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The role of high-fat diet in an APP/PS1 model of familial Alzheimer disease

Efecto de una dieta rica en grasas en ratones APP/PS1, modelo familiar de la enfermedad de Alzheimer

Dmitry Petrov

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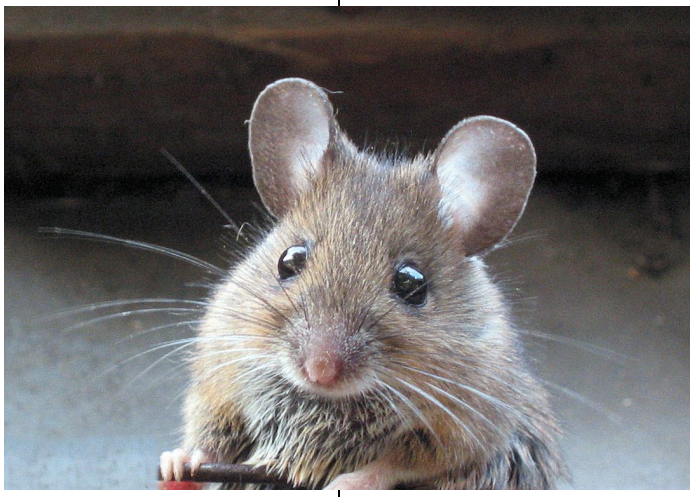

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Tesi Doctoral

THE ROLE OF HIGH-FAT DIET IN AN APP/PS1 MODEL OF FAMILIAL ALZHEIMER DISEASE

Dmitry Petrov 2015

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Memoria presentada por el licenciado en bioquímica y farmacología

DMITRY PETROV

para optar al grado de Doctor por la Universidad de Barcelona

Esta tesis ha sido inscrita dentro del programa de doctorado en investigación, desarrollo y control de medicamentos, trienio 2012-2015 de la Universidad de Barcelona. El trabajo experimental y la redacción de la presente memoria han sido realizados bajo la dirección del Dr. Antoni Camins, Catedrático de Farmacia del Departamento de Farmacia de la Universidad de Barcelona y del Dr. Jaume Folch, Profesor Titular de la Universidad Rovira i Virigili.

Barcelona, 2015

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To my loving and caring family

"How odd I can have all this inside me and to you it's just words."

David Foster Wallace, The Pale King

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INDEX

ABBREVIATIONS

3-APS	3-amino-1-propaneosulfonic acid
8-HQ	8-hydroxiquinoline
Acetyl-Coa	Acetyl coenzyme A
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
AD	Alzheimer disease
ADAS-cog	Alzheimer's Disease Assessment Scale-cognitive subscale
ADPR	Alzheimer Disease Patient Registry study
ADR	Adverse drug reaction
AGE	Advanced glycation endproducts
AKT	protein kinase B
AOE	Antioxidant enzymes
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APP/PS1	transgenic mice (a model of FAD)
APPswe/PS1dE9	transgenic mice (a model of FAD)
ARC	Activity-regulated cytoskeleton-associated protein
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BBB	endothelial blood-brain barrier
C57/Bl6	control (wild-type mice)
Ca	Calcium
CAT	choline acetyltransferase
CDK5	Cyclin-dependent kinase 5

c-Kit	proto-oncogene c-Kit; CD117
CNS	Central nervous system
COX	Cytochrome C oxidase; Complex IV
CSF	Cerebrospinal fluid
cybrid	cytoplasmic hybrid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ETC	mitochondrial electron transport chain
EU	European Union
FAD	familial Alzheimer disease
FDA	Food and Drud Administration
FOS	FBJ Murine Osteosarcoma Viral Oncogene Homolog
Fyn	Proto-oncogene tyrosine-protein kinase Fyn
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GI	Gastrointestinal
Glu	Glutamate
GSK3β	Glycogen synthase kinase 3 beta
GSK-3β	glycogen synthase kinase-3β
HF	High-fat
HFD	High-fat diet
ID	Identification number
IDE	Insulin-degrading enzyme
IFG	Impaired fasting glucose
IGF1	Insulin-like growth factor 1
IGF1-R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
IGF2-R	Insulin-like growth factor 2 receptor

IgG	Immunoglobulin G
INS	Insulin
INS-R	Insulin receptor
IPGTT	Intraperitoneal glucose tolerance test
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
JNK	c-Jun N-terminal kinase
LRP-1	Low-density lipoprotein receptor-related protein
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MAPT	microtubule-associated protein tau gene
MC	Mast cell
mGluR5	metabotropic glutamate receptor 5
MOA	Mechanism of action
mRNA	messenger ribonucleic acid
MS	Multiple sclerosis
mt	mitochondria
MTC	Methylthioninium chloride
NMDA	N-methyl D-aspartate
NMDAR	N-methyl D-aspartate receptor
NMDARi	NMDAR inhibitor
NOR	Novel object recognition
NR2B	The N-methyl-D-aspartate receptor subunit NR2B
NRF	Nuclear respiratory factor
NSAID	Nonsteroidal anti-inflammatory drug
OXPHOS	Oxidative phosphorylation
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PHF	paired helical filaments

PI3K	IRS-associated phosphatidylinositol 3–kinase
PKB	protein kinase B (AKT)
PP2A	Protein phosphatase 2
PPAR-g	Peroxisome proliferator-activated receptor g
PrPC	cell-surface prion glycoprotein C
PS1	Presenilin 1
PS2	Presenilin 2
PSD	Postsynaptic density
PUFA	Polyunsaturated fatty acids
RAGE	Receptor for advanced glycation endproducts
ROS	Reactive oxygen species
RXR	retinoid X receptor
SCF	Stem cell factor
SGSM	Soluble g-secretase modulator
STZ	Streptozotocine
SYP	Synaptophysin
T2DM	Type 2 diabetes mellitus
TD	Transdermal
TG	Triglyceride
TNFα	Tumor necrosis factor α
TZD	Thiazolidinedione
UK	United Kingdom
US	United States

INTRODUCTION

1. OVERVIEW

Global obesity is a pandemic status, estimated to affect over 2 billion people, and has resulted in an enormous strain on healthcare systems worldwide. The situation is compounded by the fact that apart from the direct costs associated with overweight pathology, obesity presents itself with a number of comorbidities, including an increased risk for the development of neurodegenerative disorders. Alzheimer disease (AD), the main cause of senile dementia, is no exception. Spectacular failure of the pharmaceutical industry to come up with effective AD treatment strategies is forcing scientific community to rethink the underlying molecular mechanisms leading to cognitive decline. To this end, the emphasis is once again placed on the experimental animal models of the disease. Research described in this doctoral thesis is focused on the effects of a high-fat diet (HFD) in C57/Bl6 Wild-type (wild-type) and APPswe/PS1dE9 (APP/PS1) mice, a well-established mouse model of familial AD.

The introductory sections which follow are aimed to inform the reader of the broader scientific context to which current work applies. Section 2 describes how fundamental science has shaped our understanding of the molecular basis of AD. Key findings and the associated hypotheses are presented in chronological order. In my opinion, by structuring the introduction in this manner, the incremental nature of scientific discovery becomes more evident. This format has also allowed me to address some of the more obscure and currently discredited hypotheses, notably the “Aluminium hypothesis”. The history of fundamental research is littered with discarded and forgotten ideas, and it was important to me to highlight this frequently overlooked aspect of basic research. In Section 3, the pharmacological approaches to AD treatment are summarized. The purpose of that section is not to go into exhaustive detail concerning any particular compound or a molecule. Rather, an attempt is made to stress that a very large number of pharmaceuticals targeting diverse pathways were considered as a possible therapy and failed. It is by no means uncommon to see Phase III clinical trials with thousands of recruited patients failing to meet the primary outcomes due to lack of efficacy. The present work will not solve the problem of AD, however it does address a specific molecular aspect of the pathology. That is the role of metabolism in the relatively early stages of the disease.

2. ALZHEIMER DISEASE: A BRIEF HISTORY

Over a century has passed since Alzheimer Disease (AD) was first identified by German physician Alois Alzheimer. In his now famous 1907 report titled "Über eine eigenartige Erkrankung der Hirnrinde" (About a peculiar disease of the cerebral cortex), Dr. Alzheimer described the case of a 51-year old patient Auguste Deter (Alzheimer, 1907). What started off as strong feelings of jealousy towards her husband, had rapidly progressed towards significant memory deterioration, cognitive impairments and hallucinations. By the time of her death, 4 and a half years post-hospitalization, the patients' health deteriorated completely as she had lost all sense of her surroundings, was incontinent and confined to bed in fetal position. The autopsy revealed what are now known as the classical morphological symptoms of AD: neurotic (now known to be composed of amyloid peptide) plaques and neurofibrillary tangles (now known to be composed of Tau protein) in the cerebral cortex (**Fig.1** on page 17). A recent re-evaluation of the DNA material extracted from the histopathological slides of Auguste Deter's brain had identified a mutation in presenilin 1 (PS1) gene as responsible for disease development in this first documented case of AD (Rupp, Beyreuther, Maurer, & Kins, 2014). This section will provide a brief historical overview of the advances that have been made in the field of AD research from the beginning of the 20th century until now.

Original 1907 report by Dr. Alzheimer had identified key morphological diagnostic criteria for AD diagnosis which are still valid today: neurotic plaques and neurofibrillary tangles. By mid-1940s a significant body of work describing AD had been published in medical journals which prompted one of the first literature reviews on the disease. In 1948, R. D. Newton published a comprehensive analysis on the state of AD field in "The journal of mental science" (Newton, 1948). This paper is of interest, as it was the first one to acknowledge that AD may occur "at any age" [*sic*]. Newton presented evidence that the rate of cognitive decline occurring in AD varies between patients. The author described that the term "Alzheimer Disease" was used when the disease occurred at a relatively young age (now called early-onset AD). When it occurred during old age with rapid progression, it was called "senile dementia", and simply "senility" when the rate of cognitive decline was slow (both conditions are referred to as sporadic AD now). In order to suggest better terminology, Newton proposed to refer to AD as "Alzheimer's dementia", a term which is still frequently used in modern literature.

In the decades that followed, no significant progress was made in identifying AD etiology until late 1970s -early 1980s. By that time, a number of theories as to the origins of AD had been proposed. These implicated autoimmunity, aluminium toxicity and infection by "slow virus" [*sic*] (Deakin, 1983). Unfortunately, none of these could account for the full spectrum of confirmed AD cases. One theory that did emerge however, became the subject of intensive research effort. In 1983, the "cholinergic hypothesis" became the dominant force in AD field (J. T. Coyle, Price, & DeLong, 1983). According to this view, selective denervation of

acetylcholine-releasing neurons, whose cell bodies are localized to the basal forebrain, may occur in AD. These cholinergic neurons innervate the cortical areas and related structures, especially the regions related to cognition and memory. It was suggested that cortical amyloid plaques primarily cluster around the cholinergic terminals, thus leading to retrograde degeneration of the related neurons and cognitive decline. The importance of this hypothesis cannot be overstated and is evidenced by the number of citations Coyle, Price and DeLong paper has received (over 2900 as of September 2015). Recent evidence suggests that cholinergic hypothesis clearly has its flaws. However, very few things in science are sufficiently "polished" when first proposed. An excellent review by Antonio Contestabile on the history of the cholinergic hypothesis points towards the advances this theory led to in AD research (Contestabile, 2011).

The history of AD research from that era will not be complete without mentioning some of the claims which are still being made today based on very limited scientific proof. Aluminium exposure hypothesis is a prime example. Aluminium as a possible trigger for AD was first described in mid-1960s (Klatzo, Wisniewski, & Streicher, 1965; Terry & Pena, 1965), and the hypothesis was under investigation by mainstream science all the way until mid-1980s. Original findings described cognitive decline in rabbits exposed to aluminium salts, together with what appeared to be cerebral neurofibrillary tangles. It is worth noting that the 1965 paper was in essence an accidental discovery, as aluminium was used as an adjuvant for immunizing the rabbits for antibody production. In the years that followed, none of the studies linking aluminium to AD were independently reproducible (reviewed by (Munoz, 1998)). Thus, the hypothesis was abandoned decades ago based on the lack of scientific evidence. Yet, despite the absence of verifiable claims, the aluminium hypothesis remains popular in the general public minds (Lidsky, 2014). In fact, a small number of articles were still being accepted for publication in scientific journals as late as 2014, which seemingly support this claim (Kawahara & Kato-Negishi, 2011; Tomljenovic, 2011; Walton, 2014). While it is not a purpose of this thesis to "debunk" questionable theories of the origins of AD, I would like to cite *verbatim* the last 2 sentences of the abstract of the Walton paper and let the reader make an informed decision: "AD is a human form of chronic aluminum neurotoxicity. The causality analysis demonstrates that chronic aluminum intake causes AD". These conclusions are in stark contrast with a similar meta-analysis published in 2015: "The findings of the present meta-analysis do not support a causative role of aluminum in the pathogenesis of AD" (Virk & Eslick, 2015).

In 1985, Wong and co-authors (Wong, Quaranta, & Glenner, 1985) have determined that the amyloid beta peptide is the main component of neuritic plaques present in the brain of AD patients. Shortly after, Nukina and Ihara (Nukina & Ihara, 1986) identified Tau protein as a principal constituent of paired helical filaments (PHF) and neurofibrillary tangles (see **Fig. 1**). These findings paved the way for a number of key discoveries which occurred in the years that followed.

Early 1990s heralded a new era in AD research. 1992 is widely considered as the year when Hardy and Higgins (J. A. Hardy & Higgins, 1992) have fully solidified an "amyloid cascade" hypothesis. Despite the claims of John Hardy that he was "one of the two (inaccurately) credited with originating the amyloid hypothesis" (J. Hardy, 2009), John Hardy is still considered as one of the "Godfathers" of the idea. The amyloid cascade hypothesis states that the amyloid peptide is key to AD pathology. The hypothesis assumes that beta amyloid deposition produces senile plaques, leading to neurofibrillary tangle formation, which cause the death of neurons leading to cognitive decline. Since then, significant research efforts on the part of academic labs and the pharmaceutical industry have largely focused on the amyloid cascade as the principal target for AD treatment. Unfortunately, none of the molecules that reached Phase II and III clinical trials targeting this pathway have proven to be more effective than placebo at treating AD (reviewed by (Schneider et al., 2014)). Nevertheless, it is abundantly clear that the amyloid cascade hypothesis has significantly furthered our understanding of the underlying disease pathology.

"Vascular hypothesis", which emerged in 1993 (J. C. de la Torre & Mussivand, 1993), proposed that abnormalities in brain microvasculature, occurring as a result of aging, may contribute to AD. It was suggested that beta amyloid may cause accelerated degeneration of brain capillaries. Disturbances in the cerebral blood flow that follow, are likely to result in reduced delivery of both the glucose and oxygen to cerebral neurons. Energy deprivation and the resultant ischemia produces tissue damage which causes inflammation and may eventually result in neurofibrillary tangle formation. Some elements of this hypothesis parallel the changes described by "oxidative stress", "mitochondrial cascade" and "neuroinflammation" hypotheses (see pages 19, 20 and 21).

Another major breakthrough which occurred during the late 1980s and early 1990s was the identification of genetic components which contribute to the disease state. Significant progress was made in the discovery of susceptibility genes for a plethora of conditions. AD was no exception. By 1995, mutations in Apolipoprotein E (APOE), presenilin 1 and 2 (PS1 and PS1) and amyloid precursor protein (APP) were definitively linked to AD pathology (Pericak-Vance & Haines, 1995). It is important to note at this point, that mutations in APP, PS1 and PS2 all result in aberrant processing of the amyloid precursor protein (**Fig. 1**), leading to amyloid plaque formation and to the development of an early-onset AD (also known as familial AD or FAD). The role of APOE mutations is not entirely clear even today, however, a particular allele of APOE (ε4) is a known risk factor for the development of late-onset AD. This was an exciting period in the field of AD research, as the possible cure seemed within easy reach. But, and such is the story of Alzheimer´s, the disease proved to be a much tougher nut to crack as was anticipated.

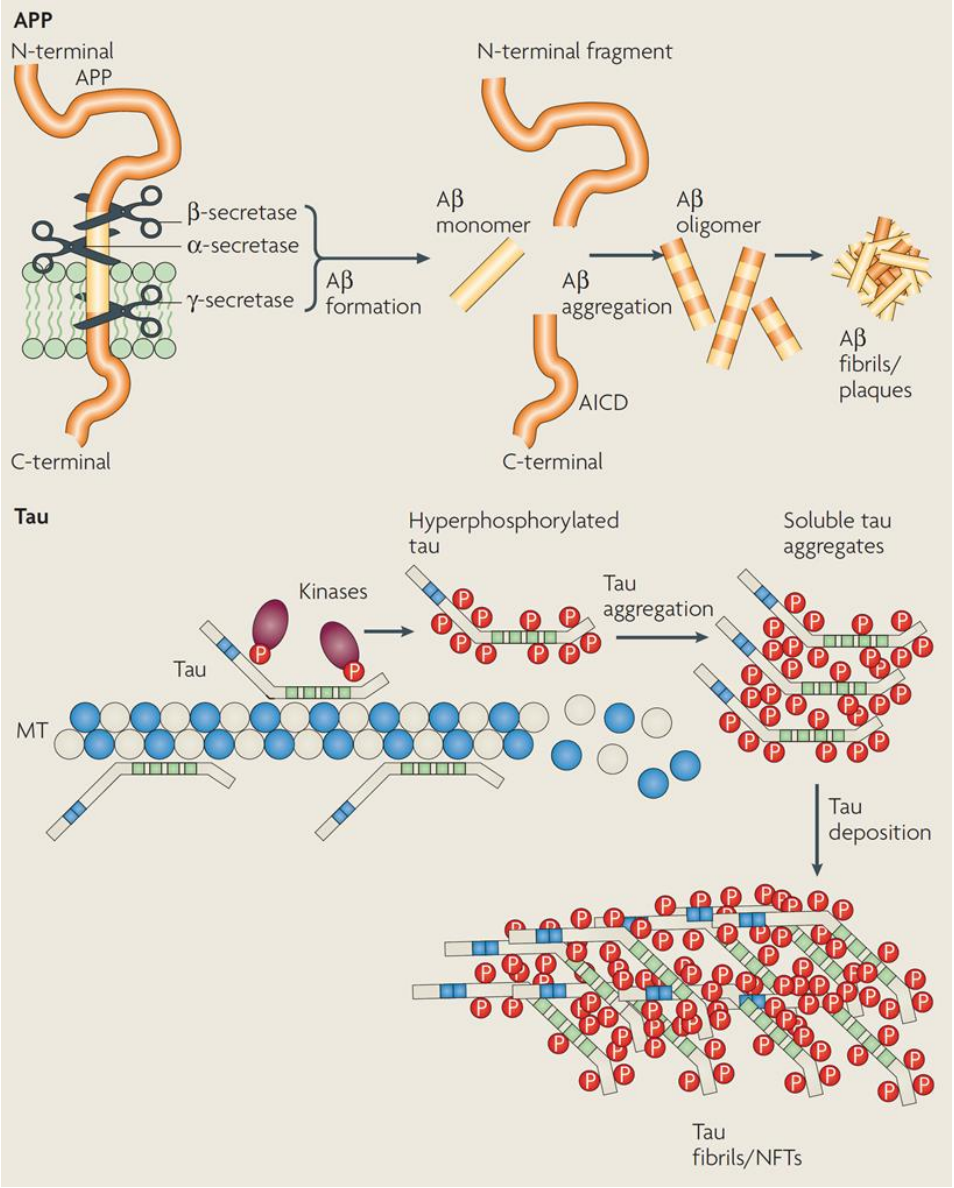


Figure 1. *(Top part) A mechanism thereby Aβ is produced from APP*: Under physiological conditions, APP is preferentially cleaved by α-secretase complex, which cuts the molecule in the middle of the Aβ fragment, thus not allowing the formation of Aβ. In a pathological state (amyloidogenic route), APP is sequentially cleaved first by β- and then by γ-secretase complexes, resulting in the generation of the amyloid beta peptides and the AICD. γ-secretase can cut APP at multiple residues resulting in the production of either Aβ (1-40) or Aβ (1-42). Aβ (1-42) is considered a more pathogenic form of the peptide. Mutations in the PS1, PS2 (proteins which form an integral part of γ-secretase complex) and APP favor Aβ (1-42) production. *(Bottom part) A mechanism thereby NFTs are generated from hyperphosphorylated tau protein*: Tau proteins physiological role is to stabilize microtubules. A process which is regulated by the phosphorylation state of the protein. Tau hyperphosphorylation results in the formation of soluble tau aggregates, which are eventually deposited as insoluble neurofibrillary tangles (see section 3.5 of the Introduction). *AICD: amyloid precursor protein intracellular domain; NFT: neurofibrillary tangles composed of paired helical filaments **Adapted from (Götz & Ittner, 2008)

Once a link between brain amyloid beta peptide deposits and AD was clearly established, the scientific community attempted to identify molecular mechanisms which could explain neurodegenerative processes. Ion channel hypothesis for Alzheimer amyloid peptide neurotoxicity was among the first to emerge (Pollard, Arispe, & Rojas, 1995). At its core, it was suggested that aberrantly processed beta amyloid peptides are capable of forming distinct (and unnatural) Ca^{2+}-conducting ion channels within neuronal membranes which could cause increased intracellular Ca^{2+} concentrations, thus disrupting neuronal homeostasis and causing excitotoxicity (neuronal damage and/or death due to overstimulation). This hypothesis faded into obscurity fairly quickly. However, the role of excessive intracellular Ca^{2+} was explored from another angle by Olney and colleagues (Olney, Wozniak, & Farber, 1997) who proposed that N-methyl D-aspartate receptor (NMDAR) signaling may be disrupted in AD ("excitotoxic neurodegeneration hypothesis"). NMDAR constitute a major class of excitatory ligand-gated ion channels in the brain. These receptors are primarily activated by the endogenous neurotransmitter glutamate (Glu). Upon activation, Ca^{2+} influx is allowed via the open channel pore. Both the Glu and the NMDAR are ubiquitous in mammalian brain, with NMDAR mainly localized to the post-synaptic membrane of excitatory synapses (Jones & Baughman, 1991). NMDAR signaling contributes to neuronal differentiation, synaptic plasticity and long-term potentiation. These receptors also play a role in glutamate neurotoxicity (Greenamyre & Porter, 1994). So how exactly does the NMDAR-mediated excitotoxicity occur? The short answer is that we still do not know. What we do know, is that excessive synaptic release of glutamate may overwhelm endogenous Ca^{2+} homeostasis regulation, leading to a significant influx of Ca^{2+} ions post-synaptically, by mechanisms which involve not only NMDAR but other receptors as well, beyond the scope of this thesis. Intracellular Ca^{2+} overload ultimately leads to neuronal death, either by apoptosis or necrosis, depending on the severity of the stimulus. During the course of researching materials for this dissertation, the most comprehensive and reliable review on calcium excitotoxicity in neurodegeneration I was able to find was published in 2003 (Arundine & Tymianski, 2003). To go back to the original "excitotoxic neurodegeneration hypothesis", one conclusion presented by Olney and colleagues is particularly significant. Briefly, the authors point out that under the circumstances of acute CNS injury, including brain trauma and stroke, NMDAR are hyper activated, which causes severe Ca^{2+} excitotoxicity. In the case of AD, however, this appeared to not be the case, because basal NMDAR activity was reduced in the brain in response to normal ageing (this observation is of particular interest when looked at from the perspective of the "synaptic dysfunction" hypothesis discussed on page 24). The most important finding of the study was that this condition of hypo-activation seemed to result in increased sensitivity of the receptors towards subsequent stimuli. That is to say, in advanced age, even weak agonist activity is capable of producing exaggerated Ca^{2+} influx. Therefore, in individuals suffering from sporadic AD or similar neurodegenerative disorders, chronic exposure to low-grade glutamatergic activation (by predisposing factors which were

unknown at the time) may produce progressive neurodegeneration as a result of continuous NMDAR-mediated excitotoxicity. In the later part of the introduction (page 33), I will go back to the concept of how chronic exposure to stimuli at levels which are generally considered insufficient to produce a biological response, may have a measurable impact on AD pathology. As a side note, recent evidence indicates that neuronal Ca^{2+} metabolism is indeed disrupted in AD, however, some authors claim that disturbances in Ca^{2+} signaling may also be neuro-protective under some circumstances (reviewed by (Supnet & Bezprozvanny, 2010)).

Another hypothesis which emerged in 1990s is the so-called "oxidative stress hypothesis". The fact that the brain is an organ with a high oxygen consumption rate coupled to its elevated lipid content and the relatively low abundance of antioxidant enzymes when compared to other tissues, gave credence to this hypothesis. Proposed by Markesbery (Markesbery, 1997), it was largely based on accumulated body of evidence indicating the presence of significant oxidative stress in the brains of AD patients. This author provided ample data to support the claim. First of all, Markesbery cited elevated levels of base metals including iron, aluminium and mercury which were present in the brains of diseased patients. Trace amounts of these elements were known to produce free radicals in multiple tissues. Second, evidence was presented that lipid peroxidation levels were increased, and this was accompanied by a simultaneous decrease in polyunsaturated fatty acids (PUFA) in AD brain. PUFA are a critical component of the phospholipid membrane and allow for additional flexibility and fluidity of cellular membranes (Rajamoorthi, Petrache, McIntosh, & Brown, 2005). PUFA are highly oxidizable and the reduction in their levels is indicative of free radical damage. In addition, increased protein and DNA oxidation in AD brains was demonstrated in literature analysis performed by Markesbery. Furthermore, the presence of advanced glycation end-products (AGEs) and related molecules was also described in affected tissue samples. Cerebral energy metabolism was apparently reduced as well. Last, but not least. Markesbery pointed out that the beta amyloid aggregates have the capacity to generate free radicals on their own. It is of little wonder then, that this hypothesis provided additional stimuli for significantly increased funding of AD research on a global level.

Due to the apparent presence of oxidative stress in AD pathology, researchers turned their attention towards mitochondria. These organelles are a central source of cellular adenosine triphosphate (ATP), the principal energy "currency" for cellular metabolism. Deficiencies in the mitochondrial Electron Transport Chain (ETC) may cause alterations in the processes of oxidative phosphorylation (OXPHOS complexes), leading to production of highly reactive free-radical superoxide, a major source of intrinsic oxidative stress. One of the first papers which linked mitochondrial DNA (mtDNA) mutations to sporadic AD cases was published by Swerdlow and coworkers in 1997 (R H Swerdlow et al., 1997). Authors utilized a cytoplasmic hybrid

(cybrid) model to demonstrate that mutations in mtDNA are largely responsible for defects in cytochrome c oxidase (COX or Complex IV) enzyme, one of the key enzymes in the ETC pathway (cybrids are created by transferring donor mtDNA to neuronal-like cells which are depleted of mitochondria *in vitro*). In that study, cybrids generated from the samples of AD patients demonstrated decreases in complex IV activity, coupled with increased production of reactive oxygen species (ROS) and increased antioxidant enzymes (AOE) activity. A comprehensive review (citing over 270 papers) evaluating the role of mitochondria in neurodegenerative diseases, including AD, was published in 1999 (Cassarino & Bennett, 1999). It took additional 5 years to recapitulate existing knowledge and to formulate a "mitochondrial cascade hypothesis" of AD (Russell H Swerdlow & Khan, 2004).

