormalities might reflect a problem in the healthiness state of the animal, which should be evaluated to guarantee its welfare. In our experiment weight is used as parameter to characterize the phenotype. Besides, it is also important because weight loss is a prominent early feature in AD patients that often precedes the cognitive decline and clinical diagnosis (Ishii et al. 2014) and correlates with disease severity and increased mortality (White et

al. 1998; Ishii et al. 2014). Although dementia causes alterations in feeding behaviour and this could, in part, explain weight loss, weight loss represents an intrinsic feature of AD that arises early in the disease process due to a problem in the metabolism. Recent studies indicate that, transgenic mice, such as Tg2576, have hypothalamic leptin signalling dysfunction leading to early body weight deficits, decreased adiposity, low plasma leptin levels and increased energy expenditure without alterations in feeding behaviour (Ishii et al. 2014). These studies suggest that low leptin state is derived from excess Aβ causing dysfunction in arcuate NPY neurons as demonstrated by abnormal transcriptional responses to the low plasma leptin levels in basal and fasting conditions, as well as in abnormal electrophysiological responses to leptin or ghrelin. Actually, treatment with leptin in APP mice has been proposed as a novel therapeutic strategy for AD since it is capable of modulating both production of Aβ and phosphorylation of tau (Greco et al. 2010) and normalize synaptic function, food intake and body weight (Tezapsidis et al. 2009; Maioli et al. 2014). However, the weight of our APP+ mice was already lower since weaning, suggesting that low body weight in early stages might be due to factors similar to those that affected perinatal survival and less due to Aβ, which is not present yet. Later on, through weeks, the increase of soluble Aβ and amyloid burden may have an effect on plasma leptin levels, and decrease dramatically body weight. This fact, together with the already present cognitive deficits that cause alterations in feeding behaviour, would lead to a downward spiral of worsening AD pathology and further weight loss (Ishii et al. 2014).

Genes of Mt-*1/2* have been identified as leptin-induced genes since it was shown that mice with targeted disrupted Mt-1/2 genes (Michalska and Choo 1993) become obese and with elevated leptin levels (Beattie et al. 1998), especially when fed with high-fat-diet (HFD). This later experiment also showed an enhanced expression of MEST (Mesoderm-Specific Transcript) a factor that regulates enlargement of adipocytes, suggesting that MT play an important role in the negative control of MEST, and may have a preventive role against HFD-induced obesity by regulating adypocite enlargement and leptin signalling (Sato et al. 2010). Also, studies with MT1&2 KO mice support the protevting role of MTs in HFD-induced weight gain, moderate insulin resistance and metabolic alterations by protecting mitochondrial functiona and energy metabolism in the hypothalamus (Lindeque et al. 2015). However there are controversial results in MTKO mice (generated by Masters et al.,) fed with control diet which do not develop obesity (Waelput et al. 2000; Manso et al. 2012a) or showed decreased effect on body weight

in other studies of our group. Thus, the number of alleles and/or the genetic background could be relevant again.

Moreover, obesity causes ER stress leading to insulin resistance, diabetes and Oxidative stress (Ozcan et al. 2004). Zinc is involved in ER function and a deficiency of this metal represents an upregulate ER stress response. As MT has a preventive role against oxidative stress and ER stress, it may regulate development of obesity, leptin resistance, and hypercholesterolemia via prevention of oxidative stress and/or ER stress (Waelput et al. 2000).

Interestingly, some groups have reported a modulating role of MTs in mitochondrial respiration (Ye, 2001) and an interaction between ATP and MT, suggesting a role of MT in the regulation of energy balance (Maret et al. 2002).

Taking all this information together what we could see in our growth curves, is that the overexpression of Mt1, despite do not have effects in APP+ mice possibly because of the gross amyloid burden, might have a preventing role against oxidative stress and obesity associated to elderly, decreasing and maintaining the body weight stable and healthier in WT mice.