By 2004, it became evident that "amyloid cascade hypothesis" cannot fully explain the pathological features of the disease. Even though the case of Auguste Deter is a clear example of FAD (due to PS1 mutation), FAD represents only a tiny proportion of patients, with the vast majority suffering from the sporadic (late-onset) form of the disease. Patients with late-onset Alzheimer do not have the mutations which result in increased beta amyloid production. Furthermore, a significant number of non-demented aged population presents with cerebral amyloid plaques discovered in post-mortem analysis. The "mitochondrial cascade hypothesis" attempts to identify the distinct pathological hallmarks of the sporadic form of AD compared to FAD, and suggests that mitochondria play a key role in the development of late-onset pathology. The hypothesis can be broken down into the following constitutive parts: 1) Basal mitochondrial ROS production depends on inherited polymorphic variations in mtDNA which affect the expression levels and activity of key enzymes participating in ETC (OXPHOS complexes); 2) Higher basal ROS production rates cause more rapid accumulation of *de novo* somatic mtDNA mutations; 3) These accumulated mutations result in additional reductions in OXHPOS metabolism, triggering further increases in ROS production. Once ROS levels reach a certain threshold, a three-part compensatory mechanism is activated. The authors termed this series of events as a "reset" (1), "remove" (2) and "replace" (3) response. During the first part of the sequence the system is "reset", whereby excess ROS in terminally differentiated neurons cause a shift in cellular redox balance, leading to aberrant activation of the plasma membrane oxidoreductase system. Resulting increase in plasma membrane ROS favors increased amyloid beta peptide production from the APP, thus contributing to amyloid plaque formation. The following "removal" phase is the elimination of the most affected neurons by apoptosis. These are the neurons in which ROS levels remain abnormally high and/or OXPHOS metabolism is significantly diminished. In the final "replacement" part of the sequence, an attempt is made to replace the destroyed neurons. It is important to note that mitochondrial homeostasis is also likely disrupted in neurons which are supposed to replace the post-apoptotic cells. However, the levels of ROS and OXPHOS perturbations are not sufficiently high in these neurons to trigger apoptosis. The exact molecular mechanism whereby "replacement" occurs is at the crux

of “mitochondrial cascade hypothesis”. The authors suggest that anaerobic glycolysis is preferentially activated in neurons in which OXPHOS metabolism is reduced. Apart from ROS generation, this also initiates hypoxic signaling pathway which activates mitotic cell-cycle re-entry program in neurons capable of proliferation. During this process, tau (hyper)-phosphorylation and neurofibrillary tangle formation may take place, providing evidence that both the tauopathy (in “replace” phase) and amyloid beta peptide production (in “reset” phase) are dependent on abnormal mitochondrial signaling. Another interesting observation made by the same authors is that it appears that the proliferation process is never completed (possibly due to the lack of ATP under these circumstances). And, therefore, the damaged neurons are never truly replaced (Russell H Swerdlow, Burns, & Khan, 2010; Russell H Swerdlow & Khan, 2004). As mentioned earlier, both the “mitochondrial cascade” and the “oxidative stress” hypotheses share some elements with the “vascular” hypothesis. In particular, cerebral ischemia results in increased oxidative stress coupled with reduced OXPHOS metabolism. Therefore, these hypotheses are not mutually exclusive.

The “two-hit hypothesis” (Zhu, Raina, Perry, & Smith, 2004) is similar to the “mitochondrial cascade hypothesis”, however it describes AD pathology from a different angle. The “mitochondrial cascade hypothesis” suggests a sequence of events starting from mitochondrial defects causing increased ROS generation, which ultimately result in disrupted mitotic signaling. In case of the “two-hit hypothesis”, authors acknowledge that both oxidative stress and cell-cycle deficiencies are early events in AD pathology. The key difference is the suggestion by the authors that either the oxidative stress or dysregulated mitotic signaling may act as *independent* triggers of the AD pathology. Once the disease process is initiated, however, it is claimed that the presence of *both* of those triggers is required for disease propagation (hence the name “two-hit”). The idea behind the hypothesis is that the neurons may be capable of adapting to a situation in which a single insult is present, but are not sufficiently equipped to respond to multiple simultaneous homeostasis disruptions. In case of AD, either one of the “hits” by itself, be it cell-cycle defects or oxidative stress, is not sufficient to cause full-blown AD. But, once one of the “hits” is already present, the whole system becomes vulnerable to the effects of the second “hit”. This model is quite elegant, with authors claiming that it can be applied to multiple neurodegenerative diseases, and not just AD.

Immune system, particularly innate immunity and inflammatory responses are additional factors which contribute to AD pathology. All the way until the mid-1980s, it was considered highly unlikely that inflammatory processes could occur within the brain due to the phenomenon of “immune privilege”. The concept of “immune privilege” is based on the observations that immune-mediated inflammation and allograft rejection rates are significantly reduced in some organs including the brain. According to Niederkorn (Niederkorn, 2006), the phenomenon was described in medical literature as early as 1873 (Graefes, 1873), with the actual term “immune privilege” created in 1948 (Medawar, 1948). It is thought

that the suppression of inflammatory responses in the CNS provides a significant evolutionary advantage. Because of the importance of the brain to the survival of the whole organism and its relative lack of regenerative capacity, immune-mediated inflammatory responses in this organ could be devastating, so it makes sense to protect the brain from the effects of generalized inflammation. It was thought that such protection is provided by means of endothelial blood-brain barrier (BBB), which was considered to be impenetrable to the cells of the immune system. Thus, the prevailing view in the 1980s was that the apparent signs of inflammation in the brains of deceased AD patients were likely artifacts and did not reflect the disease pathology. This changed with the publication of a series of papers which first described that activated T cells are capable of crossing BBB with relative ease (Wekerle, Linington, Lassmann, & Meyermann, 1986). Once across the barrier, these activated T cells were able to interact with the glial cells, particularly the astrocytes, by releasing a number of chemical factors (Wekerle, Sun, Oropeza-Wekerle, & Meyermann, 1987). In addition, it was demonstrated that severe systemic inflammation (induced by a *graft versus host* disease) resulted in T lymphocyte infiltration into the CNS (Hickey & Kimura, 1987) with the apparent involvement of perivascular microglial cells (Hickey & Kimura, 1988). These findings resulted in the re-evaluation of a possible role of immunity in CNS disorders including AD. In retrospect, it is now relatively easy to understand that beta amyloid plaques, neurofibrillary tangles and neurodegeneration could result in neuroinflammation. After all, in peripheral tissues inflammation is frequently caused by tissue damage and the chronic presence of inert foreign materials. The classical pathological features of AD could easily be such triggers in the CNS. During the 1990s, a large number of studies attempting to identify molecular mechanisms of neuroinflammation in AD were published. A working model which organized available knowledge of inflammatory pathways in relation to AD was presented by a Neuroinflammation Working Group in 2000 (Akiyama et al., 2000). It is notable that of nearly 700 studies which were cited in that article, the vast majority of papers were published during the 1990s. What emerged is a highly complex regulatory mechanism whereby immune system reacts to disease pathophysiology. To provide even a brief overview of all the molecular networks involved in neuroinflammation will take many pages, and is beyond the scope of this thesis. I would just like to point out that one of the critical findings of the study was that neuroinflammation in AD appeared to originate from within the CNS itself, with little involvement of peripherally derived monocytes and lymphocytes.

The biological pathways linking immune response to AD continue to be an area of active research. While it is clear that neuroinflammation does indeed occur in AD, the fundamental question remains: is it the cause or the consequence of the underlying pathology? At first, it appeared that the inflammatory response is a result of amyloid plaques, neurofibrillary tangles, and nerve damage (reviewed by (Akiyama et al., 2000; Heneka, Kummer, & Latz, 2014)). However, in recent years it was suggested that neuroinflammation may occur prior to the disease development and may actually lead to accelerated plaque and tangle

formation. Even if occurring in later disease stages, it may exacerbate the disease phenotype. Furthermore, inflammation can actually be a beneficial response, which slows down disease progression (Wyss-Coray, 2006). It is now recognized that in virtually all diseases that affect CNS, immune system plays a role. The key difference between neurodegenerative diseases, such as AD, and neuroinflammatory diseases, such as multiple sclerosis (MS), is the type of inflammation each of these diseases produces. In case of MS, inflammation is driven largely by the adaptive immune response, whilst in AD, innate immunity appears to be the primary contributor. In AD-driven inflammation, intrinsic CNS-resident immune cells (including microglia, perivascular myeloid cells and astroglia) appear to play a principal role (Heppner, Ransohoff, & Becher, 2015). However, peripheral/systemic inflammation caused by obesity, metabolic syndrome and type 2 diabetes mellitus (T2DM) have also been identified as risk factors for AD development (Takeda et al., 2010; Thaler et al., 2012).

A possible connection between insulin signaling molecules and AD was described by Kurochkin and Goto in 1994 (Kurochkin & Goto, 1994). The authors identified Insulin Degrading Enzyme (IDE) as being very efficient at clearing intracellular beta amyloid peptides ($A\beta_{(1-40)}$). IDE is a highly selective protease responsible for cleaving (and inactivating) small peptides, notably insulin and glucagon. Authors concluded that defects in IDE may result in excessive production of beta amyloid, thus contributing to amyloid plaque generation. That particular study linked AD to insulin signaling mostly in name alone. Just because IDE was originally identified as an insulin protease was clearly not enough to suggest that its amyloidolytic activity is also related to insulin metabolism. One of the first reports which confirmed that deletion of IDE is capable of causing both chronic elevation of cerebral amyloid beta levels and hyperinsulinemia coupled with impaired glucose tolerance *in vivo*, was performed by Farris and colleagues (Farris et al., 2003). In a follow-up study by the same group, authors point out that naturally occurring, partial loss-of-function mutations in IDE protein, are sufficient to induce a diabetic phenotype in rats together with simultaneous reductions in beta amyloid clearance in the brain (Farris et al., 2004). In the same year, seminal work was published by researchers at Mayo Clinic (2 of the co-authors of Farris paper were also from the Mayo Clinic, but in a different group) who reported increased risk for T2DM development in AD patients (Janson et al., 2004). Strikingly, either impaired fasting glucose (IFG) or T2DM was detected in 81% of patients with AD (42% in the control group). In this community-based controlled study, called the Mayo Clinic Alzheimer Disease Patient Registry (ADPR), 100 patients with AD were compared to 138 control subjects with the aim of evaluating T2DM prevalence in elderly AD population (mean age 79±9 years). Apart from the clearly increased risk of metabolic perturbations in AD, these patients presented with significantly elevated pancreatic islet levels of beta amyloid. In contrast, in subjects with T2DM who did not have AD, cerebral beta amyloid levels were not increased. When both T2DM and AD were detected, brain amyloid

deposition appeared to be correlated with the duration of diabetes. To my knowledge, this was one of the first large-scale human studies in which AD was linked to the peripheral insulin metabolism.

A second line of evidence that impaired insulin signaling contributes to AD was published by Steen, de la Monte and coworkers (Suzanne M de la Monte & Wands, 2005; Steen et al., 2005) who demonstrated that molecules involved in the insulin pathway are endogenously expressed and downregulated in AD brain. Significant hippocampal levels of mRNA transcripts of insulin (INS), insulin-like growth factors I and II (IGF1 and IGF2) and their related receptors (INS-R, IGF1-R and IGF2-R), were detected in post-mortem human tissue. In the hippocampi of AD patients, reduced expression was detected in all of these genes apart from IGF1 (which remained unchanged), when compared to the control group. Furthermore, a number of molecules involved in insulin signaling downstream of the INS-R were also affected in diseased brain. For example, mRNA levels of insulin receptor substrate (IRS), IRS-associated phosphatidylinositol 3–kinase (PI3K) and AKT (also known as protein kinase B – PKB) were reduced, with the concomitant increase in glycogen synthase kinase-3β (GSK-3β) mRNA. All of these proteins form a crucial component of the hormone-mediated growth and survival signal-transduction pathways and are known to be dysregulated in diabetes. As all of these changes were detected directly in the brain, and especially in the areas known to be most affected by senile plaques and neurofibrillary tangles, the authors proposed a hypothesis that AD may resemble what they termed “Type 3 Diabetes”. i.e AD is a form of brain-specific diabetes.

In the very same year, “synaptic dysfunction” hypothesis was proposed. Curiously, the term was coined by Rudolph E. Tanzi of Harvard University (Tanzi, 2005) in a comment to a paper published in the same issue of Nature Neuroscience by authors not affiliated with Harvard University (Snyder et al., 2005). What Snyder and colleagues observed, was the deregulation of synaptic plasticity due to NMDA receptor endocytosis caused by beta amyloid administration, resulting in the inhibition of excitatory neurotransmission. Among the key findings was the suggestion that amyloid beta oligomers, well before they reach the stage of amyloid plaques, are capable of inducing synaptic dysfunction in *in vitro* cultures of mouse neurons. As synaptic failure, and particularly reductions in Long-Term Potentiation (LTP), are associated with memory deficits (Selkoe, 2002), a conclusion was made that NMDAR-dependent synaptic deficiency may be a driver for future pathology. Because this hypothesis is clearly linked to NMDAR signaling abnormalities, these events, in my opinion, should be compared to the postulates of the “excitotoxic neurodegeneration” hypothesis, especially the part relating to the reduced basal NMDAR activity (see page 18).

One of the most recently introduced hypotheses is termed “dendritic hypothesis” and is partly based on “synaptic dysfunction” hypothesis (Cochran, Hall, & Roberson, 2013). Since the identification of synapse as a possible site of AD-related damage, additional scientific effort was put towards identifying the role the

dendrites play post-synaptically. The authors point out that dendritic abnormalities have long been observed in AD-affected brain. However, technological limitations have reduced researcher`s ability to study changes in dendrites in detail. While dystrophic neurites, reduced dendritic complexity and dendritic spine loss are all documented features of AD, it is only recently that we are beginning to understand the underlying molecular mechanisms (Cochran provided an excellent overview of the published data on the subject in the 2nd section of the paper, so I will refrain from providing source citations in this part of the introduction). In support of the dendritic role in AD pathogenesis, authors offer evidence that amyloid beta peptide is capable of direct post-synaptic binding to dendrites, can affect dendritic Ca^{2+} channel functions (beyond the scope of this introduction, but refer to “ion channel” and “excitotoxic neurodegeneration” hypotheses on page 18, as well as section 3.6 on page 43), and that Tau protein can potentially be targeted (or mis-targeted) towards the dendrites. Altogether, the data suggest that dendrites may be an additional factor contributing to AD.

What the brief and by no means exhaustive historical overview of the disease illustrates, is that over a dozen hypotheses intending to explain AD etiology have been proposed over the years. It became abundantly clear that AD is a highly complex disorder involving multiple biological pathways. It is also of little wonder then, that the pharmaceutical interventions attempted to-date have largely failed in innumerable clinical trials. In the following section of this introduction, I will attempt to summarize the clinical aspects of AD treatment, and to relate the pharmacological approaches to the disease. As in the first part of the introduction, the chronological timeline will be followed whenever possible.

3. PHARMACOLOGICAL APPROACHES TO ALZHEIMER DISEASE TREATMENT

In the 100+ years since the formal description of Alzheimer disease, a large number of treatment strategies has been attempted with mixed results. The primary goal of any pharmacological intervention is to improve the cognitive and behavioral symptoms of the disease. There are only four compounds currently approved for AD treatment on the market. 3 of these are acetylcholinesterase inhibitors, and one is an NMDAR antagonist. In this section, I will provide a brief overview of the pharmacotherapies targeting AD, loosely grouped according to the principal biological route affected by these molecules. The first 2 subsections will cover existing treatments, with the rest of the material referring to compounds which are investigated for their possible role in improving AD symptomatology. A summary of molecules discussed in section 3.1 is provided in tabulated form in **Table 1** (page 32), while **Table 2** (page 46) is a list of chemicals and clinical trial IDs for the compounds mentioned is sections 3.3-3.8.

3.1 Cholinergic system

Compounds which fall into this category all aim to restore cholinergic neurotransmission with the rationale for their use provided by the "cholinergic hypothesis" (see page 14). Acetylcholine (ACh) biosynthesis occurs pre-synaptically from the precursors Acetyl-CoA and choline and requires an enzyme choline acetyltransferase (CAT). Upon release, ACh is rapidly metabolized post-synaptically into acetate and choline by acetylcholinesterase (AChE). Thereafter, choline is transported back into the terminal (**Fig. 2**) (Purves et al., 2001). With the idea that cholinergic neurotransmission is disrupted as a result of AD pathology, this mechanism suggests a number of possible drug targets within this pathway. One of the earliest treatments which reached the clinical trials stage in humans was with lecithin. Lecithin is a complex phospholipid which is a major dietary source of choline. It was originally thought that by increasing the dietary bioavailability of choline, cerebral levels of ACh would increase as well. Cochrane Reviews published an extensive analysis of randomized clinical trials in which the efficacy of lecithin in AD was measured. In the span of 25 years (from 1978-2003) a total of 12 independent clinical trials which passed the inclusion criteria were conducted. Unfortunately, none have reported any significant benefit of lecithin administration in AD (Higgins & Flicker, 2003). (Please note that even though the Cochrane Review was published in 2003, it has been revised in 2009 with no additional changes made to the paper).

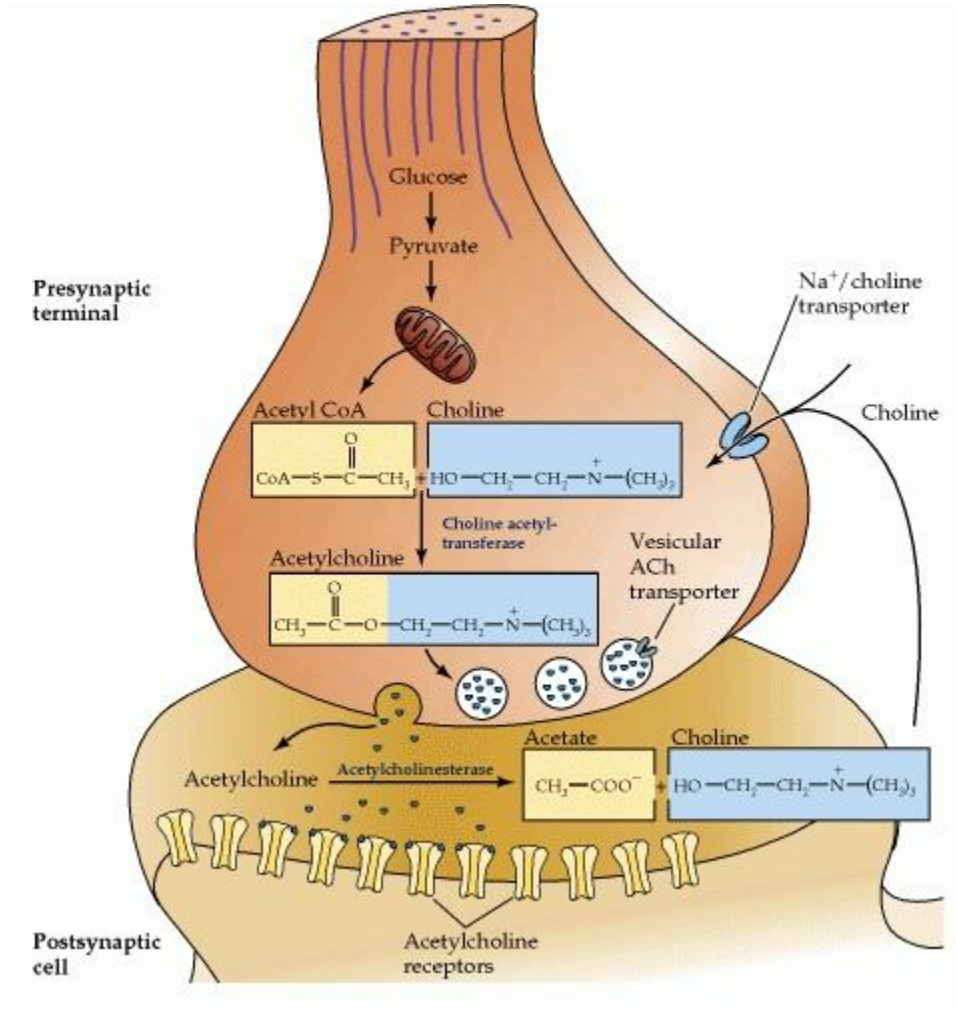


Figure 2. Acetylcholine metabolism in cholinergic nerve terminals. The synthesis of acetylcholine from choline and acetyl CoA requires choline acetyltransferase. Acetyl CoA is derived from pyruvate generated by glycolysis, while choline is transported into the terminals via a Na+-dependent transporter. After release, acetylcholine is rapidly metabolized by acetylcholinesterase and choline is transported back into the terminal. *Reproduced from (Purves et al., 2001)

Due to the very high catalytic activity of AChE (about 5000 ACh molecules per each AChE molecule per second) (Purves et al., 2001), AChE inhibition is an obvious potential target to increase ACh levels at the synaptic cleft. Physostygmine, a compound with a phenyl carbamate nucleus, was the first AChE inhibitor (AChEI) to be tried as a treatment for AD in a clinical setting. A number of papers were published starting from 1978 which evaluated physostygmine for its potential in reducing cognitive decline in AD. By 1987, however, it emerged that physostygmine treatment was just as ineffective as lecithin, possibly due to its poor absorption, very short half-life and a narrow therapeutic index (reviewed by (Orgogozo & Spiegel, 1987)). Nevertheless, research into physostygmine use in AD continued all the way until early 2000s, with some researchers attempting to overcome the half-life limitation by formulating extended-release oral forms of the compound. The possible use of physostigmine in the treatment of AD was also reviewed by Cochrane Reviews, with the latest update performed in 2008 (Coelho Filho & Birks, 2001). Of the 15 clinical trials identified, none demonstrated improved efficacy when compared to placebo. The authors reached an unequivocal conclusion that physostygmine use in AD is of purely historic interest at this point.

Tacrine happened to be the very first pharmacological treatment ever to reach the market for AD. This centrally-acting AChEI was approved by the FDA for this indication in the US in 1993 (Crismon, 1994). Tacrine exhibited significantly better pharmacokinetic (even though oral bioavailability was still low, and the half-life was just 3-6 hours) and safety profiles, when compared to physostygmine (Soares & Gershon, 1995). Furthermore, the results of 12 major clinical trials which recruited over 2000 participants in total, have demonstrated modest improvement in the rates of cognitive deterioration in treated patients. However, most of these clinical trials were short-term, with the treatment length varying between just 3-12 weeks (In only 2 trials the treatment lasted 30 and 36 weeks) (reviewed by (Nawab Qizilbash et al., 1998)). Due to the lack of long-term efficacy data and the controversy surrounding the improvement in behavioral disturbances and the functional autonomy of the treated population (it was unclear if tacrine had any effect whatsoever on these parameters), it became clear that the clinical efficacy of tacrine was far from ideal. Furthermore, possible hepatotoxicity was an additional cause for concern. Some of these issues were addressed in a review paper by William K. Summers in 2000, who defended continuing use of tacrine in AD (Summers, 2000). However, that review can hardly be considered unbiased, as Dr. Summers is the original developer of the compound. A more recent and comprehensive analysis of clinical trials involving tacrine was withdrawn in 2007 for reasons which are unclear ((N Qizilbash, Birks, Lopez Arrieta, Lewington, & Szeto, 2007). Tacrine itself was discontinued in the US market in 2012 due to safety concerns and the availability of more modern AChEIs.

A number of alternative compounds which are derivatives of tacrine were also considered as possible treatment options for AD. Velnacrine is just one example. In my opinion, velnacrine is worth mentioning

here because it illustrates the fact that the aminoacridines (a chemical class to which both tacrine and velnacrine belong) were widely recognized in the field for their hepatotoxicity potential even at the time of tacrine approval. In fact, velnacrine was developed with the specific purpose of reducing toxicity associated with tacrine treatment. A total of 4 clinical trials recruiting 899 patients were conducted with velnacrine. Unfortunately, the drug was not an improvement over tacrine in a clinical setting, and was just as toxic. As a result, FDA rejected the compound in 1994. Since then, no additional clinical trials with velnacrine as a treatment for AD were conducted (J. Birks & Wilcock, 2004). It is worth noting that there is currently renewed interest in developing new molecules which employ aminoacridine chemical structure as a basis for intelligent drug design (de Aquino, Modolo, Alves, & de Fatima, 2013; Minarini et al., 2013; Romero, Cacabelos, Oset-Gasque, Samadi, & Marco-Contelles, 2013).

Just as tacrine was the first approved pharmaceutical treatment for AD in the US, donepezil was the first symptomatic medication to gain approval in the UK (in 1997). According to Wilkinson (Wilkinson, 1999), tacrine was never considered for approval by UK regulators due to its short half-life and potential hepatotoxicity. Thus, when the clinical data on donepezil started pouring in, the regulators were understandably interested. Donepezil does not belong to an aminoacridine chemical class and is instead a piperidine-based, potent and specific non-competitive and reversible AChEI. Among its most favorable features, when compared to tacrine, is a half-life of nearly 70 hours and the apparent lack of hepatotoxicity. From the bedside perspective, the results of 24 large-scale clinical trials with nearly 6000 participants confirmed that donepezil was effective in improving not only the cognitive symptoms, but also had positive effects on behavior and functional autonomy of treated patients (compare to the tacrine studies). In addition, this time around, most of the trials were of 12-24 weeks duration, with one study lasting a full 52 weeks, with the reported benefits of delayed cognitive loss being maintained over the entire treatment period. Unfortunately, the clinical benefits of donepezil are rather modest, to the point that clinicians sometimes fail to register significant improvements in patient well-being and the treatment itself is largely palliative (J. Birks & Harvey, 2006). Donepezil is, without a doubt, a widely used drug for the symptomatic AD treatment, a higher dose of which was recently approved for severe AD (as opposed to most treatments which target mild-to-moderate AD dementia). This particular point is of interest, because all of the other compounds mentioned here were considered for relatively early disease stages, where the cognitive decline and behavioral changes were already apparent and AD diagnosis was suspected, but the patient had not yet progressed to what is considered as terminal diagnosis. It was assumed that in very advanced stages of dementia, the cholinergic neurons were damaged beyond repair, and that the inhibition of AChE would be of little benefit. In fact it was demonstrated that donepezil may lead to measurable improvement even in such circumstances. A 2011 review summarizes these findings (Curiously, the drug information package inserts were cited as legitimate scientific references in this review; see references 3 and 4) (Sabbagh &

Cummings, 2011). In a more recent paper, the authors reached similar conclusions, but the article is sadly full of grammar and stylistic mistakes (Cheewakriengkrai & Gauthier, 2013).

Rivastigmine is a phenyl carbamate derivative, just like physostigmine, however due to its pharmacological properties it is a much better drug candidate. As mentioned earlier, first carbamate derivatives suffered from poor bioavailability and a short half-life, while early aminoacridine compounds (prior to donepezil) were relatively toxic. Rivastigmine was one of the few compounds in the carbamate class which demonstrated improved CNS selectivity over physostigmine. As a result, potential toxicity and the side effects stemming from peripheral cholinergic inhibition were significantly reduced (Enz, Amstutz, Boddeke, Gmelin, & Malanowski, 1993). Rivastigmine gained approval for the treatment of mild-to-moderate AD in Switzerland in 1997 ("The Pharma Letter," 1997), the drug has since been approved in over 80 countries including all the countries in the EU, Canada and the US. A recent Cochrane Review summarized the findings of 7 major clinical trials with rivastigmine which have cumulatively recruited 3450 AD patients (J. S. Birks, Chong, & Grimley Evans, 2015). The principal clinical outcomes were similar to donepezil treatment and include reduced rates of cognitive decline in patients with mild-to-moderate AD. As with other AChEIs, the benefits were relatively modest and did not halt disease progression, but only slowed it down. The incidence of adverse events was acceptable, with most side-effects directly related to cholinergic inhibition with some adverse reactions occurring in the gastrointestinal (GI) tract. It is noteworthy that all of the AChEIs mentioned in this section were originally formulated in oral form. Rivastigmine became the first molecule in this class to gain approval as a transdermal (TD) patch (initially for mild-moderate AD). This is very significant, as transdermal administration provides similar efficacy as the oral form, with concomitant reduction in side-effects, particularly GI-related ones. This property led to the first-ever approval of a TD medication for the treatment of severe AD by the FDA in 2013 (Amanatkar & Grossberg, 2014; Jeffrey, 2013).

Galantamine is the third and latest AChEI which is still approved for the treatment of mild-to-moderate AD in the US and elsewhere. Galantamine is different from other approved drugs in this class as it is only a weak AChEI compared to other compounds described earlier. In addition to blocking AChE, galantamine is also a positive allosteric modulator of nicotinic ACh receptors (J. Coyle & Kershaw, 2001). In fact, the pharmacological properties of this alkaloid on acetylcholine signaling were described decades ago ((Ueda, Matsumura, Kimoto, & Matsuda, 1962) – this is the first article concerning galantamine pharmacology available in English language on Pubmed). Ueda and co-authors pointed out that AChE inhibition was approximately 100 times lower with galantamine, compared to physostygmine. Furthermore, the authors suggested that the effects of galantamine could not be attributed to AChE inhibition alone. In the mid-2000s, this hypothesis was validated and it was proposed that neuroprotection (and presumably clinical

benefits) afforded by all three of the currently approved drugs (donepezil, rivastigmine and galanatamine) may result from effects secondary to those of AChE inhibition (Arias, Gallego-Sandín, Villarroya, García, & López, 2005). As mentioned earlier, nicotinic receptors may be involved, however there appears to be an additional pharmacological effect of galantamine treatment which is directly relevant to the work described in the current thesis. Ali and colleagues (Ali, El-Abhar, Kamel, & Attia, 2015) demonstrated improved oral glucose tolerance together with significant reduction in diabetic phenotype in a rat model of streptozotocine (STZ)-induced T2DM when the animals were treated with galantamine. To my knowledge, this is the first study which directly demonstrates that a centrally-acting anti-AD may be successfully utilized as an anti-diabetic agent. If these data can be reproduced by other authors, then this would give additional support to the "type 3 diabetes" hypothesis of AD.

In summary, modulation of cholinergic signaling gave rise to the three pharmacological compounds (belonging to three different chemical classes) which are widely used as a treatment for AD. While AChE has long been considered a primary target for these drugs, the data described in this section suggest a more complex mechanism of action. An overview of the key features of the molecules mentioned here is provided in **Table 1**.

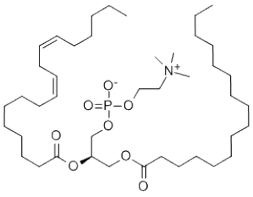
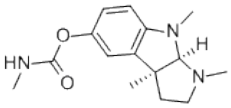
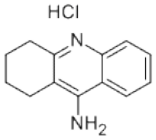
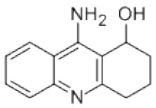
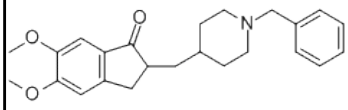
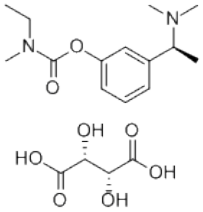
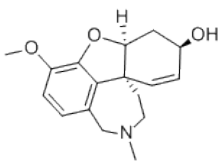
Compound	Formula	Chemical class	MOA	Approval (USA)	Page
Lecithin		a complex phospholipid composed of choline, phosphoric acid, fatty acids and glycerol	dietary source of choline	never approved	XXX
Physostigmine		phenyl carbamate derivative	AChEI	never approved	XXX
Tacrine		aminoacridine	AChEI	(mild AD) 1993 discontinued: 2012	XXX
Velnacrine		aminoacridine	AChEI	never approved	XXX
Donepezil		piperidine-based	AChEI	(mild AD) 1996 (severe AD) 2006	XXX
Rivastigmine		phenyl carbamate derivative	AChEI	(mild AD) 2000 (severe AD - transdermal patch) 2013	XXX
Galantamine		norbelladine-derived benzazepine	AChEI	(mild AD) 2001	XXX

Table 1. A list of chemical entities which affect cholinergic signaling. All of the compounds presented above were considered as a possible treatment option for AD in human clinical trials. The formulas are taken from *chemicalbook.com*

3.2 Glutamatergic approach

Disruptions in NMDAR signaling and the resultant excitotoxicity contribute to AD pathology (see "excitotoxic neurodegeneration hypothesis" on page 18). Adamantine derivative memantine is the only NMDAR inhibitor (NMDRi) used in the clinical setting for AD treatment (Danysz & Parsons, 2012). This drug is an uncompetitive NMDRi which exhibits voltage-dependency and rapid receptor blocking and unblocking kinetics. These pharmacological properties lead to a relatively good safety profile, with low likelihood of adverse drug reactions (ADRs) (Chen et al., 1992). In essence, memantine is capable of NMDAR antagonism, while still permitting physiological glutamatergic signaling, including in the processes of learning and memory (Parsons, Stöffler, & Danysz, 2007). As mentioned in section 2 (page 19), NMDAR hypo-activation may increase the sensitivity of the receptors towards subsequent stimuli. Memantine may be capable of blocking exaggerated responses precisely under such conditions.

Memantine was approved for treatment of mild-to-severe AD in 2002 in Europe and in 2003 in the USA. Cochrane Reviews (updated in 2006) had identified 12 clinical trials with memantine in various types of dementia that met the inclusion criteria. Each of these trials had recruited between 60 and 579 participants. The main conclusion reached by the authors is that memantine administration for periods of up to 6 months significantly improved the rates of cognitive decline in AD patients measured at up to 28 weeks post-treatment (McShane, Areosa Sastre, & Minakaran, 2006). A more recent meta-analysis published in 2013 (Yang, Zhou, & Zhang, 2013) reached similar conclusions. Unfortunately, the data presented in that manuscript appear to be rather unreliable. Authors claim to have used Cochrane Handbook in order to identify trials that met their inclusion criteria, with 20 mg/day memantine administration being one of those. In at least one of the cited studies, the actual daily dose of memantine was 28 mg/day (NCT00322153 – reference 36 of that paper); therefore that study should have been excluded. Considering that the number of included studies was only 12 (out of nearly 900 published papers analyzed by authors) this should not have happened. Furthermore, a number of studies were inappropriately cited and, for at least one study, the country where the trial was performed was incorrectly identified (Schmidt study (reference 30) was a single-center trial performed in Austria, and not Australia as claimed by the authors). A 2014 meta-analysis summarized a total of 23 trials and 11024 patients with AD who were treated with any of the currently available drugs (donepezil, galantamine, rivastigmine or memantine). The authors conclude that each of these drugs significantly improves AD pathology, with memantine exhibiting better side-effects profile when compared to AChEIs (Tan et al., 2014).

All in all, all 4 pharmacotherapies do lead to slight but significant improvements in patient health. However, none of these are capable of halting the course of cognitive decline or reversing disease progression. It is abundantly clear that better drugs are required. In the following sections, a brief overview of all the

additional approaches to AD treatment is provided. Unfortunately, none of the pharmaceuticals discussed below have been approved in human patients.

3.3 Amyloidogenic route

In the last two decades, the pharmaceutical industry has focused primarily on the amyloidocentric approach, devoting substantial resources to develop effective AD drugs. However, multiple failures of drug candidates in clinical trials have led researchers to question the feasibility of this strategy (Drachman, 2014). One possible reason for failure is a lack of biomarkers which could reliably identify AD in relatively early stages. It is entirely possible that the patients currently recruited for Phase III trials are in such advanced stages of AD that any attempted intervention is probably useless. Therefore, new diagnostic tools capable of early detection are sorely needed. In the meantime, there is still a number of novel treatments under development, which target the amyloidogenic route (see **Fig.1** on page 17). In order to reduce Aβ generation from the APP, γ- and β-secretase inhibition and the potentiation of α-secretase activity have been considered.

3.3.1 Inhibitors and modulators of β-secretase

β-secretase enzyme complex participates in the initial stages of the amyloidogenic APP-processing pathway. The development of β-secretase inhibitors is a challenge because besides the APP, this complex has many more substrates. To give just one example, neuregulin-1, which is involved in the myelination of CNS axons and synaptic plasticity is a target of β-secretase (Chiang & Koo, 2014). Broad range of substrates can lead to significant side-effects, even if the specific inhibition of the enzyme is achieved. Nevertheless, E2609 (clinicaltrial.gov ID# NCT01600859), MK-8931 (NCT01739348) and LY2886721 (NCT01807026 and NCT01561430) have all shown efficacy in reducing Aβ production by up to 80-90% in the cerebrospinal fluid (CSF) in humans. None of β-secretase inhibitors have reached the market so far (May et al., 2015; Vassar & Kandalepas, 2011; Yan & Vassar, 2014).

3.3.2 Inhibitors and modulators of γ-secretase

The γ-secretase complex is responsible for the final stage of amyloidogenesis, leading to the generation of Aβ(1-40) and Aβ(1-42). γ-secretase inhibition was initially considered a promising disease-modifying strategy. However, substrate promiscuity presents similar issues facing β-secretase inhibitors. Notch protein, responsible for regulating cell proliferation, development, differentiation, and cellular communication is one of the targets of the γ-secretase. Just as with the β-secretase inhibitors, off-target secondary effects are a major concern (Imbimbo & Giardina, 2011; Wolfe, 2012).

Semagacestat (LY450139) is a γ-secretase inhibitor that decreased Aβ levels in blood and CSF in humans. The clinical study results (NCT00762411, NCT01035138, and NCT00762411) which recruited over 3000 patients, are an example of the worst possible outcomes. It was reported that semagacestat treatment was associated with the worsening of cognition and the abilities to carry out the activities of daily living (ADAS-cog scale) in AD patients. Additional side effects included weight loss, increased incidence of skin cancer, and a higher risk of infection (Doody et al., 2013). Avagacestat is another γ-secretase inhibitor the development of which was discontinued as a result of a lack of efficacy (NCT00810147, NCT00890890, NCT00810147 and NCT01079819) (Dockens et al., 2012; Tong et al., 2012).

Soluble γ-secretase modulators (SGSM) may, in theory, be developed in such a way as to avoid the adverse events associated with general enzyme inhibition. The goal of such treatments is to block APP processing without interfering with other signaling pathways like Notch (D'Avanzo et al., 2015).

SGSM development began with the observation that several nonsteroidal anti-inflammatory drugs (NSAIDs) decreased Aβ(1-42) peptide levels *in vitro* and *in vivo* (Eriksen et al., 2003; Heneka et al., 2015). Examples of these drugs are ibuprofen, sulindac, indomethacin and flurbiprofen. The accepted mechanism of action (MOA) of NSAIDs is the inhibition of cyclooxygenase (COX) enzymes. While ibuprofen is a COX inhibitor, R-flurbiprofen (Tarenflurbil) is not, and its effects on the reduction of Aβ levels cannot be attributed to COX inhibition. Unfortunately, Tarenflurbil and Ibuprofen did not show efficacy for AD treatment in their respective clinical trials (Miguel-Álvarez et al., 2015; Pasqualetti et al., 2009). CHF5074, just like R-flurbiprofen, is an NSAID devoid of COX inhibitory activity. CHF5074 may inhibit Aβ production presumably by blocking γ-secretase complexes. Recent studies have reclassified this compound as a microglial modulator on the basis of its ability to reduce both amyloid burden and microglial activation (Ronsisvalle et al., 2014). Results from a Phase II trial in patients with Mild Cognitive Impairment (MCI) indicate that CHF5074 treatment leads to improvements of several cognitive measures and reduces inflammatory marker levels in the CSF (Ross et al., 2013).

The idea that the long-term use of NSAIDs could confer some protection against AD generated some interest in NSAIDs as a treatment potentially useful for reducing Aβ(1-42) levels. However, negative results reported in clinical trials with NSAIDs suggest that this hypothesis requires further refinement (Miguel-Álvarez et al., 2015).

Another example of a possible γ-secretase modulator is NIC5-15, which is a naturally occurring molecule. NIC5-15, also known as pinitol, is a natural cyclic sugar alcohol (Pitt, Thorner, Brautigan, Larner, & Klein, 2013). This compound supposedly modulates γ-secretase and is reportedly capable of reducing Aβ

production, while not affecting the substrate cleavage of Notch. No peer-reviewed data are available for this compound in human treatment, so any reported results should be considered as a forward-looking statements requiring rigorous scientific proof. However, it is claimed that the compound improves cognitive function and memory in preclinical models of AD neuropathology. If true, these data suggest that NIC5-15 may be a suitable therapeutic agent for the treatment of AD for two reasons: a) it preserves Notch activity and b) also it is potentially an insulin sensitizer. Moreover, it is supposedly being investigated as an anti-inflammatory agent because it may prevent microglia activation. Once again, these results have not yet been confirmed by independent researchers. A modest phase II trial of pinitol in AD patients has recently been completed (NCT00470418) with no results released into public domain.

3.3.3 Inhibition of β-amyloid peptide aggregation

Aβ peptide aggregates give rise to amyloid plaques. The following compounds were developed in order to prevent senile plaque formation.

The only inhibitor of Aβ aggregation that reached Phase III trials is the 3-amino-1-propaneosulfonic acid (3-APS, Alzhemed, tramiprosate) (Aisen et al., 2011; Gauthier et al., 2009). This medication was designed to interfere with or antagonize the interaction of soluble Aβ with endogenous glycosaminoglycans. Glycosaminoglycans have been shown to promote aggregation of Aβ amyloid fibril formation and deposition (Gupta-Bansal, Frederickson, & Brunden, 1995). However, the disappointing results of the above mentioned clinical trial have led to the suspension of this compound in Europe.

Colostrinin, a complex of proline-rich polypeptides present in ovine, bovine and human colostrum inhibits aggregation of Aβ and its neurotoxicity in cell assays, and improves cognitive performance in mice models (Janusz & Zabłocka, 2013). Although a phase II trial showed slight improvements in Mini Mental State Evaluation in patients with mild AD in a treatment period of 15 months, this beneficial effect was not maintained after another 15 months of continuous treatment (Bilikiewicz & Gaus, 2004).

Scyllo-inositol (ELND005) is an oral amyloid anti-aggregation agent capable of reducing Aβ toxicity in the mouse hippocampus. 18-month long Phase II clinical trial with ELND005 was conducted in participants with mild-to-moderate AD. This dose-finding, safety and efficacy trial did not meet its primary clinical efficiency outcomes (S. Salloway et al., 2011).

Clinical trials for AD treatment were also performed with metal chelating 8-hydroxiquinolines (8-HQ) compounds clioquinol and PBT2. While their mechanism of action is not completely understood, it is thought that these molecules block the interaction between the base metals and brain Aβ peptide (Matlack et al., 2014). It was suggested that increased levels of oxidative stress in the brain of AD patients may be

partially due to copper ions binding to Aβ, leading to metal-mediated generation of Reactive Oxygen Species (ROS). It was also hypothesized that 8-HQs may prevent Aβ aggregation while simultaneously restoring homeostasis in cellular levels of copper and zinc ions (Robert, Liu, Nguyen, & Meunier, 2015; Ryan et al., 2015; Wang et al., 2014). The results of the clinical trials with these compounds in AD are inconclusive (Faux et al., 2010; Jenagaratnam & McShane, 2006).

3.3.4 Compounds which promote the removal of amyloid deposits and aggregates

Another potential treatment option which is centered on the amyloidogenic pathway is to promote the clearance of existing amyloid aggregates and deposits. To achieve this, three different strategies have been evaluated:

Activation of enzymes that degrade amyloid plaques: Aggregates and amyloid plaques are degraded by multiple proteases including neprilysin, insulin-degrading enzyme (IDE), plasmin, endothelin-converting enzyme, angiotensin-converting enzyme and metalloproteinases. Protein levels of these enzymes decrease in AD, which contributes to the formation and accumulation of Aβ (Baranello et al., 2015; Higuchi, Iwata, & Saido, 2005; Nalivaeva, Beckett, Belyaev, & Turner, 2012). Despite being an attractive strategy for developing disease-modifying drugs, no compounds with this MOA have ever reached advanced clinical development due to a lack of specificity.

Modulation of β-amyloid transport between the brain and the peripheral circulation: Aβ transport between the CNS and the peripheral circulation is regulated by: 1) apolipoproteins, for example Aβ may be transported from the blood to the brain when it is bound to APOE (Giau, Bagyinszky, An, & Kim, 2015); 2) low-density lipoprotein receptor-related protein (LRP-1), which increases Aβ outflow from the brain to the blood (R. Deane, Sagare, & Zlokovic, 2008); 3) receptor for advanced glycation end products (RAGE), which facilitates the transport of Aβ across the blood-brain barrier (BBB) (R. J. Deane, 2012).

The goal of any treatment which is focused on this mechanism is to reduce cerebral amyloid load by attempting to restrict Aβ to the peripheral circulation. To this end, a number of different strategies have been proposed, notably the peripheral administration of LRP-1. However, the only drug candidates that have reached clinical stage are the inhibitors/modulators of RAGE. These include PF-0449470052, which failed in phase II trials, and TTP4000, with the Phase I trial completed in February 2013 (NCT01548430). The results of this trial have not been published.

3.4 Anti-amyloid immunotherapy

3.4.1 Active immunotherapy

Active immunotherapy is a strategy aimed to promote Aβ clearance with the objective of reducing the amyloid load in AD. Active immunization (vaccination) with either Aβ(1-42) (predominant form found in senile plaques) or other synthetic fragments has been successfully evaluated in transgenic mouse models of AD. Assays are generally based on the stimulation of B cells, T cells and immune responses through activation of the phagocytic capacity of microglia. Human tests were initially promising, however treatment with the first-generation vaccine (AN1792) has produced serious adverse events that led to the discontinuation of the Phase II trial. AN1792 consists of a synthetic full-length Aβ(1-42) peptide with a QS-21 adjuvant. As a result of a T-cell-mediated autoimmune response, 6% of patients have developed cerebral inflammation which turned out to be aseptic meningoencephalitis (Gilman et al., 2005).

Second-generation vaccines were designed using a shorter Aβ(1-6) peptide segment in an attempt to prevent nonspecific immune response seen with the full-length vaccine. CAD106, designed by Novartis, was the first second-generation vaccine that reached the clinical phases of development (Wiessner et al., 2011). A recently completed Phase II clinical trial have shown a Aβ-specific antibody response in 75% of treated patients, without causing adverse inflammatory reactions. ACC-001, developed by Janssen, has recently completed two phase II trials (NCT01284387 and NCT00479557) with an additional phase II trial still ongoing (NCT01227564). However, the pharmaceutical company has abandoned the plans for further development of this vaccine. Other vaccines, including tetra-palmitoylated Aβ(1-15) reconstituted in a liposome (ACI-24) (Muhs et al., 2007) and MER5101 (Liu et al., 2013) are currently in various stages of preclinical development.

3.4.2 Passive immunization

This therapy involves passive administration of monoclonal or polyclonal antibodies directed against Aβ. It consists of the intravenous administration of anti-Aβ antibodies to the patient. An advantage of this strategy compared to active immunization is that the pro-inflammatory T cell-mediated immune reactions should not occur. Studies in transgenic animals have shown that passive immunization reduces cerebral amyloid load and improves cognition, even when the amyloid plaque numbers are not significantly reduced. This could be attributed to the neutralization of soluble amyloid oligomers, which are increasingly recognized to play a fundamental role in the pathophysiologic cascade of AD.

Bapineuzumab and solanezumab are two monoclonal antibodies that have reached advanced stages of clinical development (Tayeb, Murray, Price, & Tarazi, 2015). However, in 2012, two Phase III clinical trials had failed because of a lack of efficacy in patients with mild-to-moderate AD. Both bapineuzumab and solanezumab are humanized monoclonal antibodies against Aβ(1-6) and Aβ(12-28) respectively. In case of bapineuzumab, significant reduction in brain amyloid plaques and phosphorylated tau in

cerebrospinal fluid was reported. However, the treatment failed to produce significant improvements of cognitive function (Stephen Salloway et al., 2014). In a solanezumab trial, infusions of 400 mg of solanezumab or placebo were administered once a month for 80 weeks in patients with mild-to-moderate AD. The results suggested that solanezumab may improve cognition in mild AD, however statistical significance was not achieved in that study (Doody et al., 2014). Currently solanezumab is in Phase III trials in patients with AD (NCT01127633 and NCT01900665) and in older individuals who have normal thinking and memory function but who may be at risk of developing AD in the future (NCT02008357).

Another monoclonal antibody, gantenerumab, is being investigated in people at risk of developing presenile AD due to genetic mutations. NCT01760005 trial is still recruiting participants and will determine the efficacy of both gantenerumab and solanezumab in the prodromal disease stages. In parallel, two additional Phase III trials of gantenerumab in patients with mild AD (NCT02051608) and prodromal AD (NCT01224106) are ongoing. Gantenerumab is a fully human IgG1 antibody designed to bind with high affinity to a conformational epitope on the β-amyloid fibres. Microglia recruitment and ensuing phagocytosis will presumably lead to amyloid plaque degradation. Experimental studies in transgenic mice support this hypothesis (Bohrmann et al., 2012; Jacobsen et al., 2014; Novakovic et al., 2013).

Crenezumab (MABT5102A) is a humanized monoclonal antibody which uses IgG4 backbone (Bouter et al., 2015). A Phase II clinical trial to assess the safety and efficacy in patients with mild-to-moderate AD (NCT01343966) was completed in April 2014, although the results are not yet publicly available. The most recent Phase II trial aiming to evaluate the safety and efficacy of crenezumab in asymptomatic carriers of E280A autosomal-dominant mutation of PS1 commenced in November 2013 (NCT01998841).

Other monoclonal antibodies against Aβ developed so far include PF-04360365 (ponezumab) which targets the free carboxy-terminal amino acids 33-40 of the Aβ peptide (Miyoshi et al., 2013); MABT5102A, which binds to Aβ monomers, oligomers and fibrils with equally high affinity (Adolfsson et al., 2012); GSK933776A, which is similar to bapineuzumab in that it binds to the N-terminal Aβ(1-5) (Novakovic et al., 2013). In addition, other passive immunotherapies mostly in Phase I clinical development include NI-101, SAR-228810 and BAN-2401 (Ghezzi, Scarpini, & Galimberti, 2013).

Intravenous immunoglobulin product Gammagard is a preparation of antibodies from human plasma. Its safety for human use had been previously demonstrated in certain autoimmune conditions. Gammagard effects were evaluated in a small number of AD patients (NCT00818662). It is believed that this mixture contains a small fraction of polyclonal antibodies against the Aβ peptide. In addition, this preparation may possess immunomodulatory properties that could potentially enhance microglial phagocytosis (Dodel et al., 2013; Relkin et al., 2009).

3.5 Strategies focused on tau proteins

Tau proteins are highly soluble and abundant in the neurons where they play a critical role in microtubule stabilization, particularly in axons. Tau hyperphosphorylation leads to the formation of insoluble paired helical filaments (PHF) which form neurofibrillary tangles (see **Fig. 1** on page 17). The loss of microtubule-binding capacity provokes cytoskeleton destabilization, which eventually causes neurodegeneration and neuronal death (Cowan & Mudher, 2013). As an alternative to amyloidocentric approaches, Tau-centered treatments aim to inhibit the phosphorylation and/or aggregation of tau protein. In addition, microtubule-stabilizing drugs could be used as a disease-modifying strategy in AD. In recent years, immunomodulation was suggested as a viable option for promoting effective clearance of tau aggregates (West & Bhugra, 2015).

3.5.1 Inhibitors of tau hyperphosphorylation

All tau proteins are a product of alternative splicing of a microtubule-associated protein tau (MAPT) gene. Phosphorylation is the primary mechanism which regulates tau binding to microtubules. Under physiological conditions the protein remains soluble, however in AD, pathological hyperphosphorylation of tau compromises its normal functions. Hyperphosphorylation occurs as a result of an imbalance between the catalytic activity of kinases and phosphatases. Increased expression of active forms of various kinases in the areas proximal to neurofibrillary tangles has been described in AD, including CDK5, GSK3β, Fyn, stress-activated protein kinases JNK and p38, and mitogen-activated protein kinases ERK1 and ERK2. Some or all of these kinases contribute to the perpetuation of the phosphorylation of tau in neurofibrillary tangles (A. V. de la Torre et al., 2012; Folch et al., 2012, 2015; Iqbal, Gong, & Liu, 2014; Kimura, Ishiguro, & Hisanaga, 2014). As a result, significant research efforts have been devoted to the development of kinase inhibitors as a possible treatment strategy for AD. For example SP600125, a widely used pan-JNK inhibitor, exerts beneficial effects on cognition and reduces neurodegeneration in an APP/PS1 transgenic mouse model of AD (Zhou et al., 2015). It has been proposed that specific inhibition of JNK3 could be sufficient to bring similar benefits as seen with SP600125 in rodent models. Human data in AD patients indicate a positive correlation between the levels of JNK3 and Aβ (1-42) in the brain. Furthermore, JNK3 upregulation was detected in the CSF and was associated with memory loss. Thus, JNK3 inhibition remains a promising target for future therapies (Gourmaud et al., 2015).