Different aspects of **mouse behaviour** such as general exploratory activity, anxiety, sensorimotor abilities (balance, reflexes and strength) and memory and learning have been studied in order to characterise the phenotype in young mice, before plaques deposits, and in old mice when plaques are present.

AD mouse models including Tg2576 have been reported to have impaired some aspects of the normal behaviour despite some inconsistences in the literature have been published. In general, mice bearing hAβPP show a less anxious and hyperactivity behaviour combined with impaired learning and memory.

Deambulations, exploratory behaviour and anxiety were evaluated with the hole board (HB) and the plus maze (PM) tests. Regarding exploratory activity and according to the literature, young AβPP+ female (but not male) exhibited a great exploratory activity since they increased the number of head dipping (HD) and spent more time doing HD than APP- mice, in the Hole Board paradigm (HB). This pattern was not consolidate in old mice in contrast with previous studies, which showed increased exploration in old APP+ mice in both sexes, heightening the importance of the genetic background in behaviour studies. Some strain differences, for example in locomotor activity, have been found to be age and gender-dependent

(Homanics et al. 1999; File 2001). In general, sex differences in these paradigms show that females' behaviour is mainly driven by activity rather than anxiety, whereas male behaviour is more characterised by anxiety (fernandes c I roser nadal?, buscar) (File 2001; Swerdlow 2007). *Mt1* overexpression partially reverse the phenotype decreasing the number and time of HD in young females and the same tendency was found in males and old mice of both sexes, in line with results observed following the administration of Zn_7MT-2A. Thus, these results indicate that endogenous and exogenous MT-1/2 isoforms are using similar mechanisms of action in exploratory activity. However, no effects were found in mice lacking MT-1/2 isoforms.

Mice carrying hAβPP also showed a less anxious behaviour in young and old mice of both sexes in plus maze paradigm, where number of visits and time spent in open arms were bigger than in APP- mice. Although hAβPP increased ambulations in HB, this decreased anxiety behaviour is not the consequence of altered ambulatory rates since the entries in the closed arms were not increased rather the opposite. *Mt-1* overexpression did not rescue the hAβPP-induced decreased anxiety in young mice, and in fact exacerbated it in old mice. Neither the administration of Zn_7MT2A nor MT-1/2 deficiency had consistent effects on anxiety, indicating that MT isoforms are not critical in this regard.

Since the motor condition of Tg2576 mice has been described to be impaired, a battery of sensorimotor tests was performed, such as the horizontal flat rod, the horizontal round rod and the coat hanger. Although no significant results were obtained in flat rod, the cylindrical rod (most difficult that flat rod) which assess balance, showed a decreased latency to fall in hAβPP mice of both sexes and ages, with the effect more marked in young than in old mice, maybe because of the deterioration of the latter. *Mt1* overexpression did not have any in effect in this context.

The Morris Water Maze (MWM) has become an important, even the most used, method to assess spatial learning and reference memory (Vorhees and Williams 2006). When first characterised by Hsiao et al, the Tg2576 was shown to be impaired in the Y-maze spontaneous alternation test at 3 and 10 months of age and to display a progressive decline in the MWM acquisition by 9-10 months (Hsiao et al. 1996). Following studies have both supported and contradicted these findings and whether the spatial reference and memory in MWM are intact until 6 months of age, coinciding with the appearance of detergent-insoluble Aβ aggregates or with soluble amyloid-b