CDK5 belongs to the family of serine/threonine cyclin-dependent kinases and is responsible for a number of physiological functions within the CNS, including neurite outgrowth and the regulation of axonal development. CDK5 catalytic activity is dependent on its direct association with p35, key regulator of CDK5 signaling. This cofactor is cleaved by a non-lysosomal protease calpain in a calcium-dependent

manner. Conversion of p35 to p25 results in prolonged activation and mislocalization of CDK5. Due to the increases in intracellular calcium levels observed in the brains of AD patients, pathological activation of CDK5 occurs, resulting in hyperphosphorylation of tau and neuronal cell death (Camins, Verdaguer, Folch, Canudas, & Pallàs, 2006; Kimura et al., 2014). CDK5 inhibition may thus also be potentially considered as a possible drug target. Currently existing CDK5 inhibitors roscovitine and flavopiridol have demonstrated neuroprotective properties in *in vitro* and *in vivo* models of excitotoxicity, ischemia and neurodegeneration (Jorda et al., 2003).

GSK3β inhibitors are arguably in the most advanced stages of clinical development for AD. Among the various drugs that are currently being studied, tideglusib, an irreversible inhibitor of GSK3β, has recently completed Phase II trials (NCT01350362). Tideglusib administration for a period of 26 weeks to patients with mild-to-moderate AD did not show clinical efficacy, and the compound has since been discontinued for this indication (Lovestone et al., 2015). Another study (NCT00948259) evaluated the safety and tolerability of a 20-week administration of NP031112 compared with placebo in patients with AD. No data has been reported for this study.

Phosphatase activation has also been considered as a possible drug target. Currently, there is only one protein phosphatase 2 (PP2A) agonist in development. Sodium selenite (VEL015) is undergoing Phase II trials in Australia (ACTRN12611001200976). Experimental studies have shown that sodium selenate reduces tau phosphorylation, both in cell culture and in mouse models of the disease. VEL015 administration to rodents have resulted in significant cognitive improvements and substantial reduction of neurodegenerative phenotype (Corcoran et al., 2010; van Eersel et al., 2010).

3.5.2 Inhibitors of tau aggregation

Hyperphosphorylated tau aggregates contribute to neurotoxicity observed in AD brain. Methylene blue dye (methylthioninium chloride - MTC) derivatives have shown some promise in inhibiting the formation of tau aggregates. Methylene blue disrupts the aggregation of tau, has the ability to inhibit amyloid aggregation, improves the efficiency of mitochondrial electron transport chain, reduces oxidative stress, prevents mitochondrial damage and is also a modulator of autophagy (Baddeley et al., 2015; Hochgräfe et al., 2015). The first-generation formulation containing methylene blue (Rember) appeared to stabilize AD progression in a clinical trial which lasted 50 weeks (Wischik, Harrington, & Storey, 2014). These results motivated researchers to develop a next-generation version of methylene blue, TRx 0237. This compound is a purified derivative of methylene blue which not only inhibits tau protein aggregation but also dissolves brain aggregates of tau. Several clinical trials are currently underway (NCT01626391, NCT01689233, NCT01689246, NCT01626378) to evaluate the potential efficacy of this drug in AD.

3.5.3 Microtubule stabilizers

Microtubule stabilization may potentially achieve a similar end-result as that seen with the inhibitors of tau hyperphosphorylation and aggregation. Paclitaxel is a microtubule-stabilizing drug currently in use in the oncology field. Unfortunately, this compound is incapable of crossing the BBB and its use is associated with serious adverse events, which limits its utility in AD (Patel, Gajbhiye, & Jain, 2015; Shemesh & Spira, 2011). In addition to paclitaxel, other microtubule-stabilizing compounds such as TPI-287 have been considered as a possible AD therapy. TPI-287 is a derivative of taxane, also used in cancer treatment. TPI 287 stabilizes the microtubules by binding to tubulin. NCT01966666 clinical trial will evaluate TPI-287 safety, pharmacokinetic properties and tolerability by intravenous infusion in mild-to-moderate AD.

Epothilone D is a microtubule-stabilizing compound which improved axonal transport, reduced axonal dystrophy, decreased tau neuropathology, and reduced hippocampal neuron loss, however drug development for AD was discontinued in 2013 after a failed clinical trial (Zhang et al., 2012).

With respect to tau, additional studies are necessary in order to better understand the exact molecular mechanisms involved in tau neurotoxicity. Recent studies comparing the neurotoxic profiles of various forms of tau suggest that a soluble form is likely the most toxic (Cowan & Mudher, 2013). This has been corroborated by a recent report specifically identifying oligomeric tau as toxic (Kumar et al., 2014). Therefore, future therapeutic strategies should be focused on targeting soluble forms of tau.

3.5.4 Anti-tau immunotherapy

Just as with the immunotherapies targeting Aβ, both passive and active immunization approaches against tau have been considered. In fact, it was demonstrated that reductions in tau aggregate formation and improved clearance of tau oligomers and insoluble aggregates could all be achieved with either active or passive immunotherapies (Anand & Sabbagh, 2015). In rodents, treatment with monoclonal antibodies directed against hyperphosphorylated tau has led to improvements in cognition and was not associated with significant adverse effects (Yanamandra et al., 2013).

In 2013 Axon Neuroscience began a Phase I trial to evaluate the safety and tolerability of AADvac-1, an active immunotherapy which consists of a synthetic peptide derived from the tau sequence coupled to keyhole limpet hemocyanin, the precise molecular nature of the antigen has not been disclosed (NCT01850238 and NCT02031198). AADvac-1 uses aluminum hydroxide as an adjuvant (Kontsekova, Zilka, Kovacech, Novak, & Novak, 2014). At the 2014 Alzheimer's Association International Conference (AAIC) in Copenhagen, good preclinical safety profile was reported for the treatment period of up to 6

months in rats, rabbits, and dogs. These early results are encouraging and it remains to be seen whether AADvac-1 will demonstrate acceptable safety and efficacy in human patients.

3.6 Dendritic Hypothesis (Aβ-PrPC–mGluR5–Fyn signaling)

Dendritic hypothesis is described in some detail on page 24. Some data suggest that soluble Aβ oligomers are the principal neurotoxic species responsible for dendritic pathology. Aβ oligomers may cause aberrant N-methyl-D-aspartate receptor (NMDAR) activation post-synaptically by forming complexes with the cell-surface prion protein (PrPC). PrPC is enriched at the neuronal post-synaptic density, where it interacts with Fyn tyrosine kinase-metabotropic glutamate receptor 5 complex (Fyn-mGluR5). Fyn activation occurs when Aβ is bound to PrPC-Fyn-mGluR5 complex. Activated in this manner, Fyn can cause tyrosine phosphorylation of the NR2B subunit of this NMDAR. This results in an initial increase and then a loss of cell-surface NMDARs (Um et al., 2013). Fyn overexpression accelerates synapse loss and the onset of cognitive impairment in the J9 (APPswe/Ind) transgenic AD mouse model, while its inhibition produces an opposite effect (Um et al., 2012). In addition, as mentioned earlier (page 40), Fyn can also contribute to tau hyperphosphorylation. Previous studies had reported elevated levels of Fyn in AD brain. Furthermore, Fyn was shown to phosphorylate tau at Tyr18 residue (Bhaskar, Yen, & Lee, 2005). Thus, Fyn appears to be a viable target in the treatment of AD pathology. Saracatinib (AZD0530) and masitinib (AB1010) are Fyn kinase inhibitors currently in Phase II and III clinical trials for mild-to-moderate AD (NCT01864655, NCT02167256, NCT00976118 and NCT01872598) (Folch et al., 2015). Both compounds are capable of blocking Fyn in a nanomolar range.

In a NCT00976118 clinical trial, oral masitinib was administered for a period of 24 weeks, concomitantly with one of the AChEIs (donepezil, rivastigmine, or galantamine) and/or memantine. In that study a significant improvement in the ADAS-Cog test response was reported. These results are encouraging, however the very small patient pool (n=26) on memantine in this Phase II trial is clearly not sufficient to draw conclusions on the potential efficacy of this compound (Piette et al., 2011). MOA of masitinib in AD is twofold. Apart from blocking Fyn, masitinib is also a stem cell factor (SCF) receptor (c-KIT) inhibitor. By inhibiting SCF/c-Kit signaling on mast cells (MCs), this compound may prevent neuroinflammation by blocking the activated MCs-microglia interactions (Folch et al., 2015).

3.7 5-HT6 receptors in Alzheimer's disease

5-HT6 receptors are expressed in areas of the CNS involved in learning and memory. Their inhibition was shown to promote acetylcholine release (Riemer et al., 2003). In AD, 5-HT6 antagonism may lead to the restoration of acetylcholine levels (Ramirez, Lai, Tordera, & Francis, 2014). This hypothesis is supported by evidence that the 5-HT6 receptor antisense oligonucleotides improve spatial learning and memory in the

Morris water maze test in normal rats (Woolley, Bentley, Sleight, Marsden, & Fone, 2001). 5-HT6 inhibitors may be useful in combination therapy, together with AChEIs. For example, Lu-AE-58054 (SGS-518) and PF-05212365 (SAM-531) are being considered as possible treatments for mild-to-moderate AD. Other compounds that are in various stages of clinical research are SUVN-502, AVN-322 and PRX-07034 (Ramírez, 2013).

3.8 Changing the concept: AD as a metabolic disorder

Clinical studies suggest that diabetes is a major risk factor that contributes to AD pathology. Results from published research indicate that there is a close link between insulin-deficient diabetes and cerebral amyloidosis (De Felice, 2013). Peripheral and central insulin signaling impairments are likely present in both diseases. As a result, "type-3 diabetes" hypothesis of AD was developed (page 24), which attempts to bridge the observed metabolic phenotypes present in diabetes and AD into a coherent framework. Insulin hormone is at a centerpiece of this hypothesis (S. M. de la Monte & Wands, 2008).

Observations made in the "Hisayama Study" indicate that altered expression of genes related to diabetes mellitus in AD brains may be a result of AD pathology, and suggest that peripheral insulin resistance, metabolic syndrome and/or full-blown diabetes may lead to worsening of cognitive symptoms. Impaired central insulin signaling in the hippocampal circuits, a key region involved in learning and memory, is likely present in AD (Hokama et al., 2014). Glucose toxicity, insulin resistance, oxidative stress, elevated levels of advanced glycation end products and cytokine-mediated neuroinflammation are among the proposed mechanisms by which diabetes could increase the risk of AD development. In a recent study, Clarke and colleagues demonstrated that intracerebroventricular infusion of soluble Aβ oligomers initiates neuroinflammatory cascades which eventually cause disturbances in peripheral glucose homeostasis (Clarke et al., 2015). Tumor necrosis factor α (TNFα) quite possibly plays an important role in this process (Lourenco et al., 2013).

As molecular mechanisms causing AD and T2DM pathologies are possibly related, it is logical to assume that drugs used in T2DM treatment may have a neuroprotective effect in AD. Thiazolidinediones (TZDs) are an example of anti-diabetic compounds whose possible role in AD was investigated. TZDs are agonists of peroxisome proliferator-activated receptor γ (PPAR-γ), which act by promoting PPAR-γ heterodimerization with the retinoid X receptor (RXR). PPAR-γ/RXR heterodimer is a transcription factor which regulates expression of genes involved in lipid and glucose metabolism. TZDs improve insulin sensitivity and reduce cytokine-dependent inflammation. Rosiglitazone and pioglitazone are used as anti-diabetic drugs which regulate glucose homeostasis by increasing insulin sensitivity, reducing blood glucose levels and improving lipid metabolism. Both compounds have also been studied as potential therapeutics

for AD treatment, with reported improvements in mitochondrial oxidative metabolism (Blalock et al., 2010; Gold et al., 2010; Risner et al., 2006). In animal models, pioglitazone modified various indices of brain aging but did not slow down the cognitive decline (Blalock et al., 2010). Rosiglitazone and pioglitazone also induce the expression of peroxisome proliferator-activated receptor-γ co-activator 1 alpha (PGC-1α), a molecule that plays multiple roles in mitochondrial biogenesis, energy metabolism and mitochondrial antioxidants expression. Previous studies have demonstrated that in human brain tissues, the expression of PGC-1α decreases with progression of AD dementia (Qin et al., 2009). Thus, PGC-1α upregulation may improve the mitochondrial energy metabolism and AD pathology (Katsouri, Parr, Bogdanovic, Willem, & Sastre, 2011).

Pioglitazone treatment improved memory and cognition in patients with mild-to-moderate AD in a small clinical trial (Sato et al., 2011). A much larger clinical trial demonstrated improvements in memory retention and attention with rosiglitazone treatment (6 months) in patients who did not possess an ApoE4 allele (Risner et al., 2006). However, another major phase III trial using rosiglitazone failed to show efficacy in AD (NCT00550420) (Gold et al., 2010). It is important to note that in these trials rosiglitazone was administered alone at dosages that were much lower than those needed to exert a beneficial effect on AD pathophysiology in rodent models of the disease. NCT00348140 is a recently completed clinical trial in which rosiglitazone was administered in combination with AChEIs in patients with mild-to-moderate AD. No results have yet been reported.

Intranasal insulin was also considered as a treatment option for AD. This particular route of administration is attractive as it bypasses the BBB. This is very significant because insulin transport to the brain from the periphery is dependent on active transport mechanisms which may become disrupted in AD. In addition, the probability of possible adverse events in peripheral tissues is minimized. In theory, insulin delivery directly to the brain will activate cerebral insulin signaling leading to improvements in memory processing and will result in neuroprotection (Claxton et al., 2015; Freiherr et al., 2013; Hölscher, 2014). A currently ongoing clinical trial NCT01767909 is evaluating long-term (12 months) efficacy of intranasal insulin (Humulin RU-100) in mild AD.

Other pancreatic hormones such as amylin may also play a role in AD. Adler and colleagues reported that patients with AD have reduced concentrations of plasma amylin. In transgenic animal models of AD, amylin and pramlintide (amylin analog) decreased brain Aβ levels and improved cognition. Interestingly, amylin also inhibited β-secretase enzyme, while pramlintide did not (Adler et al., 2014; Qiu & Zhu, 2014).

Section	MOA	Compound	Clinicaltrials.gov Trial ID # (if mentioned)
3.3.1	β-secretase inhibition	**E2609**	NCT01600859
		MK-8931	NCT01739348
		LY2886721	NCT01807026; NCT01561430
3.3.2	γ-secretase inhibition and modulation	**LY450139 (Semagacestat)**	NCT00762411; NCT01035138; NCT00762411
		Avagacestat	NCT00810147; NCT00890890; NCT00810147; NCT01079819
		NIC5-15 (pinitol)	NCT00470418
	NSAID; possible γ-secretase inhibition	**R-flurbiprofen (Tarenflurbil)**	
		Ibuprofen	
		CHF-5074	
3.3.3	GABAergic; binds soluble Aβ	**3-APS (Alzhemed, tramiprosate)**	
	Possible immunomodulator	**Colostrinin**	
	Aβ aggregation blocker?	**ELND005 (Scyllo-inositol)**	
	antifungal; metal chelator?	**Clioquinol**	
		PBT2	
3.3.4	RAGE inhibition/modulation	**PF-0449470052**	
		TTP4000	NCT01548430
3.4.1	Active anti-Aβ vaccine	**AN1792**	
		CAD106	
		ACC-001	NCT01284387; NCT00479557; NCT01227564
		ACI-24	
		MER5101	
3.4.2	anti-Aβ antibodies / passive immunization	**Bapineuzumab**	
		Solanezumab	NCT01127633; NCT01900665; NCT02008357; NCT01760005
		Gantenerumab	NCT01760005; NCT02051608; NCT01224106
		MABT5102A (Crenezumab)	NCT01343966; NCT01998841
		PF-04360365 (Ponezumab)	
		GSK933776A	
		NI-101	
		SAR-228810	
		BAN-2401	
	Intravenuos IgG product	**Gammagard**	NCT00818662
3.5.1	CDK5 inhibition	**Roscovitine**	
		Flavopiridol	
	GSK3β inhibition	**Tideglusib**	NCT01350362
		NP031112	NCT00948259
	PP2A agonism	**VEL015 (Sodium selenate)**	ACTRN12611001200976 *** Australian clinical trial
3.5.2	Tau aggregation inhibition	**Rember**	
		TRx 0237	NCT01626391; NCT01689233; NCT01689246; NCT01626378
3.5.3	Microtubule stabilization	**Paclitaxel**	
		TPI-287	NCT01966666
		Epothilone D	
3.5.4	Active anti-Tau vaccine	**AADvac-1**	NCT01850238; NCT02031198
3.6	Fyn inhibition	**AZD0530 (Saracatinib)**	NCT01864655; NCT02167256
	Fyn + c-KIT inhibition	**AB1010 (Masitinib)**	NCT00976118; NCT01872598
3.7	5-HT6 receptor inhibition	**Lu-AE-58054 (SGS-518)**	
		PF-05212365 (SAM-531)	
		SUVN-502	
		AVN-322	
		PRX-07034	
3.8	PPAR-g + PGC-1α agonism	**Rosiglitazone**	NCT00550420; NCT00348140
		Pioglitazone	
	Intranasal insulin	**Humulin RU-100**	NCT01767909

Table 2. A list of drug candidates which were considered as a possible AD treatment in humans

3.9 Summary

Data described in this section offer ample evidence that AD is a highly complex disease. AChEIs and memantine are the only drugs currently approved for this condition. Nevertheless, the field of AD research does not stand still and a multitude of treatment options are considered. Unfortunately, based on historical evidence, the vast majority of pharmaceuticals described here are likely to fail. Hopefully, a targeted therapy capable of reversing the course of AD will be developed some time in the future. In the meantime, both the scientific community and the pharmaceutical industry are likely to remain very busy identifying key targets responsible for this devastating disease. In our opinion, a truly successful therapy will need to take into account the convergence of multiple factors which form an integral part of the disease process. A combination of early detection in the prodromal stages of AD, the role of metabolism and the possibility of combined pharmacotherapies should be considered (**Fig. 3**).

DISCLAIMER: Large portions of text presented in Sections 3.3-3.8 were recently accepted for publication to Neural Plasticity Journal (Hindawi Publishing Corporation) (supplementary Publication on page 111).

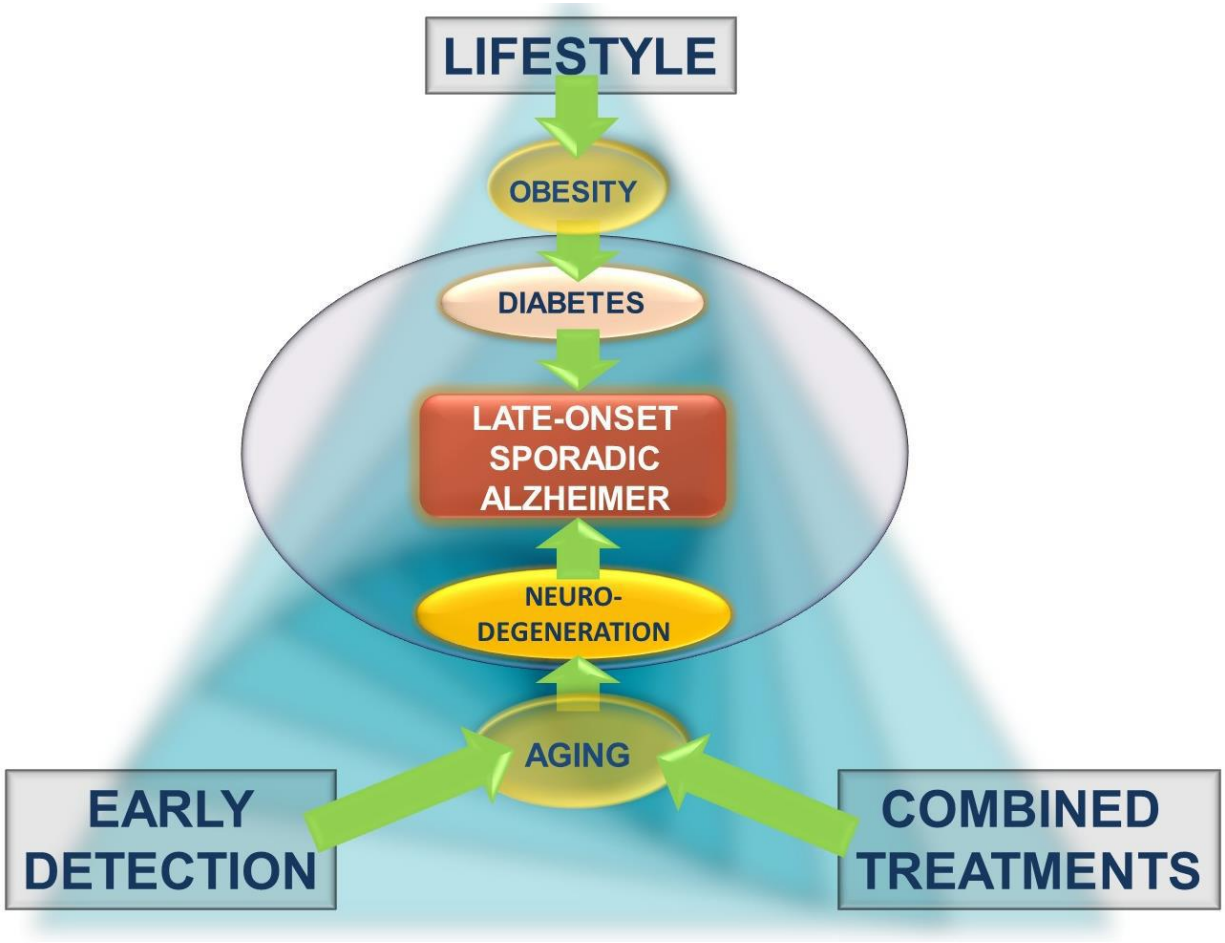


Figure 3. Future strategies in the treatment of late-onset and sporadic forms of AD could be centered on three main points: avoidance of habits and lifestyle leading to obesity and diabetes; early detection of AD biomarkers or structural alterations in pre-symptomatic individuals; and combined therapies in early phases of cognitive loss. *Accepted, Neural Plasticity (Hindawi Publishing Corporation)

OBJECTIVES

OBJECTIVES

Overall objectives of the present doctoral thesis are to determine the changes which occur at the molecular level as a result of a high-fat diet administration in 3- and 6-month old APPswe/PS1dE9 mice, a transgenic model of familial Alzheimer disease. More specifically:

1. Characterization of the peripheral and central metabolic phenotypes at 3 and 6 months of age in APPswe/PS1dE9 versus the wild-type mice on a standard chow in order to:
    A) Determine if transgenic status has an effect on peripheral carbohydrate metabolism
    B) Determine if hippocampal insulin signaling is altered in mice predisposed to Alzheimer disease development
    C) Identify molecular pathways which are altered in early stages of AD-like pathology, prior to amyloid plaque development and memory loss in hippocampal circuits

2. Characterization of the peripheral and central metabolic phenotypes in 6-month-old APPswe/PS1dE9 versus the wild-type mice on a high-fat diet. In addition to the 1A and 1B described above, the following objectives are considered:
    A) Determine if high-fat diet treatment differentially affects wild-type and transgenic mice
    B) Determine if chronic hypercaloric diet administration affects memory
    C) Determine if high-fat diet accelerates AD-like pathology
    D) Determine if hypercaloric diet affects hippocampal signaling molecules traditionally associated with AD

RESULTS

Publication 1

EARLY ALTERATIONS IN ENERGY METABOLISM IN THE HIPPOCAMPUS OF APPSWE/PS1DE9 MOUSE MODEL OF ALZHEIMER'S DISEASE

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Summary

APP/PS1 transgene causes CNS-directed overexpression of both a chimeric mouse/human APP and the exon 9-deleted variant of PS1. As a result, elevated levels of "humanized" Aβ protein are secreted in the brain of transgenic mice. In this paper, we determine peripheral metabolic status (by IPGTT and ITT) and the changes which occur in the hippocampus in 3- and 6-month-old animals. NOR test indicates that memory is not significantly affected in younger mice. At this age, neither diffuse nor fibrillar cerebral plaque deposits were detectable and no significant differences in glucose and insulin tolerance were observed. However, a significant increase in the insoluble Aβ (1-42) was detected in the cortical extracts of 3-month-old APP/PS1 mice. Furthermore, a number of genes related to memory (FOS, ARC) and insulin signaling (INS-R) were downregulated in the hippocampus of young transgenic animals, compared to wild-type. In addition, tau hyperphosphorylation, disruptions in mitochondrial OXPHOS and reduced expression of molecules responsible for mitochondrial biogenesis (PGC-1α, NRF1 and NRF2) were also detected. By the age of 6 months, transgenic mice developed readily visible senile plaques, pronounced memory loss, impaired glucose and insulin tolerance, continued disruptions in OXPHOS complexes and mitochondrial biogenesis signaling, as well as significantly increased levels of insoluble Aβ(1-40) and Aβ(1-42). These data indicate that hippocampal insulin signaling and mitochondrial metabolism deficiencies occur prior to the appearance of senile plaques, memory loss and peripheral carbohydrate metabolism alterations in an APP/PS1 mouse model of FAD.

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Early alterations in energy metabolism in the hippocampus of APPswe/PS1dE9 mouse model of Alzheimer's disease

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ABSTRACT

The present study had focused on the behavioral phenotype and gene expression profile of molecules related to insulin receptor signaling in the hippocampus of 3 and 6 month-old APPswe/PS1dE9 (APP/PS1) transgenic mouse model of Alzheimer's disease (AD). Elevated levels of the insoluble Aβ (1–42) were detected in the brain extracts of the transgenic animals as early as 3 months of age, prior to the Aβ plaque formation (pre-plaque stage). By the early plaque stage (6 months) both the soluble and insoluble Aβ (1–40) and Aβ (1–42) peptides were detectable. We studied the expression of genes related to memory function (*Arc*, *Fos*), insulin signaling, including insulin receptor (*Insr*), *Irs1* and *Irs2*, as well as genes involved in insulin growth factor pathways, such as *Igf1*, *Igf2*, *Igfr* and *Igfbp2*. We also examined the expression and protein levels of key molecules related to energy metabolism (PGC1-α, and AMPK) and mitochondrial functionality (OXPHOS, TFAM, NRF1 and NRF2). 6 month-old APP/PS1 mice demonstrated impaired cognitive ability, were glucose intolerant and showed a significant reduction in hippocampal *Insr* and *Irs2* transcripts. Further observations also suggest alterations in key cellular energy sensors that regulate the activities of a number of metabolic enzymes through phosphorylation, such as a decrease in the *Prkaa2* mRNA levels and in the pAMPK (Thr172)/Total APMK ratio. Moreover, mRNA and protein analysis reveals a significant downregulation of genes essential for mitochondrial replication and respiratory function, including PGC-1α in hippocampal extracts of APP/PS1 mice, compared to age-matched wild-type controls at 3 and 6 months of age. Overall, the findings of this study show early alterations in genes involved in insulin and energy metabolism pathways in an APP/PS1 model of AD. These changes affect the activity of key molecules like NRF1 and PGC-1α, which are involved in mitochondrial biogenesis. Our results reinforce the hypothesis that the impairments in both insulin signaling and energy metabolism precede the development of AD amyloidogenesis.