assemblies (Aβ*56) or it is not impaired until 19 months of age (Lesné et al. 2006) is still under investigation. In any case, these discrepancies about cognition could be related on differences in the test protocol or genetic background of the Tg which have been described to influence MWM and behavioural performance in general (falten ref)(Lesné et al. 2006). In our previous studies with young APPM*t1&2* KO we confirmed that hAβPP significantly do induced impairment in spatial memory tasks in females and so Aβ is interfering yet in this ages. Unfortunately when the test was realized in old APPmt3ko mice we do not obtain satisfactory, since the advanced age dilute the effect of hAβPP-induced learning impairment and might mask the putative role of the deficiency of MT-3 isoform. Taking all this into account, in the present study, we performed MWM in young mice to evaluate the role of *Mt1* on hAβPP-induced learning alterations. APPWT female mice displayed impaired spatial learning process since they needed more time to find the submerged platform than WT. *Mt1* overexpression rescued this phenotype matching to learning levels of WT females, suggesting that MT-1/2 isoforms could be involved in the mechanisms underlying this type of learning. However deficiency of MT-1/2 did not worsen the phenotype of Tg2576 against what we support. This could be due differences in the genetic background features, as when MT-1&2 KO mice (in 129Sv background) were backcrossed with C57Bl/6J mice, they had baseline impairments ins spatial learning in the MWM (McAuliffe et al. 2008), which are in line with the present results obtained in Tg2576 mice crossed twice with B6 mice. Furthermore, the rest of the tests of the MWM, such as probe trial and the learning of a new location of the platform were essentially normal regardless of the genotype.

Learning was also assessed in other paradigms such as operant conditioning test (Skiner) and classical conditioning test (eye-blink). In the first case young females learn to get food pellets by pressing a lever. Along the sessions all the genotypes increased the percentages of lever presses and especially APPTgMT females showed the highest percentage of lever presses in the last two days of the conditioning. Although the results did not achieved significant differences when analysed together within the 4 genotypes, a significant *Mt1* overexpression x day interaction is obtained when compared APPWT and APPTgMT groups. In the eyeblink test studied in old female mice learning was severely impaired in APP+ mice as is observed with the increase of the CRs, consistent with other studies (Kishimoto et al. 2012). *Mt1* overexpression partly rescued this phenotype as well as in WT mice, indicating that this is a general benefit of MT-1 rather than something related specifically to the Tg2576 phenotype.

Altogether, the data from behaviour analysis suggests that the overexpression of Mt1 do have an important impact reverting the exploratory activity and slightly decreasing the anxiety associated to hAβPP phenotype of Tg2576. Also, *Mt1* it has been shown to be implicated in spatial memory and some learning tasks, albeit we are aware that behaviour is susceptible to gender, age and genetic background differences.

In Tg2576 mice soluble Aβ peptides are present through life and at 6 months of age, insoluble forms start appearing. By 9-10 months of age, amyloid plaques are prominent.

In order to **assess the amyloidosis** we use on the one hand western blotting techniques in young and old mice to check the amyloid cascade, and on the other hand immunohistochemistry to quantify the amyloid burden in old mice. As expected, amyloid plaques in AβPP+ old mice were prominent and widely distributed throughout the hippocampus and cortex, areas most affected by cerebral amyloidosis in Tg2576 mice. Mt-1 overexpression increased slightly but significantly the amyloid plaque burden in the hippocampus, but not in cortex, in both sexes. These results are in line with the tendency observed in old mice injected chronically with Zn_7MT2-A and with APPMTKO mice which showed a significantly decreased amyloid load (Manso et al. 2011, 2012a). These findings evidenced that Mt-1/2 isoforms *in vivo* boost the amyloid deposits especially in the hippocampus which is robustly affected by plaques. Despite expecting *Mt1* to decrease the amyloid burden, this fact do not necessary means that Mt1 is deleterious, as the formation of amyloid plaques could represent a protective process against oligomeric Aβ species (Lee, 2005), which have been reported to play a central role in neurotoxicity and correlate better with disease severity instead of amyloid plaques burden (Minati, 2009 and Adlard 2014). Perhaps, there may be several reasons holding this effect of MT-1/2 on amyloid burden such as changes in the expression of hAβPP, in its processing, in glial activity, the type of metal interacting with Aβ, among others.