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1. Introduction

Alzheimer's disease (AD) is the most common cause of senile dementia and the incidence rates of the disease are increasing exponentially due to the amount of aged population. AD diagnosis is based on the detection of senile amyloid-β (Aβ) plaques and neurofibrillary tangles in the brain [1]. Although the exact mechanisms triggering neurodegeneration in AD remain unclear, a number of hypotheses have been proposed [2–11].

In recent years, several studies have focused on the potential relationship between AD and metabolic disorders [14–17]. Obesity and diabetes significantly increase the risks of cognitive decline and AD, suggesting that brain glucose metabolism impairments [18–25] may be linked to AD pathogenesis [14]. Both the AD and type 2 diabetes mellitus (T2DM) are associated with peripheral and central insulin signaling abnormalities, including alterations in brain insulin and insulin-like

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growth factor (IGF) levels [19–22]. Pathological changes in these signaling pathways affect neuronal survival, energy homeostasis, gene expression and memory processes [23–30]. For instance, insulin and IGF1 regulate the expression and phosphorylation of Tau protein through activation of kinases [26–36].

Therefore, AD could be considered as a "type 3 diabetes" metabolic disorder, with insulin providing the link connecting both chronic diseases [19–22]. Several studies have reported a role for insulin in the control of neuronal function in cortical and hippocampal areas, which are involved in memory processing and cognitive functions [24–26]. Thus, insulin directly influences neurons, modulates neurotransmitter release, neuronal outgrowth, neuronal survival, as well as synaptic plasticity [19]. Moreover, it has been demonstrated that soluble Aβ oligomers alter insulin signaling because they bind to insulin receptors in hippocampal neurons, thereby inducing receptor mobilization from the membrane and into the cell [15].

Another molecule implicated both in diabetes and AD is the insulin-degrading enzyme (IDE). IDE is capable of degrading both insulin and Aβ, however it binds insulin with a much higher affinity. In an animal model of T2DM, elevated levels of circulating insulin resulted in competitive inhibition of IDE, thus causing an increase in Aβ levels. Additionally, mice lacking IDE have lower rates of Aβ and insulin degradation, and develop hyperinsulinemia and Aβ deposits in the brain [19–21].

AD etiology is complex and Aβ by itself is unable to account for all aspects of AD [1–6,37–41]. In order to identify the underlying causes of the disease, it is of utmost importance to understand the potential correlations between Aβ oligomers and hippocampal metabolism in early disease-stages, prior to plaque deposition. Most of AD research is currently undertaken in animal models that have increased Aβ levels compared to controls, and while Aβ pathology is mimicked in these models, many other factors associated with AD are not. Transgenic mice that carry an APP and presenilin 1 (PS1) mutations show AD-like pathology and memory impairment, and are useful for studying AD and testing possible treatments [42]. The current study, carried out in an APP/PS1 model of AD, aimed to identify the metabolic pathways responsible for the onset of AD, with the main focus on the early disease-stages, prior to the formation of senile plaques and memory loss. For this purpose, we examined behavioral phenotype and mRNA expression and protein levels of genes related to insulin receptor and mitochondria signaling in 3 and 6 month-old APP/PS1 mice. Three month-old animals were chosen because at this age, neither significant cognitive loss, nor brain Aβ plaques are detectable compared to the six month-old mice, which exhibit high brain Aβ content and memory loss [12–14].

2. Materials and methods

2.1. Animals

Male APPswe/PS1dE9 and C57BL/6 mice were used in this study. APP/PS1 animals co-express a Swedish (K594M/N595L) mutation of a chimeric mouse/human APP (Mo/HuAPP695swe), together with the human exon-9-deleted variant of PS1 (PS1-dE9), allowing these mice to secrete elevated amounts of human Aβ peptide. Both mutations are associated with AD, are under control of the mouse prion protein promoter, directing both mutated proteins mainly to the CNS neurons, and result in age-dependent amyloid plaque depositions in mouse brain. The APPswe-mutated APP is a favorable substrate for β-secretase, whereas the PS1dE9 mutation alters β-secretase cleavage, thereby promoting overproduction of Aβ42. The animals were kept under controlled temperature, humidity and light conditions with food and water provided ad libitum. Mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya. Every effort was made to minimize animal suffering and to reduce the number of animals used. Fifty animals, divided into four groups, were used for the present study, with at least 10 wild-type and 10 APP/PS1 transgenic mice of 3 and 6 months of age, per group. Following in vivo testing, the animals were sacrificed and at least 6 mice in each group were used for RNA and protein extract isolation, with an additional 4 mice for immunohistochemistry.

2.2. Glucose and insulin tolerance tests

Intraperitoneal glucose tolerance tests (IP-GTT) and insulin tolerance tests (ITT) were performed in accordance with the previously published guidelines [72]. For IP-GTT, mice were fasted overnight for 16 h. The test was performed in a quiet room, preheated to +30 °C. The tip of the tail was cut with the heparin-soaked (Heparina Rovi, 5000 IU/ml; Rovi S.A.; Madrid, Spain) scissors, 30 min prior to 1 g/kg intraperitoneal glucose injection (diluted in H_2O). Blood glucose levels of the tail vein were measured at −30, 0, 5, 15, 30, 60 and 120 min after the glucose injection with the Ascensia ELITE blood glucose meter (Bayer Diagnostics Europe Ltd.; Dublin, Ireland). ITT was performed in similar conditions with the 0.25 IU/kg of human insulin, diluted in saline (Humulina Regular, 100 IU/ml/Lilly, S.A.; Madrid, Spain), except that the mice underwent a 5-hour morning fast. Blood glucose levels were measured at −30, 0, 15, 30, 45 and 60 min after the insulin administration. If, during this time, blood glucose levels dropped to below 20 mg/dl, 1 g/kg glucose was administered to counteract the effects of insulin, in order to reduce animal suffering.

2.3. Novel object recognition test

The test was conducted as described previously [43] in a 90° two arm, 25 cm long, and 20 cm high maze. The light intensity in the middle of the field was 30 lx. The objects to be discriminated were plastic figures (object A: 5.25 cm high, object B: 4.75 cm high). First, mice were individually habituated to the apparatus for 10 min a day, for two days. On the third day, they were submitted to a 10 min acquisition trial (first trial) during which they were placed in the maze in the presence of two identical novel objects (A + A, or B + B) placed at the end of each arm. A 10 min retention trial, with the objects (A + B) (second trial) occurred 2 h later. The amount of exploration time each animal spent on objects A and B during the acquisition trial varied between 5 and 20 s, depending on the individual mouse. Total exploration time between the 2 objects when calculated for each individual animal indicated the absence of the object preference bias (Fig. 1C) (n = 5–9 per group). During the retention trial, the times that the animal took to explore the new object (tn) and the old object (to) were recorded. A discrimination index (DI) was defined as (tn − to) / (tn + to). In order to avoid further object preference biases, objects A and B were counterbalanced so that half of the animals in each experimental group were first exposed to object A and then to object B, whereas the other half saw first object B and then object A. The maze, the surface, and the objects were cleaned with 96° ethanol between animals, so as to eliminate olfactory cues.

2.4. Immunohistochemistry

For detection of Aβ deposits, free-floating coronal sections, 20 μm thick, were rinsed with 0.1 mol/l PB, pH 7.2, and pre-incubated in 88% formic acid. Then, sections were treated with 5 ml/l H_2O_2 and 100 ml/l methanol in PBS and pre-incubated in a blocking solution (100 ml/l of FBS, 2.5 g/l of BSA and 0.2 mol/l of glycine in PBS with 5 ml/l of Triton X-100). After that, sections were incubated overnight (O/N) at 4 °C with the primary mouse anti-human beta-amyloid clone 6F/3D antibody (1:100; DakoCytomation, Denmark). Then, sections were incubated with the biotinylated secondary antibody (1:200; Sigma-Aldrich) followed by the avidin–biotin–peroxidase complex (ABC; 1:200; Vector, Burlingame, CA). Peroxidase reaction was developed with 0.5 g/l diaminobenzidine in 0.1 mol/l PB and 0.1 ml/l H_2O_2, and immunoreacted

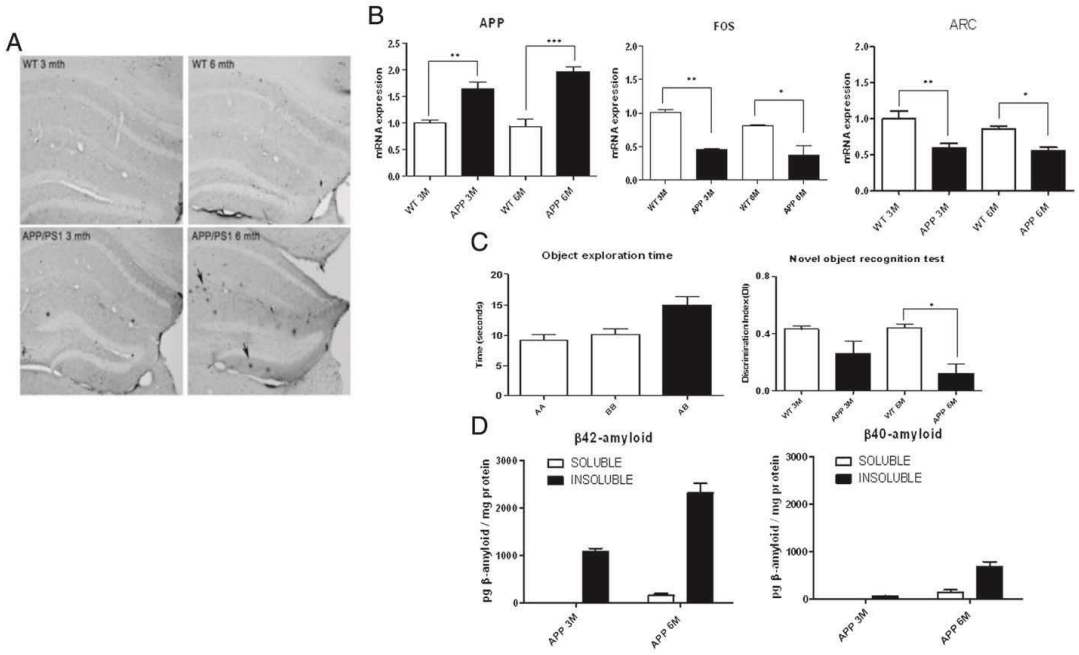

Fig. 1. Representative immunohistochemical staining with the Aβ-specific 6F/3D antibody, in 3 and 6 month-old mice, demonstrating Aβ plaque deposits in the hippocampus of 6 month-old APP/PS1 animals (A). mRNA expression profile of app, fos and Arc in the hippocampal extracts ($n = 6$) (B). The results of the 2 object novel object recognition test, demonstrating an absence of the object preference bias and a significant memory loss in 6 month-old APP/PS1 animals, compared to wild-type controls ($n = 7–12$) (C). Concentrations of the soluble and insoluble human Aβ (1–40) and Aβ (1–42) peptides in the cortical extracts in 3 and 6 month-old APP/PS1 mice, expressed as pg/mg of total protein as determined by ELISA ($n = 5–6$) (D). (Statistical analysis was performed with one-way ANOVA, with Tukey's post-hoc test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$.)

sections were mounted on gelatinized slides. Stained sections were examined under a light microscope (Olympus BX61).

2.5. Western blot analysis

Aliquots of hippocampus homogenate containing 15 mg of protein per sample were analyzed using the Western blot method. In brief, samples were placed in a sample buffer (0.5 m Tris–HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95–100 °C for 5 min. Samples were separated by electrophoresis on 10–15% acrylamide gels. Following this, the proteins were transferred to PVDF sheets using transblot apparatus. Membranes were blocked overnight with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris; 1.5% NaCl, 0.05% Tween 20, pH 7.5). They were then incubated with primary antibodies, as detailed in Table 1. After O/N incubation, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG secondary antibody (1:2000). Immunoreactive protein was detected using a chemiluminescence-based detection kit. Protein levels were determined by densitometry, using Chemi doc XRS + Molecular Imager detection system (Bio-Rad), with ImageLab image analysis software. Measurements are expressed as arbitrary units. All results are normalized to GAPDH, unless stated otherwise.

2.6. Serum insulin ELISA

Heart puncture was used to collect whole blood samples from 3 and 6 month-old wild-type and APP/PS1 mice, following a 5-hour morning fast, at the point of sacrifice. Blood samples were transferred to Serum-Gel Z microcentrifuge tubes (Sarstedt, Numbrecht, Germany), for serum separation. The samples were collected and kept at room temperature, and the serum was separated by centrifugation for 10 min at 5000 ×*g*. Serum insulin levels were measured with Rat/Mouse Insulin ELISA kit (Cat #: EZRMI-13K; EMD Millipore; St. Charles, MO, USA), according to manufacturer's instructions, utilizing 10 μl of mouse serum.

Table 1
A list of antibodies used for the immunoblotting experiments.

Protein	Antibody
pAMPK (Thr 172)	#2531 (Cell signaling)
AMPK	#2532 (Cell signaling)
PGC1A	101707 (Cayman chemical)
TFAM	DR1071b (Calbiochem)
NRF1	Sc-28379 (Santa Cruz biotech)
IDE	Ab32216 (Abcam)
pGSK3B (Tyr 216)	Ab74754 (Abcam)
pGSK3B (Ser 9)	#9336 (Cell signaling)
GSK3B	#9315 (Cell signaling)
pCDK5 (Tyr 15)	ab63550 (Abcam)
CDK5	Sc-173 (Santa Cruz biotech)
P35	#2680 (Cell signaling)
pTAU (Ser 199)	44734G (Life Technologies)
pTAU (Thr 205)	44738G (Life Technologies)
pTAU (Ser 396)	44752G (Life Technologies)
pTAU (Ser 404)	44748G (Life Technologies)
TAU5	AHB0042 (Biosource)
PSD-95	Ab18258 (Abcam)
SYP	M0776 (DakoCytomation)
OXPHOS	MS604 (MitoSciences)
GAPDH	MAB374 (Millipore)
2nd–ary Anti-Mouse	170-5047 (Biorad)
2nd–ary Anti-Rabbit	NA934V (GE Healthcare)

2.7. Measurement of Aβ peptides in brain tissues by ELISA

Aβ 1–40 and Aβ 1–42 were measured in cortical extracts according to a previously published procedure [44]. In brief, the samples were weighed and homogenized in a 8× volume of PBS with AEBSF protease inhibitor cocktail set (Cat # 539131; Calbiochem; La Jolla, CA, USA). The soluble fraction was separated by centrifuging the samples for 10 min at 4000 ×g. The pellets containing insoluble Aβ peptides were solubilized in a 5 M guanidine HCl/50 mM Tris HCl solution by incubating for 3.5 h on an orbital shaker at room temperature in order to obtain insoluble fraction. The levels of soluble and insoluble Aβ 1–40 and Aβ 1–42 were determined employing the commercially available human ELISA kits (Cat # KHB3481 and KHB3441; Invitrogen, Camarillo, CA, USA). Data obtained from the cortical homogenates are expressed as picograms of Aβ content per milligrams of total protein (pg/mg).

2.8. RNA extraction and quantification

Total RNA was isolated from the hippocampi of wild-type and APP/PS1 transgenic mice, as described previously [73]. Briefly, the tissue was homogenized in the presence of Trizol reagent (Life Technologies Corporation). Chloroform was added and the RNA was precipitated from the aqueous phase with isopropanol at 4 °C. RNA pellet was reconstituted in RNAse-free water, with the RNA integrity determined by Agilent 2100 Bioanalyzer.

2.9. Quantitative RT-PCR

First-strand cDNA was reverse transcribed from 2 μg of total RNA from the hippocampi of 3 and 6 month-old mice, using the High Capacity cDNA Reverse Transcription kit, according to manufacturer's protocol (Applied Biosystems). Equal amounts of cDNA of each individual animal were subsequently used for qRT-PCR, and each sample was analyzed in triplicate for each gene. TaqMan gene expression assays (Applied Biosystems) as detailed in Table 2 were used to determine transcription levels of individual genes. qRT-PCR was performed on the StepOnePlus Real Time PCR system (Applied Biosystems) and normalized to the average transcription levels of gapdh and tbp, using the delta–delta Ct method.

2.10. Statistical analysis

All data are presented as means ± SEM, and differences are considered significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$. Differences between samples/animals were evaluated using either one-way ANOVA, with Tukey's post-hoc test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$ and with the student's t-test, where $ denotes $p < 0.05$, $$ denotes $p < 0.01$ and $$$ denotes $p < 0.001$. Both the statistical analyses and the graphs presented here were created with the GraphPad InStat software V5.0 (GraphPad Sofware Inc., San Diego, CA, USA).

Table 2
A list of probes used for qRT-PCR analyses.

Gene	TaqMan probe
app	Mm01344172_m1
arc	Mm00479619_g1
fos	Mm00487425_m1
gapdh	Mm99999915_g1
igf1	Mm01228180_m1
igf2	Mm00439564_m1
igfbp2	Mm00492632_m1
igf1r	Mm00802831_m1
ins1	Mm01950294_s1
insr	Mm01211875_m1
irs1	Mm01278327_m1
irs2	Mm03038438_m1
nrf1	Mm01135606_m1
nfe2l2	Mm00477784_m1
Ppargc1a	Mm01208835_m1
prkaa1	Mm01296700_m1
prkaa2	Mm01264789_m1
tbp	Mm00446971_m1
tfam	Mm00447485_m1

3. Results

3.1. Early phenotypical signs of amyloidogenesis in APP/PS1 mice

In order to determine the extent of the amyloid deposition in the brains of APP/PS1 mice, hippocampal sections were stained with the 6F/3D monoclonal antibody, which is specific for the human form of the Aβ peptide. It was previously reported that the amyloid plaque deposits first start to appear at the age of 6 months in this model [13,14]. In agreement with the earlier studies, our immunohistochemical results demonstrated neither diffuse nor fibrillar plaque deposition in the brains of APP/PS1 mice at 3 months of age. In contrast, Aβ protein aggregates were clearly visible by the age of 6 months (Fig. 1A). In addition, a significant increase in the mRNA levels of the app (utilizing a probe which recognizes both human and mouse transcripts) was detected in hippocampal extracts of both 3 and 6 month-old animals. Likewise, we observed a significant reduction in the mRNA levels of *Arc* and *fos*, which play a role in memory function, in the brains of APP/PS1 mice [45] (Fig. 1B). Moreover, we have characterized the progression of cognitive impairment, utilizing a 2 object novel object recognition test. Our results showed a significant memory loss in 6 month-old APP/PS1 mice (Fig. 1C).

Interestingly, we have detected significant levels of the insoluble Aβ (1–42) peptides in the cortical homogenates of 3 month old APP/PS1 mice (>1000 pg/mg), at an age when Aβ plagues are not yet detectable by immunohistochemistry. The amyloid burden was further increased in 6 month old transgenic animals, with the detectable levels of both soluble and insoluble Aβ (1–40) (>140 and >680 pg/mg) and of soluble and insoluble Aβ (1–42) (>150 and >2300 pg/mg) (Fig. 1D).

3.2. Glucose and insulin tolerance tests and peripheral insulin levels

Since a connection between AD and T2DM has been established during the past decade, we intended to identify any metabolic perturbations in glucose metabolism in the APPswe/PS1dE9 strain [18]. In fact, as shown in Fig. 2, APP/PS1 mice exhibited impaired fasting glucose and insulin tolerance, following IP-GTT and ITT, respectively. Interestingly, the biggest differences in blood glucose levels, between the wild-type and transgenic animals, were detected from 30 to 120 min following i.p. glucose administration in IP-GTT or insulin administration in ITT. In addition, a slight increase in fasting peripheral insulin levels, determined by ELISA, was observed in 6 month-old APP/PS1 mice, compared to age-matched controls. Having confirmed the existence of the peripheral metabolic phenotype in the APP/PS1 mice, we then proceeded to study the expression of genes related to insulin metabolism in the brain, with a particular focus on hippocampal insulin receptor signaling pathway.

3.3. Identification of differentially expressed genes related to insulin receptor

Previous studies demonstrated alterations in brain insulin signaling in AD, but the onset and the severity of this impairment are unclear [43–46]. For this reason, we evaluated mRNA expression of preproinsulin 1 (*Ins1*), insulin receptor (*Insr*), insulin receptor substrates 1 (*Irs1*) and 2 (*Irs2*), insulin-like growth factors I (*Igf1*) and II (*Igf2*), IGF receptor (*Igfr*), as well as insulin-like growth factor-binding protein 2 (igfbp2), at 3 and 6 months of age (Fig. 3). We detected a small, but significant reduction

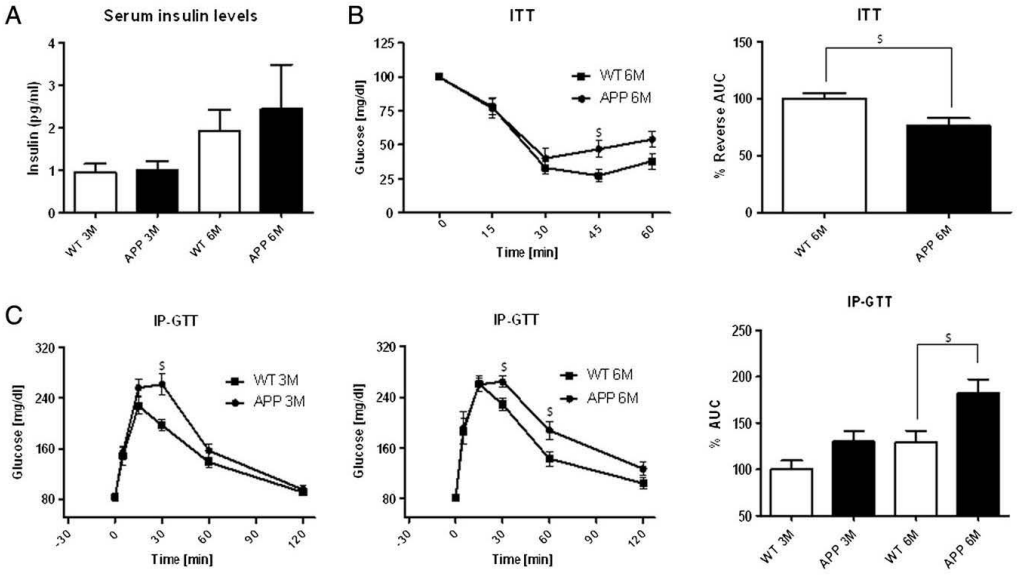

Fig. 2. Fasting serum insulin levels ELISA (n = 5–7) (A), insulin tolerance test (n = 5–7) (B) and intraperitoneal glucose tolerance test (n = 5–12) (C) in 3 and 6 month-old wild-type and APP/PS1 mice. For the ITT and the IP-GTT, AUC data were calculated from the time point 0 till the end of the experiment (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$.)

in *insr* and *irs2* transcripts in the hippocampal extracts of 3 month-old APP/PS1 mice, compared to age-matched controls. Interestingly, the expression of both *Igf2* and *Igfbp2* transcripts was significantly increased at 6 months in transgenic animals.

3.4. Energy metabolism is impaired in the early stages of the amyloidogenesis

AMP-activated protein kinase (AMPK) is a sensor of cellular stress that maintains energy homeostasis by promoting mitochondrial

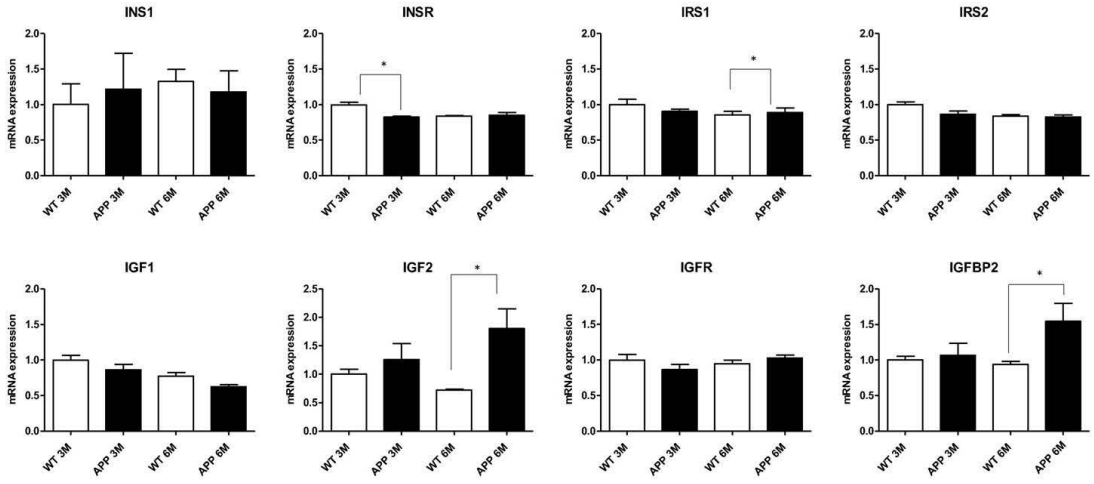

Fig. 3. mRNA expression profile of genes related to insulin signaling pathway in the hippocampal extracts of 3 and 6 month-old wild-type and APP/PS1 mice (n = 4–6). (Statistical analysis was performed with one-way ANOVA and with Tukey's post-hoc test, where * denotes $p < 0.05$.)

biogenesis through transcriptional coactivator peroxisome proliferator activated receptor-γ coactivator 1α (PGC-1α) signaling pathway [47–54]. We detected a significant reduction in the mRNA levels of the alpha 2 (*Prkaa2*), but not of the alpha 1 (*Prkaa1*) isoform of the catalytic subunit of AMPK, in the hippocampi of 3 month-old APP/PS1 mice, compared to wild-type controls (Fig. 4A). This observation is consistent with the slight reduction in the protein levels of phosphorylated AMPK (pAMPK (Thr172)/Total APMK ratio) (Fig. 4B). Thus, our data suggest alterations in a key cellular energy sensor that regulates the activities of a number of molecules involved in cellular metabolism.