We assessed the expression of the full length hAβPP and its proteolytic fragments by western blot. In old mice, hAβPP levels were significantly increased in hippocampus and cortex of APPTgMT mice compared to APPWT mice in both sexes. Moreover, hAβPP levels were also increased by *Mt1* overexpression in the hippocampus of young male (but not female) mice. Higher levels of hAβPP means more substrate for α- and β-secretase proteolytic cleavage and may thereby increase the levels of the different proteolytic fragments derived from its sequential processing such as CTF-β

and Aβ (mainly $A\beta_{1\text{-}40}$), and all together, contribute to the higher amyloid load observed in APPTgMT mice. Despite higher hAβPP levels and hAβPP processing in APPTgMT mice, in cortex and hippocampus, increased amyloid burden was only seen in hippocampus. Interestingly, previous studies with *Mt1&2* KO mice showed no differences in young mice but a higher hAβPP levels in the hippocampus (but not the cortex) of old females, even with showing less amyloid plaques, suggesting that the proteolytic process from the protein precursor to amyloid plaques formation is subjected to other factors and that therefore, more hAβPP is not always mandatory to have more amyloid deposits.

Furthermore, γ-secretase will cleavage CTF-β and will produce Aβ peptides which can oligomerise or eventually precipitate to form plaques. In the cortex, *Mt1* overexpression did not influence SDS-soluble Aβ monomeric and trimeric peptides, in line with the results obtained in M*t-1&2* KO mice, except for a small increase in trimeric form in males (). In contrast, in the hippocampus, *Mt1* overexpression had opposed effects regarding Aβ monomers, increasing them in males and decreasing them in females. Importantly, this is consistent with the phenotype observed in APPM*t-1&2* KO mice. When Aβ was analysed by ELISA in the hippocampus of old mice, *Mt1* overexpression significantly decreased the $A\beta_{1\text{-}40}$ and exhibit the same tendency in $A\beta_{1\text{-}42}$. Although the ratio $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ is a little more favourable to amyloidogenic pathway than non-amyloidogenic, total levels of both $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ were significantly lower in APPTgMT in comparison to APPWT mice. Also, trimers were decreased in APPTgMT female hippocampus but increased in males and with no effects in CTX. Trimers have been proposed as the fundamental Aβ assembly unit *in vivo* and its great resistance to denaturation support this idea (Lesné et al. 2006). Studies of Aβ-mediated inhibition of LTP describe trimers as the main toxic specie of Aβ (Walsh et al. 2002; Selkoe 2009). Although overall Aβ levels have been reported to affect accumulation of pathogenic oligomers, there are studies that suggest that formation of oligomers and formation of Aβ fibrils may follow distinct pathways *in vivo* (Haass and Selkoe 2007; Necula et al. 2007; Meilandt et al. 2009; Di Carlo 2010). In fact, in a hippocampal dependent test such as MWM, APPTgMT females, which showed decreased levels of soluble oligomers and monomers of Aβ in the hippocampus, performed the test better than APPWT females, evidencing on the one hand the correlation between increased levels of Aβ oligomers and the degree impairment of APP+ mice (Selkoe 2009) and on the other hand the effect of Mt1 overexpression in reducing the neurotoxicity of soluble oligomers in the hippocampus.

Recent studies (Siddiq et al. 2015) have demonstrated an inhibitory effect of MT-1/2 isoforms on the *in vitro* activity of one of the α-secretase (ADAM17/TACE) of the non-amyloidogenic pathway. This effect presumably would favour the amyloidogenic pathway increasing the levels of AB peptides. Interestingly, MT-3 isoform has been reported to increase the activity of another α-secretase such as ADAM10, in the mouse neuroblastoma Neuro2A (Park et al. 2014) Swedish APP cells, suggestive of MT isoform-specific roles.

Also, Zn_7MT-2A *in vitro* is capable to decrease Aβ neurotoxicity of cultured cortical neurons presumably due to a metal swap between Zn_7MT-2A and Cu(II)-Aβ (Chung et al. 2010) preventing the toxicity from Cu mediated aggregation of $A\beta_{40}$ and $A\beta_{42}$.

As mentioned in the introduction, MTs and specially MT-1 and MT-2 isoforms have been consistently reported to be increased in AD brains (Duguid et al. 1989; Adlard et al. 1998; Zambenedetti et al. 1998; Carrasco et al. 2006; Hidalgo et al. 2006) and in Tg AD models, showing in the latter a prominent up-regulation in the vicinity of amyloid plaques, one of the major hallmarks in AD (Carrasco et al. 2006; Hidalgo et al. 2006), suggesting a role of these proteins in amyloidogenic pathway.

Assessing the levels of *Mt1* in our transgenic overexpressing model, we found that *Mt1* levels quantified by *in situ* hybridisation were significantly increased in all brain areas of TgMT mice studied but were not significantly increased by hAβPP expression, despite Mt1 signal was increased in plaque areas compared to areas without plaques in APPWT and APPTgMT, as is reported in previous studies (). In line with these results, the immunostaining of MT-1/2 showed an increased levels of these isoforms in TgMT mice regardless of APP expression. When comparing WT and APPWT mice, MT-1/2 immunostaining significantly increase in the latter in male hippocampus and the same pattern was found in females and in the cortex, apparently because of the amyloid plaques and the associated neuroinflammation, although it is difficult to find prominent effects due to the high basal expression of MT-1/2. In contrast, when comparing TgMT and APPTgMT mice, MT-1/2 isoforms tended to decrease in the latter in both cortex and hippocampus, when what we expected was more staining of MT-1/2 in APPTgMT coincident with prominent amyloidosis. This effect might be related to differences between the regulation of the endogenous MT-1/2 genes and the transgenic gene. The transgenic gene of *Mt1* is minimally mutated and expressed about 50% less on per basis.

It shows a normal tissue distribution and responds normally to inducers such as heavy metals, dexamethasone, and lipopolysaccharide (LPS), which essentially suggest that the transgene is regulated in a similar fashion to endogenous Mt1 gene (Palmiter et al. 1993).

Interestingly, a caudal-frontal gradient in MT-1/2 IHC was observed in the cortex that could be associate to the prominent expression of MT-1/2 in astrocytes, since these cells tend to show that pattern of gradient expressing GFAP as we observed in our assays (data not shown) and has been reported previously (Campbell et al. 1993; Chiang et al. 1994) (l ultima entrar manual).

Inflammation is an early phenomenon closely related to AD pathogenesis and particularly to amyloid plaques where the presence of activated microglia and astrogliosis have been reported (Agostinho et al., 2010; Glass et al., 2010; Lee and Landreth, 2010). MT-1/2 proteins have been shown to affect gliosis in different contexts of brain injury and stress (Penkowa et al. 1999; Molinero et al. 2003; Chung et al. 2004; Fitzgerald et al. 2007). In the present study, immunohistochemistry of GFAP showed an elevated level of reactive astrocytes surrounding the amyloid plaques compared to areas devoid of plaques. Nevertheless, and in line with previous studies using M*t1&2* KO mice, *Mt1* overexpression did not significantly affect GFAP levels in either the hippocampus or cortex. However, increased astrogliosis has been found after chronic injection of Zn_7MT-2A to old Tg2576 females concomitantly with increased amyloid burden, concluding that on the one hand the enhanced inflammatory response is not beneficial once the plaque pathology is stablished, and on the other hand the presumably different mechanisms of action between exogenous and endogenous MT-1/2 proteins. Similarly, microgliosis, assessed with Iba-1 immunostaining, was detected mainly around amyloid plaques, particularly with dense cored ones (Apelt and Schliebs 2001) with little signal in areas without plaques. *Mt1* overexpression, however, did not affect microgliosis in cortex and hippocampus. In contrast, with western blotting techniques using a more representative sample of the entire hippocampus of one hemisphere, APPTgMT male mice showed increased Iba-1 levels; the same trend was observed in female mice and it is consistent with the western blot results found in M*t1&2* KO mice. In young mice, since amyloid deposits are not yet present, Mt1 overexpression significantly decreased both microglia and astroglia in the hippocampus and the same tendency was found in the cortex. Remarkably this was occurring again only in male mice. Satisfactory, the results are generally consistent with those found in MT1&2 KO mice.