PGC-1α is involved in energy homeostasis and glucose metabolism, as well as in mitochondrial metabolism and biogenesis [49,50]. In the current study, we detected a significant reduction in PGC-1α mRNA and protein (Fig. 4) levels in the hippocampi of 3 and 6 month-old APP/PS1 mice, compared to control animals. Since PGC-1α regulates the transcriptional activity of genes essential for mitochondrial replication and respiratory function, such as estrogen-related receptor α (ERR-α), nuclear respiratory factor (NRF) and mitochondrial transcription factor A (TFAM), we proceeded to study their mRNA and protein levels.

3.5. The impairment of mitochondrial biogenesis is involved in the early stages of the amyloidogenesis

It has been hypothesized that mitochondrial dysfunction could be a trigger of AD [49–54]. In fact, mRNA expression analysis of genes, downstream to PGC-1α, confirmed significant reductions in *Nrf1* and *Nrf2* transcripts in the hippocampi of 3-month old APP/PS1 animals, compared to controls. mRNA levels of *Tfam* were also slightly reduced at this age, although the observed changes did not reach statistical significance (Fig. 4A). These data suggest that reduced mitochondrial biogenesis is an early event in the hippocampus of APP/PS1 mice.

3.6. Mitochondrial OXPHOS expression

Deregulation in OXPHOS signaling is indicative of mitochondrial function impairment and has been previously reported in the brains of 3 and 6 month old APP mice [46,47]. In agreement with the above mentioned studies, we have detected a significant reduction in OXPHOS complexes I, II, III, and IV in the hippocampi of the 3 month old APP/PS1 mice (Fig. 5).

3.7. Tau phosphorylation and Tau kinase levels

Since Tau expression is regulated by insulin/IGF-I, and also by AMPK, increased Tau phosphorylation could be an early event in the brains of APP/PS1 mice. It is well-known that Tau is a microtubule-binding protein participating in neuronal cytoskeletal dynamics maintenance and axonal transport [55–59]. Evaluation of several Tau phosphoepitopes by Western blotting, revealed a global increase in Tau phosphorylation in the hippocampal extracts of APP/PS1 mice, both at 3 and 6 months of age (Fig. 6).

3.8. Involvement of GSK-3β and CDK5 kinases in the early stages of the amyloidogenesis and Tau phosphorylation

Insulin and IGFI signaling regulate the expression and phosphorylation of Tau proteins, as impaired insulin function leads to the overactivation of GSK-3β, a kinase capable of Tau phosphorylation. Our results show a significant increase in p35 content and pCDK5 (Tyr15)/CDK5 ratios in the hippocampus of APP/PS1 animals, compared to controls, both at 3 and 6 months of age. In contrast, the protein expression levels of GSK-3β, phosphorylated at Ser9 (inactive form), and Tyr216 (active form), as well as of IDE, remained unchanged (Fig. 7).

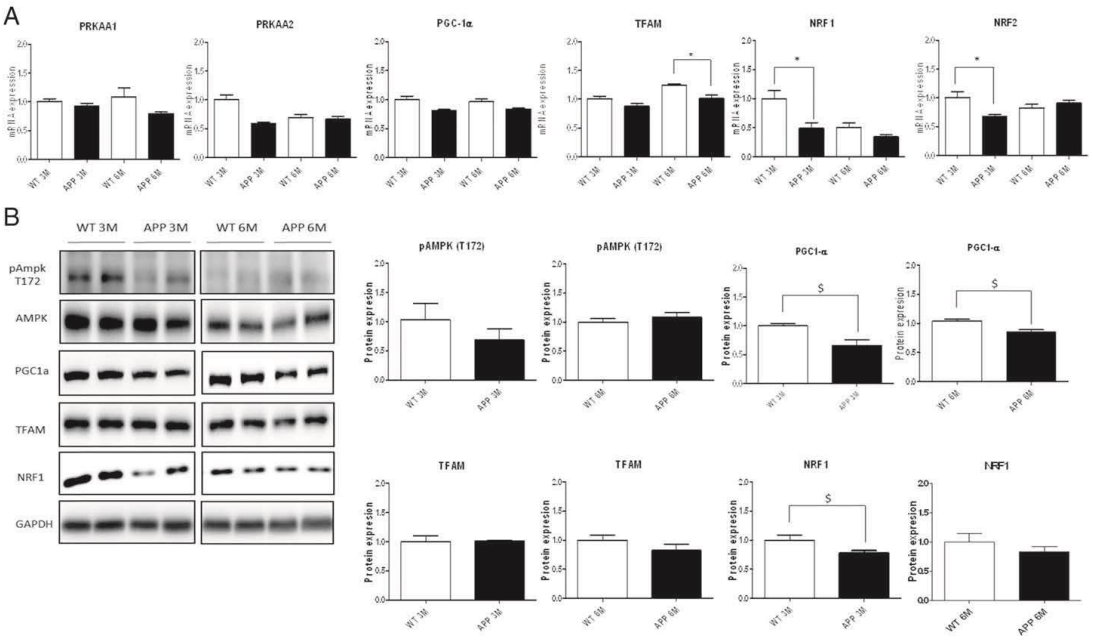

Fig. 4. mRNA expression profile (n = 4–6) (A) and representative immunoblot images and quantification (n = 4–6) (B) of the molecules related to energy metabolism and mitochondrial biogenesis in the hippocampal extracts of 3 and 6 month old wild-type and APP/PS1 mice. pAMPK (T172) is normalized to total AMPK levels, with the rest of the proteins normalized to GAPDH. (Statistical analysis was performed with one-way ANOVA and with Tukey's post-hoc test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$, and with the student's t-test, where $ denotes $p < 0.05$).

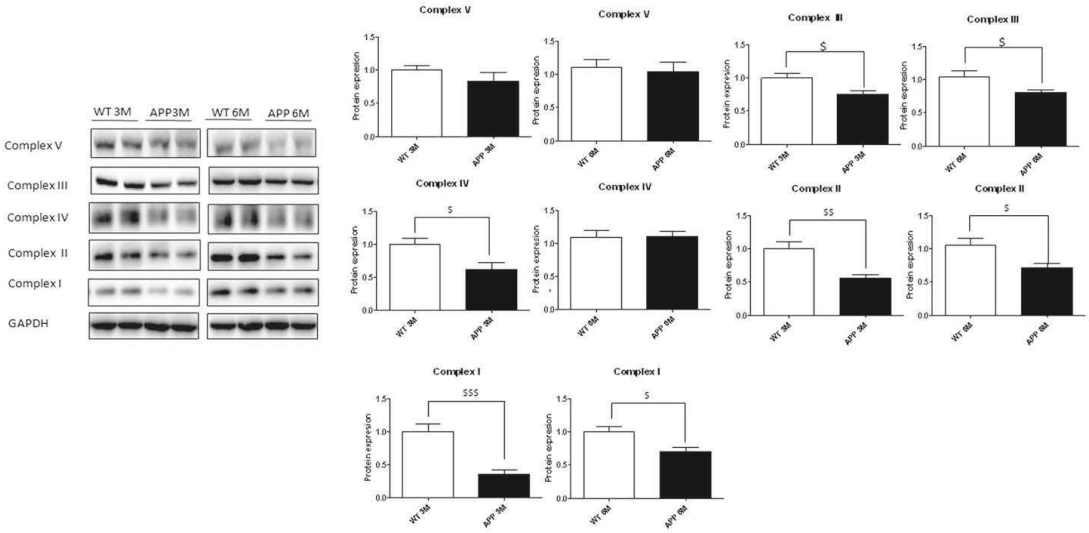

Fig. 5. Representative immunoblot images and quantification of various OXPHOS complexes, normalized to GAPDH protein levels, in the hippocampal extracts of 3 and 6 month old wild-type and APP/PS1 mice (n = 4–6). (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$, $$ denotes $p < 0.01$, and $$$ denotes $p < 0.001$.)

3.9. Changes in synaptic protein levels

Because Aβ oligomers induce synaptic loss in AD we evaluated protein expression levels of representative pre- and post-synaptic proteins (synaptophysin (SYP) and PSD-95 respectively). As shown in Fig. 8, there were no significant changes in their levels in the hippocampus of APP/PS1 mice, relative to the control tissue. Our results are in agreement with a previous study by Minkeviciene et al. who did not detect any changes in synaptic protein levels in 17 month-old APP/PS1 mice [48].

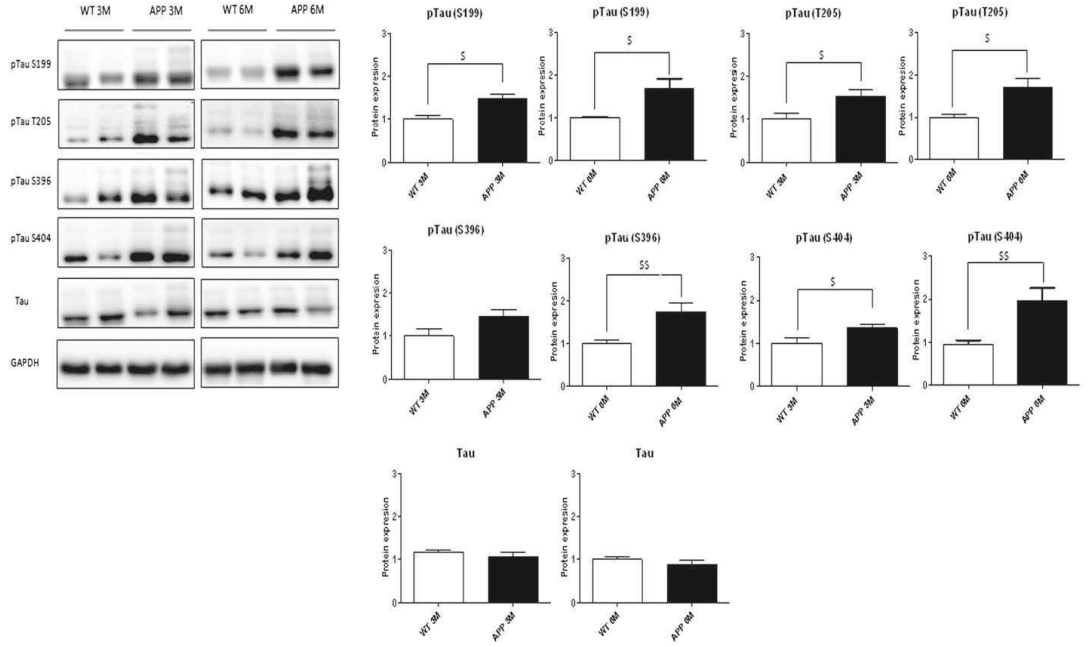

Fig. 6. Representative immunoblot images and quantification of various Tau phosphoepitopes, normalized to total Tau protein levels, in the hippocampal extracts of 3 and 6 month old wild-type and APP/PS1 mice (n = 4–6). (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$ and $$ denotes $p < 0.01$.)

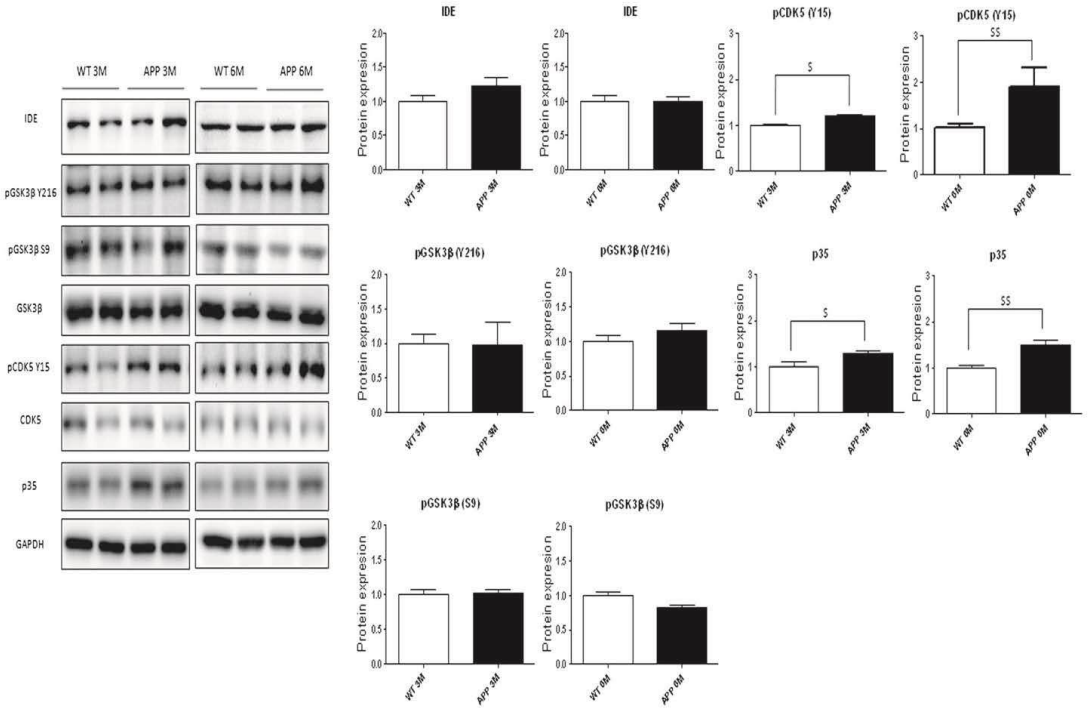

Fig. 7. Representative immunoblot images and quantification of molecules implicated in insulin signaling and Tau phosphorylation, in the hippocampal extracts of 3 and 6 month old wild-type and APP/PS1 mice (n = 4–6). pCDK (Y15), pGSK3β (Y216) and pGSK3β (S9) are normalized to their respective total unphosphorylated protein levels, whereas p35 and IDE are normalized to GAPDH. (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$ and $$ denotes $p < 0.01$.)

4. Discussion

In the current study, we employed an integrated approach consisting of the analyses in both the periphery and at the CNS levels, in order to identify potential changes that occur in the early stages of the amyloidogenic process, prior to amyloid plaque formation in a mouse model of AD. We investigated several key metabolic routes related to glucose uptake and insulin signaling, cellular energy homeostasis, mitochondrial biogenesis and Tau phosphorylation.

In previous studies it was demonstrated that an increase in Aβ levels in APP/PS1 mice is accompanied by plaque deposition in the brain and memory loss, clearly evident at the age of 6 months [11–15]. Thus, APP/PS1 mice are commonly used in AD research [12,14,42,49]. In agreement with these studies, we detected a significant amyloid peptide deposition and the presence of Aβ aggregates in 6 month-old APP/PS1 mice, compared to age-matched wild-type controls. In addition, the 2 object novel object recognition test has revealed a significant cognitive impairment at the age of 6 months in this model. A novel finding in our study is the detection of the insoluble Aβ (1–42) in the brains of 3 month old APP/PS1 mice. This data is intriguing, as Aβ plaques were not detectable by immunohistochemistry at such an early age. Our results suggest that the formation of the insoluble Aβ "proto-fibrils" is

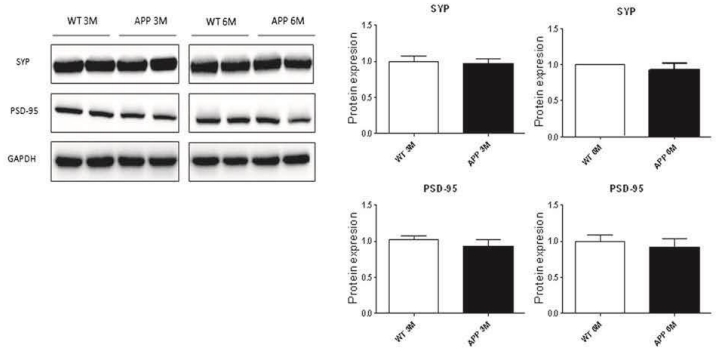

Fig. 8. Representative immunoblot images and quantification of synaptophysin and PSD-95, in the hippocampal extracts of 3 and 6 month old wild-type and APP/PS1 mice (n = 4–6).

an early event which leads to plaque formation by the age of 6 months. Elevated levels of the insoluble Aβ (1–42) were previously reported in brain homogenates of 6 month old APP/PS1 animals [50]. However, the authors of that study did not detect any Aβ in 4 month old mice. The discrepancy with this study could be explained by the differences in experimental methodologies. As expected, by the age of 6 months we have detected a further increase in the levels of insoluble Aβ (1–42) together with the appreciable levels of both soluble and the insoluble Aβ (1–40) as well as senile plaques,

Moreover, in the present research, we demonstrate early changes affecting insulin signaling in the preplaque APP/PS1 mice. These mice display impairment in genes involved in glucose metabolism and mitochondrial function, such as OXPHOS. Thus, as mentioned above, AD neuropathology can be explained, in part, through alterations in glucose metabolism. It was previously published that in 5–7 week-old APP/PS1 mice, glucose and insulin tolerance were not impaired [16]. In our study, we had used older animals and our results demonstrated a tendency towards altered glucose tolerance already at 3 months of age, with the 6-month-old animals exhibiting significantly impaired glucose and insulin tolerance. In the same model, but employing female mice, Hiltunen et al. reported significantly impaired glucose tolerance at 7 months of age in APP/PS1 animals, compared to wild-type controls. Interestingly, the authors did not detect any changes in ITT, but this could be explained by the fact that the ITT was terminated 20 min after the insulin injection. In our study, the biggest differences in ITT between the wild-type and transgenic animals occurred between 30 and 60 min, following i.p. insulin administration. In addition, Hiltunen and colleagues generated triple transgenic mice by cross-breeding APP/PS1 with the mice overexpressing pancreatic IGF2 [49]. While IGF2 single mutants showed impaired glucose and insulin tolerance, in triple APP/PS1/IGF2 animals, this phenotype was further exacerbated. In fact, our results clearly show a significant age-dependent increase in the IGF2 content in the hippocampus, supporting the role of IGF2 signaling in the metabolic perturbations affecting APP/PS1 mice.

Recent studies suggest that there is a close link between insulin-deficient diabetes and cerebral amyloidosis in the pathogenesis of AD [25,51–59]. Using a streptozotocin (STZ)-induced diabetic APP/PS1 mouse model, it has been shown that the diabetic condition promoted the processing of APP, resulting in increased Aβ generation, neuritic plaque formation, and spatial memory deficits [24]. Patients with AD show a remarkable deposition of Aβ peptide in the brain, whereas patients with T2DM present Islet Amyloid Polypeptide (IAPP) deposition in pancreatic β-cells [17]. Then, AD and T2DM share common key molecular alterations in Aβ peptide processing and insulin signaling, in a poorly understood interplay [15–17,51,52,60–65]. Chua and colleagues have suggested that an increase in brain Aβ42 levels in 15 month-old female APP/PS1 mice, may be dependent on impaired brain insulin signaling [53]. However, Sadowski and colleagues demonstrated a correlation between the hippocampal amyloid plaque levels and glucose utilization at 22 months of age. It is of note, that the majority of published studies focus on very late stages of the disease, when Aβ plaques are fully developed [66].

Therefore, the question would be: Are metabolic disorders the cause or the consequence of the AD? It is known that brain glucose metabolism defects are strongly associated with memory impairment in AD brain. Human brain imaging studies indicate that impaired glucose utilization precedes the onset of cognitive deficits in AD, suggesting causality [21]. In this context, the binding of insulin, IRS1 and IRS2 to the INSR, could modulate hippocampal synaptic plasticity and memory consolidation [19–22]. In agreement with this hypothesis, we detected a small, but significant reduction in the hippocampal *Arc*, *Fos*, *Insr* and *Irs2* transcripts in 3 month-old APP/PS1 mice, compared to wild-type controls. By the age of 6 months, APP/PS1 mice develop impaired glucose and insulin tolerance, accompanied by a significant increase in *Igf2* and *Igfbp2* transcripts. Interestingly, we did not detect any changes in the transcription of *Igf1*, which is involved in development, cognitive functions and aging processes, and the alterations of which had been linked to AD pathology.

Downstream of insulin signaling, we focused on mitochondrial markers. Structural and functional perturbations of mitochondria in AD have been recognized for some time, and led Swerdlow and Khan to propose the mitochondrial cascade hypothesis [5]. This hypothesis states that inherited mutations in mitochondrial DNA determine the basal functional ability of mitochondria to respond to, and to recover from stress-induced signaling. The physiopathology of AD develops when the mitochondria lose their functional capacity, and includes neuronal apoptosis, Aβ deposition, and neurofibrillary tangles [7,59–65]. Here, we report a significant downregulation in mitochondrial OXPHOS complexes in the brains of 3 month old APP/PS1 mice. Likewise, we detected reduced mRNA expression levels of genes related to mitochondrial biogenesis and the regulation of energy metabolism, including *Prkaa2* subunit of AMPK, *Pgc-1α*, *Nrf1* and *Nrf2*. NRF1, through its interaction with PGC-1α, regulates mitochondrial biogenesis directly, and is a key transcriptional regulator of IDE [62–64]. The vast majority of IDE protein is localized to the cytosol, with the small amounts present in the mitochondria, where it participates in Aβ degradation. Mitochondrial localization is dependent on the long isoform of ide mRNA transcripts, the expression of which was found to be positively correlated with *Pgc-1α* and *Nrf-1* transcripts in the brains of non-demented human patients. Interestingly, the correlation was weaker in the brains of AD patients, suggesting an impairment of this route [49]. The observed reduction of *Pgc-1α* and *Nrf-1*, both at the mRNA and protein levels, in the hippocampi of young APP/PS1 animals in our study, supports this hypothesis. The lack of changes in IDE protein levels can be explained by the phenomenon of eclipsed distribution [63]. As the dominant, short isoform of IDE is ubiquitously expressed in the cytosol, any changes at the mitochondrial level would be masked by this dominant isoform.

PGC-1α is a member of a family of transcriptional coactivators that plays a central role in the regulation of cellular energy metabolism. It stimulates mitochondrial biogenesis and participates in the regulation of both carbohydrate and lipid metabolism in peripheral disorders such as obesity and diabetes, however its role in the CNS is less clear [55–60]. In addition to the direct effects on mitochondrial gene expression, PGC-1α is also involved in the regulation of genes that protect neuronal cells from oxidative stress, such as mitochondrial superoxide dismutase. PGC-1α is regulated by several metabolism-responsive elements like AMPK which, when activated by elevated AMP/ATP ratios, can phosphorylate it directly [59]. Recent reports indicate that PGC-1α could be a potential biomarker of AD disease, as reduced PGC-1α mRNA and protein levels had been detected in AD brains [58–61]. In agreement with this, we detected significant reductions in PGC-1α mRNA and protein levels in APP/PS1 brains, compared to wild-type controls, at 3 and 6 months of age. We also observed a decrease in a ratio of activated pAMPK (Thr172)/Total APMK in 3 month old APP/PS1 mice, supporting the role of mitochondrial biogenesis impairment in the early stages of AD. PGC-1α remains an attractive target for AD therapeutic intervention [59].

AMPK is a cellular energy sensor conserved in all eukaryotic cells. It regulates the activities of a number of key metabolic enzymes and protects cells from stresses that cause ATP depletion, by switching off ATP-consuming biosynthetic pathways [58]. It AMPK can also phosphorylate substrates like Tau proteins, thereby causing their hyperphosphorylation. Tau hyperphosphorylation occurs both as a result of elevated levels of Aβ and genetic mutations in Tau proteins, and causes microtubule disassembly, which leads to the formation of neurofibrillary tangles and synaptic loss. In a mouse model overexpressing a P301L-mutated version of human Tau (rTgP301L transgene), Tau hyperphosphorylation resulted in its accumulation in the still functional dendritic spines. Significantly, these observations were reported in relatively young 4.5 month-old animals, at an age when cognitive impairments were already evident, but neither the neuronal, nor synaptic loss was detectable [61].

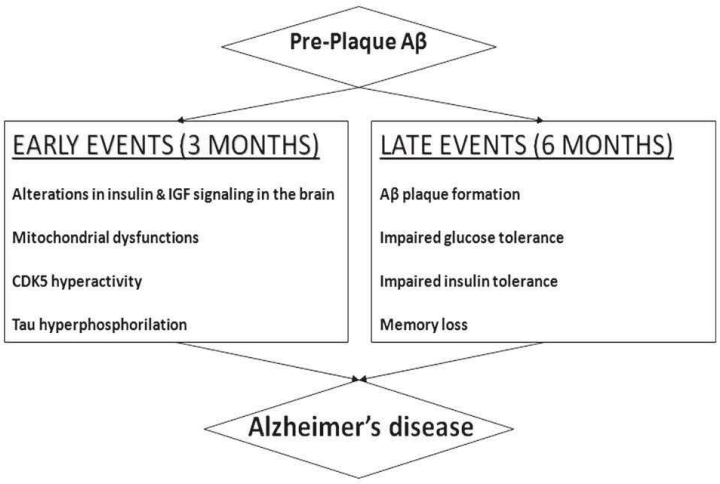

Fig. 9. Summary of the events, leading to the progressive amyloid plaque deposition and memory loss in an APP/PS1 mouse model of FAD.

We detected a significant increase in phosphorylation of several Tau phosphoepitopes in the hippocampi of APP/PS1 mice, compared to controls, including p-Tau (Ser199); p-Tau (Ser 205); p-Tau (Ser 396); and p-Tau (Ser404). At the same time, the protein levels of phosphorylated forms of AMPK and GSK-3β remained unchanged, suggesting that Tau phosphorylation occurs via an alternative pathway in our model. Several studies have suggested that AD and T2DM may share a common pathway to pathology: the hyperactivation of CDK5 [67–69]. CDK5 activation may cause aberrant phosphorylation of cytoskeletal components like Tau and neurofilaments. Results from our research demonstrated an increase in p(Y15) CDK5 phosphorylation in 3 and 6 month-old APP/PS1 mice, suggesting that CDK5 may be the kinase involved in Tau phosphorylation [62,64–71].

In summary, our results show an early downregulation of glucose, insulin signaling and energy metabolism pathways in an APP/PS1 mouse model of FAD. An overview of the key events, occurring between 3 and 6 months of age in our model, is presented in Fig. 9. These changes affect the activity of key molecules involved in memory processes (Arc, Fos) and mitochondrial regulation, such as OXPHOS, PGC-1α and NRF1, as well as Tau phosphorylation. The data presented here reinforces the hypothesis that the preceding events in the amyloidogenenic process in AD are related to both insulin signaling and energy metabolism impairment. Finally, we demonstrate an increase in the levels of pCDK5, which may be responsible for Tau phosphorylation and NFT formation in the hippocampi of the APP/PS1 mice.

Acknowledgments

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Publication 2

HIGH-FAT DIET-INDUCED DEREGULATION OF HIPPOCAMPAL INSULIN SIGNALING AND MITOCHONDRIAL HOMEOSTASIS DEFICIENCES CONTRIBUTE TO ALZHEIMER DISEASE PATHOLOGY IN RODENTS

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Summary

Continuous HFD administration starting at the time of weaning was previously demonstrated to cause relational memory loss in a subpopulation of wild-type mice by 5 months of age. In order to determine if molecular mechanisms leading to memory defects are significantly different between the HFD-treated control animals and mice predisposed to AD development, we have administered HFD to wild-type and APP/PS1 mice until they reached 6 months of age. HFD treatment resulted in significant body-weight increase, increases in plasma insulin and triglyceride levels, significantly impaired glucose and insulin tolerance and memory loss in both groups. HFD-exposed transgenic mice demonstrated significant worsening of peripheral metabolic parameters when compared to HFD-fed control animals. Both the cerebral senile plaque size and quantity remained unaffected by HFD treatment. On a molecular level, HFD produced changes in hippocampal expression of some genes related to insulin signaling (INS1, INS-R, IGF1-R) in transgenic mice only, while some other molecules, notably IGF1 and pIRS2, exhibited different response in treated APP/PS1 vs. wild-type. Furthermore, OXPHOS deficiencies were detected in treated wild-type animals, as well as in both untreated and treated APP/PS1 mice. Interestingly, we have observed increases in Tau phosphorylation and elevated expression of associated kinases in HFD-treated wild-type mice. Taken together, our data suggest that hypercaloric diet is capable of inducing changes in the hippocampal signaling networks, some of which parallel those observed in mice predisposed to AD. Conversely, transgenic mice on a normal diet demonstrate some of the deficiencies which only appear in response to HFD in wild-type animals. AD-like pathology appears to amplify HFD-induced metabolic perturbations.

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High-fat diet-induced deregulation of hippocampal insulin signaling and mitochondrial homeostasis deficiences contribute to Alzheimer disease pathology in rodents

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ABSTRACT

Global obesity is a pandemic status, estimated to affect over 2 billion people, that has resulted in an enormous strain on healthcare systems worldwide. The situation is compounded by the fact that apart from the direct costs associated with overweight pathology, obesity presents itself with a number of comorbidities, including an increased risk for the development of neurodegenerative disorders. Alzheimer disease (AD), the main cause of senile dementia, is no exception. Spectacular failure of the pharmaceutical industry to come up with effective AD treatment strategies is forcing the broader scientific community to rethink the underlying molecular mechanisms leading to cognitive decline. To this end, the emphasis is once again placed on the experimental animal models of the disease. In the current study, we have focused on the effects of a high-fat diet (HFD) on hippocampal-dependent memory in C57/Bl6 Wild-type (WT) and APPswe/PS1dE9 (APP/PS1) mice, a well-established mouse model of familial AD. Our results indicate that the continuous HFD administration starting at the time of weaning is sufficient to produce β-amyloid-independent, hippocampal-dependent memory deficits measured by a 2-object novel-object recognition test (NOR) in mice as early as 6 months of age. Furthermore, the resulting metabolic syndrome appears to have direct effects on brain insulin regulation and mitochondrial function. We have observed pathological changes related to both the proximal and distal insulin signaling pathway in the brains of HFD-fed WT and APP/PS1 mice. These changes are accompanied by a significantly reduced OXPHOS metabolism, suggesting that mitochondria play an important role in hippocampus-dependent memory formation and retention in both the HFD-treated and AD-like rodents at a relatively young age.
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1. Introduction

Over the last couple of decades a global nutrition transition from undernourishment to overconsumption has taken place. Replacement of traditional diets with cheap and easily available processed foods rich in refined carbohydrates, animal fats and edible oils resulted in a global obesity pandemic. While usually considered the plight of the developed world, obesity is also an emerging public health concern among the growing middle classes in poorer countries [1]. Overweight and moderate obesity (defined as Body Mass Index (BMI) of between 25 and 35) may not have a major impact on life expectancy *per se* [2], however, excessive weight significantly increases the risks of developing a number of pathological conditions. These include metabolic syndrome, diabetes, non-alcoholic steatohepatitis, coronary heart disease, stroke, gallbladder disease, osteoarthritis, some types of cancers [3], cognitive decline and Alzheimer disease (AD) [4–7].

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AD is the most common cause of senile dementia, accounting for between 60 and 80% of all dementias. According to recent estimates, the number of cases of AD worldwide is projected to rise from approximately 30 million in 2010 to 40 million by 2020 and to 100 million by 2050. Apart from the genetic component and old age, seven primary preventable environmental risk factors contributing to AD have been identified: diabetes mellitus, midlife hypertension, midlife obesity, depression, physical inactivity, smoking and cognitive inactivity [8,9]. Thus, it is becoming increasingly evident that most of the prognostic preventable AD risk factors may also be linked to obesity and resulting comorbidities, including metabolic syndrome and diabetes. Even though the epidemiological data suggest an existing relationship between AD and energy metabolism, molecular mechanisms behind this relationship are poorly understood. Because AD is a multifactorial disorder with complex etiology which takes decades to fully develop, it is especially challenging to identify the precise disease mechanisms. For example, in a patient with dementia it is not always easy to tell if the underlying pathology is that of a specific brain disease or whether it is also associated with vascular components, metabolic alterations or additional factors (ie. traumatism). Such difficulties notwithstanding, recent years saw a number of breakthroughs in AD research field which contribute to a greater understanding of the molecular dynamics of this devastating condition.

A classical, but currently hotly debated "amyloid cascade" hypothesis [10,11] states that cognitive decline and memory loss in AD are caused by the formation of large, insoluble beta amyloid plaques in the brain, which result in neuronal death and produce characteristic disease symptoms. However, it is necessary to differentiate between the insoluble plaques and soluble amyloid molecules. Recently emerged alternative theories suggest that the β-amyloid monomers, fibrils, or oligomers, and not the plaques, may in fact be the primary neurotoxic species in the brain, responsible for AD development and progression [12]. Apart from amyloid beta itself, mounting evidence suggests that impaired glucose and insulin signaling and metabolism in the brain play a key role in AD. The discovery of brain-specific insulin signaling deficiencies in the very early stages of AD pathogenesis has led some authors to propose that AD may be termed "type 3 diabetes" [13–15]. This hypothesis is further strengthened by a recent study of diabetes-related genes in the brains of post-mortem AD patients and in a mouse model of AD [16]. Microarray analysis has demonstrated significant alterations in the mRNA expression profiles of genes related to insulin signaling, obesity and diabetes in the frontal cortex, temporal cortex and hippocampus in both species. Interestingly, the biggest differences were observed in the hippocampus, a key area related to memory.

Deficiencies in Tau processing may provide yet another link between diabetes and AD. Hyperphosporylated Tau protein is a principle constituent of neurofibrillary tangles (NFT) [17] which, alongside amyloid beta plaques, have long been considered key histopathological hallmarks of AD. Abnormalities in Tau phosphorylation have been detected in cortex and the hippocampi of both type 1 (streptozotocin-induced) and type 2 (*db/db*) mouse models of diabetes [18,19].

Prior research has established a clear relationship between obesity, insulin resistance, diabetes and dementia (reviewed in [20]). Results from published research indicate that there is a close link between insulin deficient diabetes and cerebral amyloidosis in the pathogenesis of AD [21–24]. Epidemiological, clinical, and basic studies have shown a relationship between AD and Type 2 Diabetes Mellitus (T2DM), and that the main physiological link between both conditions is peripheral and central insulin signalling impairment [25,26]. In fact, results from the so called "Hysayama Study" indicate that altered expression of genes related to diabetes mellitus in AD brains is a result of AD pathology, which may thereby be exacerbated by peripheral insulin resistance or diabetes mellitus [16]. These cognitive deficits associated to T2DM have been argued to be due in large part to an impaired central insulin modulation in the hippocampus, which is a critical region for memory processing [27]. Furthermore, a number of recent pilot clinical trials have demonstrated an improvement in AD symptoms in patients upon administration of both the intranasal insulin and Glucagon-like peptide-1 (GLP1) analogues. It has been suggested that these compounds may affect synaptogenesis, neurogenesis, cell repair and inflammation processes, and may additionally help to reduce cerebral β-amyloid load (reviewed in [28]).

As it is especially difficult to study long-term effects of hypercaloric diet in human subjects, we have chosen a mouse model in order to further investigate the underlying molecular events linking brain energy metabolism to AD. A well-established experimental approach to induce insulin resistance in peripheral organs of rodents consists of a high-fat diet (HFD) treatment, which results in obesity [29–31]. We have characterized the neuropathological effects of a HFD in 6-months-old male APPswe/PS1dE9 (APP/PS1) mice in comparison to the nontransgenic C57BL/6 (non-Tg; WT) control animals.

2. Materials and methods

2.1. Animals

Male APPswe/PS1dE9 and C57BL/6 mice were used in this study. APP/PS1 animals co-express a Swedish (K594M/N595L) mutation of a chimeric mouse/human APP (Mo/HuAPP695swe), together with the human exon-9-deleted variant of PS1 (PS1-dE9), allowing these mice to secrete elevated amounts of human Aβ peptide. Both mutations are associated with AD, are under control of the mouse prion protein promoter, directing both mutated proteins mainly to the CNS neurons, and result in age-dependent amyloid plaque depositions in mouse brain. The APPswe-mutated APP is a favorable substrate for β-secretase, whereas the PS1dE9 mutation alters β-secretase cleavage, thereby promoting overproduction of Aβ42. The mice were fed for 5 months with a high-fat diet consisting of 25% fat (45 kcal %), mainly from hydrogenated coconut oil, 21% protein (16 kcal %), and 49% carbohydrate (39 kcal %); Cat# D08061110 (Research Diets Inc, New Brunswick, USA). Body weight was recorded weekly. The animals were kept under controlled temperature, humidity and light conditions with food and water provided *ad libitum*. Mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya. Every effort was made to minimize animal suffering and to reduce the number of animals used. Fifty animals, divided into four groups, were used for the present study, with at least 10 wild-type and 10 6-month-old APP/PS1 transgenic mice, per group. Following *in vivo* testing, the animals were sacrificed at the age of 6 months and at least 6 mice in each group were used for RNA and protein extract isolation, with an additional 4 mice used for immunofluorescence.

2.2. Total blood cholesterol and triglycerides measurements

Total blood cholesterol and triglyceride levels were measured following 4-hour-long fast at the point of sacrifice with Accutrend Plus meter (Roche Diagnostics, Switzerland).

2.3. Glucose and insulin tolerance tests

Intraperitoneal glucose tolerance tests (IP-GTT) and insulin tolerance tests (ITT) were performed in accordance with the previously published guidelines [32]. For IP-GTT, mice were fasted overnight for 16 h. The test was performed in a quiet room, preheated to +30 °C. The tip of the tail was cut with the heparin-soaked (Heparina Rovi, 5000 IU/ml; Rovi S.A.;Madrid, Spain) scissors, 30 min prior to 1 g/kg intraperitoneal glucose injection (diluted in H2O). Blood glucose levels in the tail vein were measured at −30, 0, 5, 15, 30, 60 and 120 min after the glucose injection with the Ascensia ELITE blood glucose

meter (Bayer Diagnostics Europe Ltd.; Dublin, Ireland). ITT was performed in similar conditions with the 0.25 IU/kg of human insulin, diluted in saline (Humulina Regular, 100 IU/ml/Lilly, S.A.;Madrid, Spain), except that the mice underwent a 4-5 hour-long morning fast. Blood glucose levels were measured at −30, 0, 15, 30, 45 and 60 min after the insulin administration. If during this time blood glucose levels dropped to below 20 mg/dl, 1 g/kg glucose was administered to counteract the effects of insulin, in order to reduce animal suffering.

2.4. 2-Object novel object recognition test (NOR)

The test was conducted as previously described by us and others [33]. In brief, a 90°, 25 cm long, and 20 cm high L maze was used. The light intensity in the middle of the field was 30 lx. The objects to be discriminated were plastic figures (object A: 5.25 cm high, object B: 4.75 cm high). First, mice were individually habituated to the apparatus for 10 min a day, for two days. On the third day, they were submitted to a 10 min acquisition trial (first trial) during which they were placed in the maze in the presence of two identical novel objects (A + A, or B + B) placed at the end of each arm. A 10 min retention trial, with the objects (A + B) (second trial) occurred 2 h later. The amount of exploration time each animal spent on objects A and B during the acquisition trial varied between 5 and 20 s, depending on the individual mouse. Total exploration time between the 2 objects when calculated for each individual animal indicated the absence of the object preference bias (Fig. 1C) (n = 5–9 per group). During the retention trial, the times that the animal took to explore the new object (tn) and the old object (to) were recorded. A discrimination index (DI) was defined as (tn − to) / (tn + to). In order to avoid further object preference bias, objects A and B were counterbalanced so that half of the animals in each experimental group were first exposed to object A and then to object B, whereas the other half were exposed to object B first, and then to object A. The maze, the surface, and the objects were cleaned with 96° ethanol between animals, so as to eliminate olfactory cues.

2.5. RNA extraction and quantification

Total RNA was isolated from the hippocampi of wild-type and APP/PS1 transgenic mice utilizing Trizol-based extraction (Life Technologies Corporation; Carlsbad, Ca, USA), as described previously [34]. Briefly, the tissue was homogenized in the presence of Trizol reagent (Life Technologies Corporation; Carlsbad, CA, USA). Chloroform was added and the RNA was precipitated from the aqueous phase with isopropanol at 4 °C. RNA pellet was reconstituted in RNAse-free water, with the RNA integrity determined by Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA).

2.6. Real-time-PCR

First-strand cDNA was reverse transcribed from 2 μg of total RNA using the High Capacity cDNA Reverse Transcription kit, according to manufacturer's protocol (Applied Biosystems). Each sample was analyzed in duplicate for each target. TaqMan probes (Applied Biosystems), as detailed in Table 2, were used to determine transcription levels of individual genes. Reaction was performed on the StepOnePlus Real Time PCR system (Applied Biosystems; Carlsbad, CA, USA) and the values were normalized to *gapdh* and *tbp*.

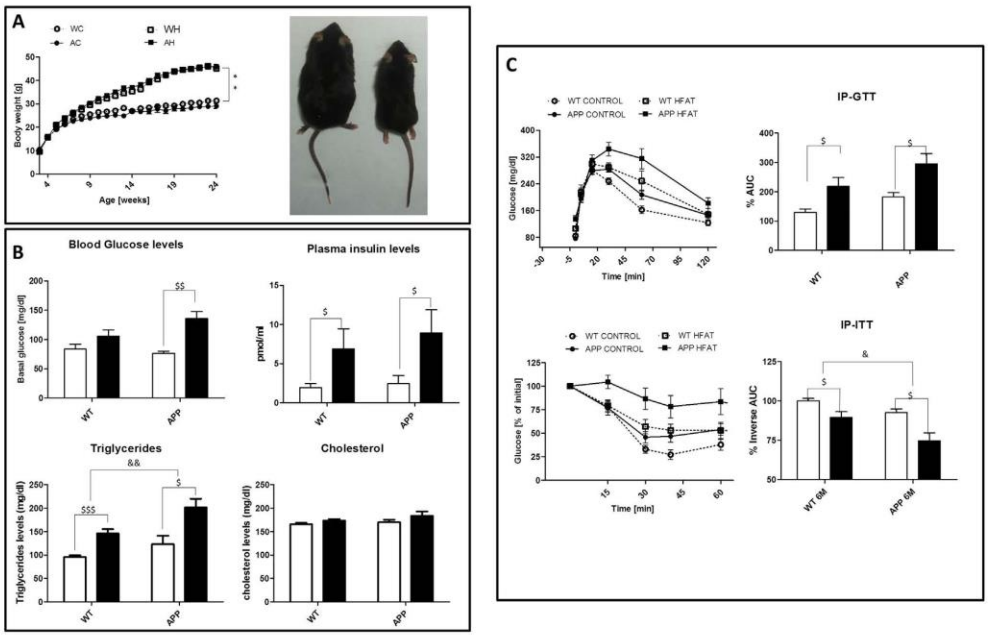

Fig. 1. Peripheral metabolic parameters in HFD-fed WT and APP/PS1 mice. (A) Body weight, (B) fasting blood glucose , fasting serum insulin levels ELISA, total blood triglycerides and cholesterol in 6-month-old animals (n = 5–12 independent samples per group). (C) Intraperitoneal glucose (16 hour fast) and insulin (4 hour fast) tolerance tests in 6-month-old mice (n = 5-12 independent samples per group). For the ITT and the IP-GTT, AUC data were calculated from the timepoint 0 till the end of the experiment. (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$, $$ denotes $p < 0.01$, $$$ denotes $p < 0.001$; regular 2-way ANOVA, where & denotes $p < 0.05$, && denotes $p < 0.01$) *WC*: Wild-type control diet, *AC*: APP/PS1 control diet, *WH*: Wild-type high-fat diet, *AH*: APP/PS1 high-fat diet; open bar: chow, closed bar: high-fat diet.

Table 1
A list of antibodies used for immunoblotting and immunofluorescence.

Protein	Antibody
Akt	#9272 (Cell signaling)
pAkt (S473)	#4060P (Cell signaling)
CDK5	Sc-173 (Santa Cruz biotech)
pCDK5 (Y15)	ab63550 (Abcam)
ERK1/2	#9102 (Cell signaling)
pERK1/2 (T202/Y204)	#9101 (Cell signaling)
GSK3B	#9315(Cell signaling)
pGSK3B (Y216)	Ab74754 (Abcam)
IDE	Ab32216 (Abcam)
IRβ	Sc-20739 (Santa Cruz biotech)
pIRβ (Y1150/1151)	Sc-81500 (Santa Cruz biotech)
IRS1	#2382 (Cell signaling)
pIRS1 (S612)	#2386S (Cel signaling)
IRS2	Sc-1555 (Santa Cruz biotech)
pIRS2 (S723)	Ab 3690 (Abcam)
JNK	#9252 (Cell signaling)
pJNK (Y183/T185)	#9251 (Cell signaling)
Neprelysin	Ab951 (Abcam)
NRF1	Sc-28379 (Santa Cruz biotech)
OXPHOS	MS604 (MitoSciences)
p35	#2680(Cell signaling)
PGC1A	101707 (Cayman chemical)
PPARa	Ab8934 (Abcam)
PPARg	#2430 (Cell signaling)
TAU	AHB0042 (Biosource)
pTAU (S404)	44748G (Life Technologies)
TFAM	DR1071b(Calbiochem)
GAPDH	MAB374 (Millipore)
Thioflavin S	Thioflavin S (Sigma-Aldrich)
2nd –ary Anti-Mouse	170-5047 (Biorad)
2nd –ary Anti-Rabbit	NA934V (GE Healthcare)

2.7. Immunofluorescence, thioflavin S and Hoechst staining

Slides were allowed to defrost at room temperature and then were rehydrated with Phosphate-buffered saline (PBS) for 5 min. Later, the brain sections were incubated with 0.3% Thioflavin S (Sigma-Aldrich; St. Louis, MO, USA) for 20 min at room temperature in the dark. Subsequently, these were submitted to washes in 3-min series: with 80% ethanol (2 washes), 90% ethanol (1 wash), and 3 washes with PBS. Finally, the slides were mounted using Fluoromount (EMS), allowed to dry overnight at room temperature in the dark, and stored at 4C. Image acquisition was performed with an epifluorescence microscope (BX41; Olympus, Germany).For plaque quantification, similar and comparable histological areas were selected, focusing on having the hippocampus and the whole cortical area positioned adjacently [35].

Table 2
A list of Taqman probes used for real-time PCR analysis.

GENE	TaqManProbe
app	Mm01344172_m1
essra	Mm00433143_m1
gapdh	Mm99999915_g1
igf1	Mm01228180_m1
igf2	Mm00439564_m1
igfbp2	Mm00492632_m1
igf1r	Mm00802831_m1
ins1	Mm01950294_s1
insr	Mm01211875_m1
irs1	Mm01278327_m1
irs2	Mm03038438_m1
nrf1	Mm01135606_m1
nrf2 (nfe2l2)	Mm00477784_m1
Ppara	Mm00440939_m1
pparg	Mm01184322_m1
ppargc1a	Mm01208835_m1
prkaa1	Mm01296700_m1
prkaa2	Mm01264789_m1
tbp	Mm00446971_m1
tfam	Mm00447485_m1

2.8. Immunoblot analysis

Aliquots of hippocampal homogenates containing 15 mg of protein per sample were analyzed using the Western blot method. In brief, samples were placed in a sample buffer (0.5 m Tris–HCl, pH 6.8, 10% glycerol, 2%(w/v) SDS,5%(v/v) 2-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling at 95–100 °C for 5 min. Samples were separated by electrophoresis on 10–15% acrylamide gels. Following this, the proteins were transferred to PVDF sheets using transblot apparatus. Membranes were blocked overnight with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris; 1.5% NaCl, 0.05% Tween 20, pH 7.5). They were then incubated with primary antibodies, as detailed in Table 1. After O/N incubation, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG secondary antibody (1:2000). Immunoreactive protein was detected using a chemiluminescence-based detection kit. Protein levels were determined by densitometry, using Chemidoc XRS + Molecular Imager detection system (Bio-Rad Laboratories Inc.; Hercules, CA, USA)), with ImageLab image analysis software. Measurements are expressed as arbitrary units. All results are normalized to GAPDH, unless stated otherwise.

2.9. Measurement of β-amyloid peptides in cortical tissues by ELISA

Soluble and insoluble β-amyloid (βA) βA_{1-40} and βA_{1-42} were measured in cortical extracts employing the commercially available mouse and human ELISA kits (Cat # KMB3481, KMB3441, KHB3481 and KHB3441; Invitrogen, Camarillo, CA, USA) according to manufacturer´s guidelines. The soluble fraction was separated by centrifuging the samples for 10 minutes at 4000xg. The pellets containing insoluble Aβ peptides were solubilized in a 5 M guanidine HCl/50 mM Tris HCl solution by incubating for 3.5 hours on an orbital shaker at room temperature in order to obtain insoluble fraction. Data obtained from the cortical homogenates are expressed as picograms of Aβ content per milligrams of total protein (pg/mg).

2.10. Data analysis

All data are presented as means ± SEM, and differences are considered significant at $p < 0.05$. Differences between samples/animals were evaluated using student's t-test, and either one-way or 2-way ANOVA, with Tukey's post-hoc test. Both the statistical analysis and the graphs presented here were created with the GraphPad InStat software V5.0 (GraphPad Sofware Inc., San Diego, CA, USA).

3. Results

3.1. HFD treatment increases body-weight gain, insulin and triglycerides levels in blood and provokes impaired glucose and insulin tolerance in both WT and APP/PS1 mice

In order to determine the effects of a high-fat hypercaloric diet on metabolic parameters in a mouse model of AD, male WT and APP/PS1 animals were fed either standard chow or a HFD. Mice were divided into 4 groups (n = at least 10 per group): chow-fed WT control (WC), HFD-fed WT (WH), chow-fed transgenic APP/PS1 (TC) and HFD-fed APP/PS1 (TH). Treatment commenced at the time of weaning (21 days) and lasted until the animals reached 6 months of age. This specific time point was chosen as at six months-old APP/PS1 mice present with AD-like neuropathology, including readily detectable βA plaques and memory loss [33] (Fig. 2). As expected, HFD treatment produced progressive diet-induced obesity, with body weight at completion of the experiment reaching 144% in WH vs. WC ($P < 0.0001$),

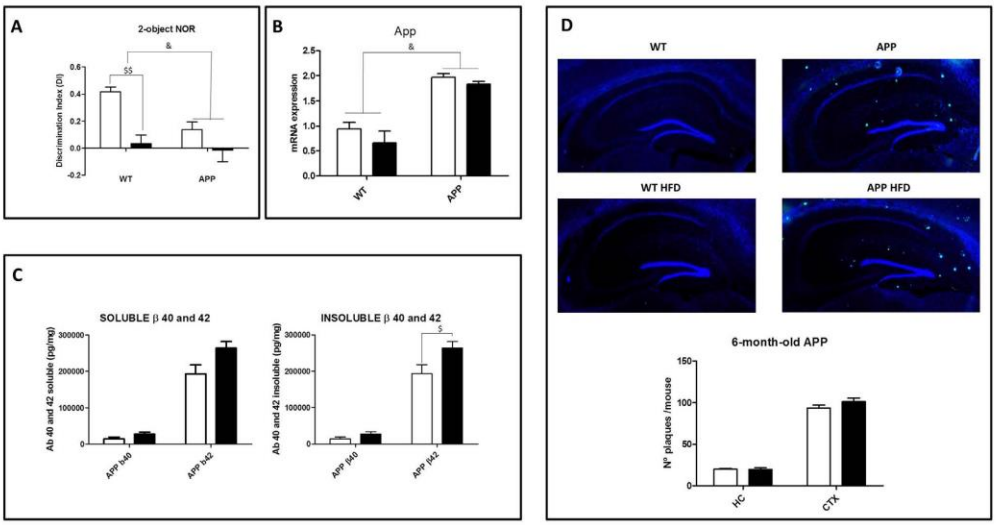

Fig. 2. (A) The results of the 2-object Novel Object Recognition test (NOR), demonstrating significant memory loss as a result of a HFD treatment in 6 month-old wild-type, as well as in chow-fed and HFD-fed APP/PS1 animals, compared to chow-fed wild-type mice (n = 7–12 independent samples per group). (B) Hippocampal mRNA expression of the *app* with the probe recognizing both mouse and human forms of *app* in 6-months-old mice (n = 10-12 independent samples per group, with 3 technical replicates per sample). (C) Concentrations of the soluble and insoluble human βA_{1-40} and βA_{1-42} peptides in the cortical extracts in chow- and HFD-fed 6-month-old APP/PS1 mice, expressed as pg/mg of total protein as determined by ELISA (n = 5–8 independent samples per group, with 3 technical replicates per sample). (D) Immunofluorescence analysis of β-amyloid plaque numbers in the brains of 6-month-old WT and APP/PS1 mice. No plaques were observed in WT animals (n = 4–6 independent samples per group, with at least 5 slices analyzed per sample). Hoechst staining in blue / Thioflavin S staining in green (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$, $$ denotes $p < 0.01$; regular 2-way ANOVA, where & denotes $p < 0.05$, && denotes $p < 0.01$); open bar: chow, closed bar: high-fat diet.

and 158% in TH vs. TC ($P < 0.0001$) (Fig. 1A). This weight increase was accompanied by fasting hyperinsulinemia, with plasma insulin concentrations of 6.88 pM/ml in WH (1.92 in WC; $P = 0.0484$) and 8.92 pM/ml in TH (2.44 in TC; $P = 0.0332$) (Fig. 1B). An additional sign pointing to the possible presence of metabolic syndrome in 6-months-old HFD-fed mice was an increase in fasting triglycerides levels with 146 mg/dl in WH (96 in WC; $P < 0.001$) and 202 mg/dl in TH (123 in TC; $P = 0.0119$). Interestingly, a 2-way ANOVA demonstrated significant differences between WT and APP/PS1 groups as a whole, with higher triglycerides concentrations in transgenic animals, suggesting exacerbated phenotype in AD-like mice (Fig. 1B). Fasting blood glucose levels support this observation, as the HFD treatment resulted in a significant increase of this parameter in TH vs. TC group (136 vs 76 mg/dl; $P = 0.0046$), but not in WH vs. WC (106 vs 84 mg/dl; $P = 0.1452$). Fasting blood cholesterol levels were not affected in any of the groups (Fig. 1B).