Therefore, *Mt1* overexpression in old mice might control the amyloid plaque deposition but the effect of the amyloidosis is more potent than the effect of Mt1 in gliosis, whereas in young males MT-1/2 attenuated the Aβ-stimulated inflammatory response, consistent with previous reports (Kim et al. 2012; Manso et al. 2012a).

The inflammatory response characterised by the activation of microglia and astroglia, including production of inflammatory mediators as proinflammatory cytokines and TNF-a has a consequent increase in oxygen free radicals and ROS production followed by oxidation and/or nitration of lipids, proteins, DNA, etc. (Inoue et al. 2009), and further causing cellular death (Vasto et al. 2008). Oxidative stress induces the release of zinc from MTs via NO, promoting the expression of antioxidant enzymes, including MT-1/2 itself and reducing the oxidative damage. Zinc *per se* may be a strong inducer of oxidative stress by promoting mitochondrial and extra-mitochondrial production of ROS and its concentration is influenced by proinflammatory cytokines and by MTs homeostasis, which are in turn affected by proinflammatory cytokines (Vasto et al. 2008). Altogether, the neuroprotective properties of MT-1/2, result from the combination of its scavenger of ROS action and zinc binding proteins (Santos et al. 2012).

Moreover, several studies suggest that oxidant agents and oxidation products increase APP synthesis and intracellular and secreted Aβ (Yan 1995; Frederikse et al. 1996; Cheng and Trombetta 2004; Shineman et al. 2008) and it has been proposed that increased AβPP mRNA stability is underlying these increased AβPP and Aβ levels (Shineman et al. 2008). Despite some studies have proposed an antioxidant role of both $A\beta_{1\text{-}40}$ and to a lesser extent $A\beta_{1\text{-}42}$ consistent with oxidative stress inducing APP and its proteolytic fragments (Andorn 2000). In the Tg2576 mice is generally accepted that oxidative stress is present yet in early ages and mice overexpressing antioxidants enzymes such as SOD, CAT (catalase) or being administered by antioxidants treatments (curcumin, Vitamine E, etc.), diminished oxidative stress and AD-like pathology. In contrast, mice with pro-oxidant diets (with vitamin deficiency and selenium and high Fe), increase oxidative stress and exacerbate the AD-like pathology. However, the effects on amyloidosis are diverse and while some of these different models altered the amyloidosis burden without affecting the soluble or insoluble Aβ forms and vice versa, other affect both pools or none.

Although in the present study we have not directly measured oxidative stress, we would expect an attenuated Tg2576 phenotype with decreased

levels of amyloid burden in APPTgMT mice. Only in the hippocampus, females overexpressing *Mt1*, showed in general, decreased levels of the products of the proteolytic processing of the precursor. This reduction in Aβ levels, together with the less anxiety phenotype, which has been directly correlated with oxidative stress (lower antioxidants→ less anxiety), suggest that before the amyloid deposits, *Mt1* is contributing to more antioxidant and less neurotoxic environment in the hippocampus.

By the way, since MTs are Zn and Cu-binding proteins, and these metals have been widely reported to participate in amyloid-β peptide aggregation (Bush, 1995, manual) and in ROS production (Barnham, 2004, manual), we analysed the metal composition of the cortex and hippocampus by ICP-MS technique. AD brains exhibit abnormalities in metal homeostasis, such as increased Fe, Zn and Mn levels (Maynard et al. 2002), and decreased Cu levels and Cu-dependent enzymes. Studies in transgenic mouse models of AD suggest that Cu deficiency may be a direct consequence of Aβ/AβPP overproduction and may further facilitate ab accumulation and amyloid formation. Also, aging, main risk factor for AD, displays consistent changes in the metabolism of Fe and Cu (Maynard et al. 2002).

In the present study, **aging had different effects on metals**, depending on the area. In the cortex, aging increased slightly Zn levels and had more robust effect in Cu levels. This effect was partially mitigated in APP+ mice, which is consistent with previous studies (Maynard et al. 2002; Manso et al. 2012a). Conversely, in the hippocampus, aging increased Cu levels but decreased Zn levels, while both metals were increased by AβPP expression. Interestingly, copper and iron levels increased with aging, have been proposed as a mechanism to explain the age-dependent onset of amyloid neuropathology in Tg2576 mice, and more considering hippocampal neuropathology, where APP+ mice have even higher levels of Cu with aging. Experiments with APP+ mice show a beneficial effect of increased Cu bioavailability on life span, either by supplementing it with the diet (Bayer et al. 2003; Schäfer et al. 2007) or by using transgenic mice (Phinney et al. 2003). Of notice, increased Cu bioavaility may be linked to decreased Aβ trimers (Crouch et al. 2009). Nevertheless the overexpression of *Mt1* had minor effects on metal analysis and the more remarkable changes are those associate to the phenotype APP+.

It should be noted that the analysis of metals by ICP-MS has technique limitations. The measures of Zn and Cu are referred to the total content and thus we cannot rule out specific effects of *Mt1* on free metal ion levels and/or bound metal levels.

In old APP+ mice, we observed a neuronal loss in the CA1 of hippocampal area with a clear thinning of the pyramidal layer, as revealed by Nissl staining. Neuron loss is a known hallmark in transgenic mice carrying the Swedish mutation (Calhoun et al. 1998) and selective neuron loss in CA1 area of the hippocampus has been demonstrated in AD patients (West et al. 1994). However, *Mt1* overexpression did not significantly influence neuronal survival (Penkowa et al. 2005; Asmussen et al. 2009; Eidizadeh et al. 2015). Whether or not this is related to different neuronal susceptibilities, to *Mt1* levels, to the specific experimental model causing neurodegeneration or simply because the *Mt1*-induced plaque load which are disrupting the pyramidal layer, remains to be established.

Conclusions

1. The phenotype of the AD mouse model bred in our laboratory is consistent with that described in the literature, despite some variability. Our model showed **elevated premature mortality more pronounced in males than in females**; **lower body weight** initiated at early stages and following throughout the experiment; **Aβ accumulation with concomitant inflammation** and **changes in metal content** and **hippocampal neuronal loss**. Behaviour analysis, which exist more conflicting data in the literature, showed **hyperactivity** and **decreased anxiety**, **impairment in motor coordination in young mice** and **no important differences in strength**. **Spatial memory and learning was deficient in some tasks** of different paradigms.

Regarding *Mt1* overexpression:

2. **significantly prevented from hAβPP-induced mortality in both sexes at perinatal stages.** However, did not affect the survival in adult mice, even increasing the mortality rates in male mice in APP- and APP+.
3. **in line with survival, did not have any effect in the hAβPP-related lower body weight in both sexes** and slightly reduced the weight in WT mice.
4. **modulated some behavioural characteristic traits of the Tg2576 mice,** reverting the hyperactivity bu do not recued the APP-induced decreased anxiety in young mice and exacerbate in old males.
5. **spatial memory and learning were modulated in young female** which reverted the inability of de Tg2576 to switch to adequate searching strategy in the hidden platform of the MWM and have slightly effect in other learning tests.
6. **favoured the amyloid deposition in the hippocampus of old mice and in general increased the levels of the proteolytic processing of hAβPP, included.** In the hippocampus of females, decreased the Aβ oligomers.
7. **MT-1/2 immunostaining was significantly increased in TgMT and APPTgMT mice, but tended to decrease in the latter** despite the amyloidosis and inflammation, which suggest differences in regulation between endogenous and transgenic Mt1.

8 **astrogliosis and microgliosis were decreased in young males, before the deposit of amyloid plaques.** Once plaques were present, Mt1 **might have a direct role inhibiting microglia response** while having an **indirect stimulatory role in the formation of deposits** in AβPP+ mice.

9 **did not show any effect in the hAβPP associated hippocampal neuronal loss.**

10 **metals such as Zinc and Copper were poorly affected**, maybe masked by AβPP phenotype and aging.

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