As our initial screening has not only demonstrated clear alterations in peripheral glucose metabolism in response to HFD, but also significant differences between the WT and APP/PS1 animals, we performed additional glucose and insulin tolerance tests. Predictably, IP-GTT has shown impaired glucose tolerance in 6-months-old WH and AH groups, when compared to their respective controls ($P = 0.02$ and $P = 0.0436$) (Fig. 1C). The results of the ITT were intriguing. While the test results indicated impaired insulin tolerance in both WH and AH ($P = 0.0106$ and $P = 0.0346$) we, once again, detected a more severe phenotype in an APP/PS1 model ($P = 0.0239$, with 2-way ANOVA) (Fig. 1C). Taken together, our data indicate a possible acceleration of a HFD-induced peripheral metabolic phenotype in APP/PS1 animals compared to control mice. In the following steps, we proceeded to study the effects of HFD on CNS and attempted to identify molecular pathways related to insulin metabolism in the brain, with a particular focus on hippocampal metabolic and insulin signaling.

3.2. High-fat diet contributes to increased cerebral β-amyloid levels and memory loss

We have employed a 2-object Novel Object Recognition (NOR) test as a means of evaluating the impact of HFD on cognitive performance. Interestingly, our results demonstrate that HFD treatment has a significant impact on memory function in both the WT and transgenic animals (Fig. 2A). In order to determine if the resulting memory loss is dependent on the increased cerebral βA load, we have measured hippocampal expression of the APP, cortical levels of the βA_{1-40} and βA_{1-42} and assessed the numbers of senile plaques in the brain.

Because APP/PS1 mouse model expresses a human form of the APP and the βA, it is necessary to quantify the combined expression of both endogenous and transgenic protein. At the mRNA level, a probe recognizing both human and mouse versions of *app* was selected for Real-time PCR analysis. We have detected approximately a 2-fold increase in *app* transcripts in the hippocampal extracts of APP/PS1 mice, compared to WT controls (Fig. 2B). Hypercaloric diet did not influence mRNA expression of this target in either group. Elevated levels of soluble and insoluble forms of βA_{1-40} and βA_{1-42} peptides were detected in cortical homogenates of APP/PS1 animals. However, HFD treatment resulted in a significant increase in insoluble βA_{1-42} levels only, and only in TH versus TC group (~266 compared to ~195 ng/mg) (Table 3 and Fig. 2C). Surprisingly, this increase did not have an effect on the total number of plaques in the hippocampal and cortical areas of the brain (Fig. 2D). Furthermore, there appeared to be an increase in the concentrations of the soluble βA_{1-42} in WH animals, but it only affected a subset of this group, rendering the data unsuitable for statistical analysis (Table 3). Thus, our results suggest that alterations in cerebral amyloid levels do not play a critical role in HFD-induced memory loss in 6-month-old mice.

Table 3
Concentrations of the soluble and insoluble mouse and human βA_{1-40} and βA_{1-42} peptides in the cortical extracts in chow- and HFD-fed 6-month-old wild-type and APP/PS1 mice, expressed as pg/mg of total protein as determined by ELISA (n = 5–8 independent samples per group, with 3 technical replicates per sample). (Statistical analysis was performed with the Student's t-test; ± is S.E.M.). *WC*: Wild-type control diet, *AC*: APP/PS1 control diet, *WH*: Wild-type high-fat diet, *AH*: APP/PS1 high-fat diet, *m*: mouse, *h*: human, *t*: total (mouse + human), *p*: p value (t-test) *N/A*: not applicable, *N/D*: not detected.

Sol. (pg/mg)	m βA 40	h βA 40	t βA 40	m βA 42	h βA 42	t βA 42
WC	N/D	N/A	N/A	10 ± 10	N/A	10 ± 10
WH	N/D	N/A	N/A	1132 ± 632	N/A	1132 ± 632
AC	N/D	649 ± 262	649 ± 262	1120 ± 706	1088 ± 168	2208 ± 863
AH	N/D	879 ± 260	879 ± 260	1976 ± 1121	1244 ± 266	3220 ± 1346
WC vs WH (p)	N/A	N/A	N/A	0.1431	N/A	0.1431
AC vs AH (p)	N/A	0.558	0.558	0.5215	0.588	0.5305
WC	460 ± 158	N/A	460 ± 158	6 ± 6	N/A	6 ± 6
WH	331 ± 17	N/A	331 ± 17	270 ± 270	N/A	270 ± 270
AC	1276 ± 320	14075 ± 5195	15352 ± 4231	1722 ± 655	193310 ± 24590	194688 ± 24396
AH	1350 ± 190	27338 ± 5954	28688 ± 6138	2502 ± 633	263797 ± 18173	265882 ± 18194
WC vs WH (p)	0.3878	N/A	0.3878	0.3024	N/A	0.3024
AC vs AH (p)	0.8467	0.1476	0.1114	0.4245	0.043*	0.041*

3.3. HFD affects expression of genes involved in insulin signaling in the hippocampus

We evaluated mRNA expression profiles of preproinsulin 1 (*Ins1*), insulin receptor (*Insr*), insulin receptor substrates 1 (*Irs1*) and 2 (*Irs2*), insulin-like growth factor I (*Igf1*), and IGF receptor (*Igfr*) in the hippocampus of 6 months-old mice (Fig. 3A). A modest increase in *ins1* transcripts was accompanied by a small but significant reduction in *insr* and *igfr* levels in the TH vs. TC group, while HFD treatment did not have an effect on these molecules in WT brains. Conversely, *igf1* was upregulated in WH vs. WC group, but remained unchanged in transgenic animals. We did not detect significant differences in total IRS1 and IRS2 expression in any of the groups, both at the mRNA and protein levels (Fig. 3A, B). As post-translational modifications and especially ligand-mediated tyrosine$^{1150/1151}$ autophosphorylation of the signal-transducing catalytic β-subunit of IR play a major role in receptor activation, we have measured protein levels of both total IRβ and pTyr$^{1150-1151}$-IRβ. Immunoblotting analysis revealed no changes in the ratios between the phosphorylated and total IRβ protein in the hippocampal extracts at 6 months of age (Fig. 3B), suggesting that receptor functionality is unaffected. Functional IR is necessary for downstream signaling which is controlled in large part by IRS1 and IRS2 adaptor molecules. Autologous (insulin-mediated) and insulin independent Ser/Thr phosphorylations of IRS may both potentiate and attenuate IR signaling. Mouse pSer^{612}-IRS1 and pSer^{723}-IRS2 (corresponding to human pSer^{616}-IRS1 and pSer^{731}-IRS2) are amongst the better known negative regulators of the IR-IRS pathway. Thus, an insulin-dependent increase in the phosphorylation state of these residues may lead to

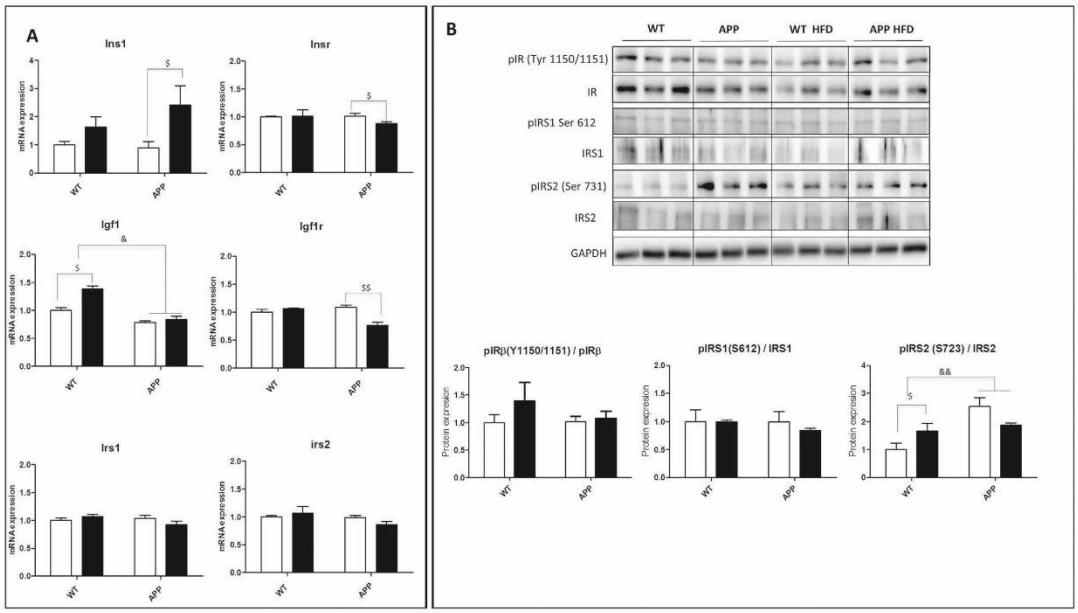

Fig. 3. (A) mRNA expression profile (n = 4–6 independent samples per group, with 3 technical replicates per sample) and (B) representative GAPDH-normalized immunoblot images and quantification (n = 4–6 independent samples per group) of molecules related to proximal insulin signaling in the hippocampal extracts of chow- and HFD-fed 6-month-old wild-type and APP/PS1 mice. Mouse pSer^{612}-IRS1 and pSer^{723}-IRS2 correspond to human pSer^{616}-IRS1 and pSer^{731}-IRS2 (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$; $$ denotes $p < 0.01$; regular 2-way ANOVA, where & denotes $p < 0.05$, && denotes $p < 0.01$); open bar: chow, closed bar: high-fat diet.

desensitization of proximal insulin signaling. We did not detect differences in IRS1 phosphorylation, however, our data demonstrated a significant increase in pSer^{723}-IRS2 in WH vs. WC ($P = 0.039$) and in APP/PS1 vs. WT (2-way ANOVA, $P = 0.0078$) mice (Fig. 3b), indicating a potential role of IRS2 in response to both the hypercaloric diet treatment and in AD-like phenotype.

3.4. Effects of HFD on signaling kinases and tau phosphorylation

Having determined that the proximal insulin signaling is likely perturbed in the hippocampi of HFD-exposed and APP/PS1 transgenic animals, we turned our attention to the signaling kinases implicated both in insulin signaling and senile plaque formation. Apart from their diverse cellular roles, these kinases (except for CDK5) have also been shown to phosphorylate IRS proteins directly, thus modulating the activity of these adaptor molecules. We measured protein levels of pSer^{473}-Akt, pTyr^{216}-Gsk3β, pThr^{183}/pTyr^{185}-JNK1, pThr^{202}/pTyr^{204}-ERK1/2, pTyr^{15}-CDK5 and its activator molecule p35 in the hippocampal extracts of 6-month-old animals. All of the above mentioned phosphorylations result in the activation of the respective kinases. Interestingly, we have observed significant differences in the activation state in all of the kinases tested when compared to at least one of the groups (Fig. 4). For example, ERK1/2 and CDK5/p35 were overactivated in WH vs. WC group only, while JNK1 activity was enhanced in WH vs. WC and TH vs. TC hippocampi. Basal GSK3β activation was higher in the TC vs. WC groups, with the HFD resulting in additional activation in WH group only. In contrast, Akt activity was inhibited in all of the groups when compared to WC (WH vs. WC; TC vs WC; TH vs. TC, as well as in APP/PS1 vs. WT – 2-way ANOVA, $P = 0.0085$). As kinase-mediated Tau hyperphosporylation is one of the principal diagnostic criteria of AD, we have also measured the phosphorylation state of the Tau protein at Ser^{404}. HFD treatment resulted in a significant increase in pSer^{404}-Tau in the WH vs. WC group, an increase which was comparable to the levels observed in the hippocampus of the APP/PS1 animals (Fig. 4). Collectively, our data suggest that the HFD treatment shares some of the features of the distal insulin signaling abnormalities observed in AD-like model.

3.5. Amyloid degrading enzymes (ADE) and HFD

Peripheral hyperinsulinemia and insulin resistance disrupts insulin transport into the CNS, resulting in the reduction of brain insulin levels. Under the normal circumstances, excess insulin is removed by Insulin Degrading Enzyme (IDE), increased expression of which forms a part of a negative feedback loop triggered by insulin itself. Thus, low local insulin levels will result in the reduced expression of IDE. As expected, we have observed a significant reduction in the protein levels of IDE in the hippocampi of both WH and TH mice (Fig. 5). However, IDE may also participate in β-amyloid clearance, and the reduction in its levels may potentially exacerbate APP/PS1 phenotype. Neprilysin is another well-known ADE that had been implicated in Aβ degradation. Interestingly, we detected a significant upregulation of neprilysin levels in TH vs. TC group (Fig. 5), suggesting a possible compensatory mechanism to counteract elevated amyloid load in HFD-treated APP/PS1 mice.

3.6. Mitochondrial metabolism is altered in the hippocampi of HFD-fed WT and APP/PS1 mice

So far, we have mainly focused on the insulin route and have not discussed the implications of altered insulin signaling on mitochondrial homeostasis. As mitochondria are the principal organelles involved in cell metabolism, we measured hippocampal mRNA and protein expression levels of a number of molecules associated with mitochondrial energy status and biogenesis. We did not detect changes in the mRNA expression of α- catalytic subunits of one of the major kinases activated in response to ATP depletion – AMPK (*Prkaa1* and *Prkaa2* levels were not affected in any of the groups). However, we observed a marginal but significant reduction in response to HFD in a key transcriptional

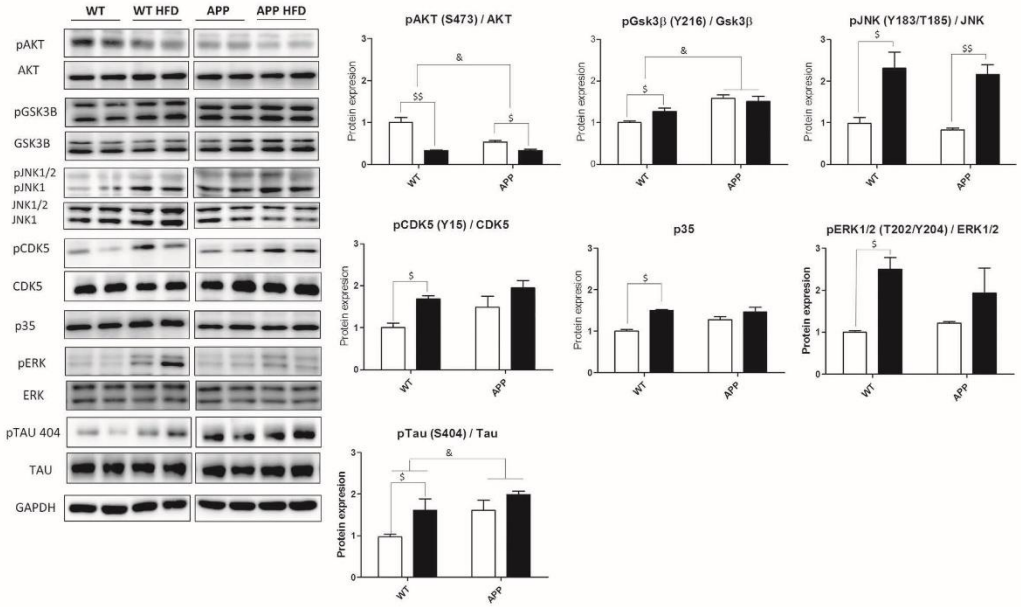

Fig. 4. Representative GAPDH-normalized immunoblot images and quantification (n = 4–6 independent samples per group) of molecules related to distal insulin signaling in the hippocampal extracts of chow- and HFD-fed 6-month-old wild-type and APP/PS1 mice. (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$; $$ denotes $p < 0.01$; regular 2-way ANOVA, where & denotes $p < 0.05$); open bar: chow, closed bar: high-fat diet.

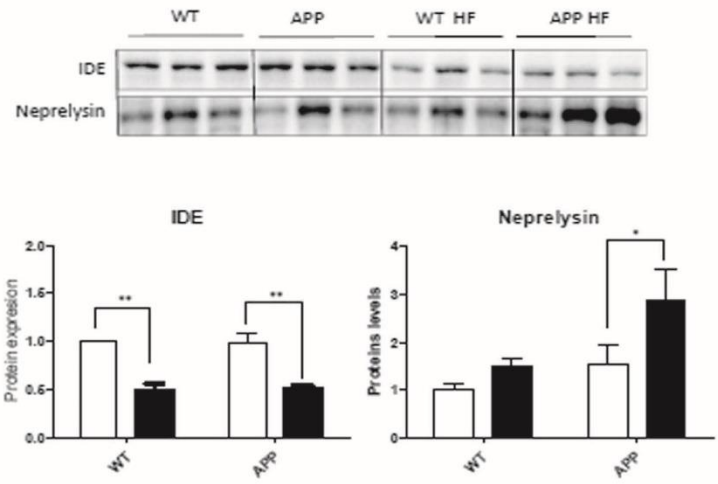

Fig. 5. Representative GAPDH-normalized immunoblot images and quantification (n = 4–6 independent samples per group) of enzymes implicated in β-amyloid degradation in the hippocampal extracts of chow- and HFD-fed 6-month-old wild-type and APP/PS1 mice. (Statistical analysis was performed with one-way ANOVA with Tukey's post-hoc test, where * denotes $p < 0.05$, ** denotes $p < 0.01$); open bar: chow, closed bar: high-fat diet.

co-regulator of mitochondrial biogenesis PGC-1α, which is partly regulated by AMPK (*ppargc1a* was reduced in APP/PS1 vs. WT groups; 2-way ANOVA, P = 0.0144; PGC-1α was reduced in WH vs. WC and TH vs. TC, P = 0.0044 and P = 0.0022, respectively) (Fig. 6A, B).

We have also determined the expression levels of PGC-1α co-regulated transcription factors, including peroxisome proliferator-activated receptors α (PPAR-α) and γ (PPAR-γ), mRNA and protein levels of which remained unchanged (Fig. 6A, B). Differences in the mRNA expression of the nuclear respiratory factors 1 (*nrf1*) and 2 (*nrf2*) between the groups were very small, even though statistically significant, and were comparable to the changes observed in estrogen-related receptor α (*esrra*) and mitochondrial transcription factor A (*tfam*) transcripts (Fig. 6A). At the protein level, we detected a significant reduction of TFAM in WH vs. WC and TH vs. TC groups (Fig. 6B).

In addition, we evaluated mitochondrial function impairment via immunoblotting analysis of OXPHOS complexes. Our results demonstrated significant reduction in OXPHOS I, II, III and IV in the hippocampi of 6-months-old WH vs. WC mice. All of the OXPHOS complexes were down regulated in the basal state in TC vs. WC animals, and were not further reduced in response to HFD treatment (Fig. 7). Taken together, our data indicate significant perturbations in cellular energy metabolism in the brains of HFD-treated and APP/PS1 mice.

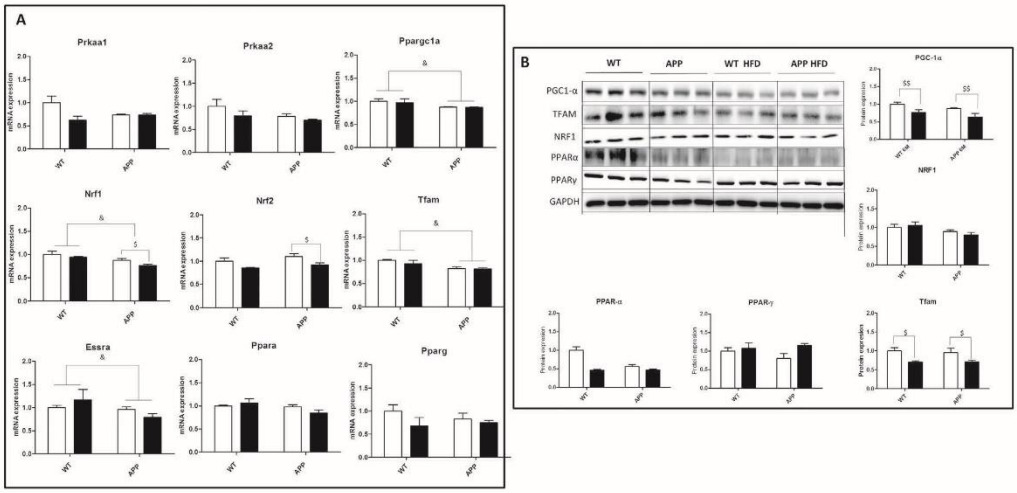

Fig. 6. (A) mRNA expression profile (n = 4–6 independent samples per group, with 3 technical replicates per sample) and (B) representative GAPDH-normalized immunoblot images and quantification (n = 4–6 independent samples per group) of molecules related to distal insulin signaling and mitochondrial homeostasis in the hippocampal extracts of chow- and HFD-fed 6-month-old wild-type and APP/PS1 mice. (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$; regular 2-way ANOVA, where & denotes $p < 0.05$); open bar: chow, closed bar: high-fat diet.

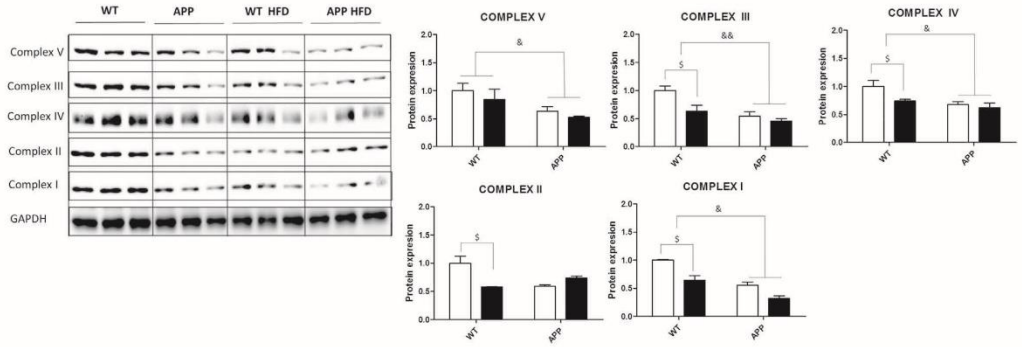

Fig. 7. Representative GAPDH-normalized immunoblot images and quantification (n = 4–6 independent samples per group) of mitochondrial OXPHOS complexes in the hippocampal extracts of chow- and HFD-fed 6-month-old wild-type and APP/PS1 mice. (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$, $$ denotes $p < 0.01$; regular 2-way ANOVA, where & denotes $p < 0.05$, && denotes $p < 0.01$); open bar: chow, closed bar: high-fat diet.

4. Discussion

The original "amyloid cascade" hypothesis postulated over 20 years ago in a seminal *Science* paper by Hardy and Higgins [10] has undoubtedly led to major breakthroughs in understanding the pathophysiology of AD. At its core, the hypothesis defined the cascade as a sequence of events starting from abnormal β-amyloid peptide processing leading to β-amyloid deposition, tau phosphorylation and neurofibrillary tangle formation, all of which ultimately result in cellular dysfunction and neuronal death [11]. Since then, significant research efforts on the part of academic labs and the pharmaceutical industry have largely focused on the amyloid cascade as the principal target for AD treatment. Unfortunately, none of the molecules that reached Phase II and III clinical trials targeting this pathway have proven to be more effective than placebo at treating AD (reviewed in [36]). Mounting criticism of the amyloid cascade hypothesis has recently prompted John Hardy himself to address the issues plaguing AD drug development [37]. The general failure of the amyloidocentric approach in pharmaceutical development strongly suggests that the β-amyloid is unlikely to be solely responsible for AD progression.

It has been long suspected that mitochondria play a significant role in neurodegenerative diseases [38–40]. By 2004, Swerdlow & Khan [41] proposed a comprehensive model which takes into account mitochondrial alterations occurring during the course of AD. The "mitochondrial cascade" hypothesis suggests that the cause and effect relationship in AD are reversed when pitted against an "amyloid cascade" hypothesis. The theory implies that the damage to the mitochondria occurs prior to the β-amyloid accumulation. This view has recently been expanded [42–44] and is supported by a number of studies (reviewed in [45]) which classify AD as a primarily metabolic disorder. According to this view, dementia develops as a result of the diminished ability of the brain to efficiently utilize the available glucose, leading to reduced neuronal plasticity which affects cerebral capacity to form and retain memories.

In the present paper, we present results that reinforce the hypothesis that AD may be viewed as a metabolic disorder, with the disease neuropathology at least partially related to insulin signaling failure and energy depletion in hippocampal neurons. Our data notwithstanding, before we continue the discussion regarding the implications of insulin signaling and mitochondrial regulation on hippocampal memory, we would like to address some of the discrepancies in peripheral metabolic parameters in our results compared to previously published studies. Ramos-Rodriguez and colleagues recently reported that a similar HFD treatment did not result in an increase in basal fasting blood glucose levels, nor in plasma triglycerides, but instead increased plasma cholesterol in 6 months-old APP/PS1 animals [46]. Our data clearly demonstrate elevated basal blood glucose in TH group, an increase in total blood triglyceride levels in WH vs. WC and in TH vs. TC groups, and no changes in cholesterol levels. Regarding blood glucose measurements, our methodology is very similar with 16 hours-long fast applied in both studies. However, the glucose values approximating 60 mg/dl detected by Ramos-Rodriguez *et al*, suggest that the measurements were taken at room temperature, which may explain the observed differences in the basal glucose levels in WC animals (~80 mg/dl in our study), as our tests were conducted at 28-29C [32]. As for the differences in cholesterol and triglycerides levels, these can be easily explained by the different diets used (60% Kcal obtained mainly from animal fat (lard) in Ramos-Rodriguez *et al*, vs. 45% Kcal mainly from hydrogenated coconut oil (vegetable fat) in our study). Interestingly, increases in plasma insulin levels in mice treated with either diet were comparable. Taken together, our data indicate that metabolic syndrome is indeed present in our model.

The connection between AD, obesity and type-2 diabetes has been known by epidemiologists for some time now. In fact, as early as 2004, in a study carried out at the Mayo clinic, investigators reported either impaired fasting glucose or diabetes in 81% of human subjects suffering from AD [47]. However, it is only recently that we are beginning to understand the pathophysiological processes linking peripheral metabolic abnormalities and neuronal dysfunction. Defective insulin signaling and mitochondrial redox imbalances appear to play a major role in the development of both the metabolic syndrome and AD. Crucially, it has been suggested that the insulin-dependent signaling in the brain may potentially be regulated via the pathways independent of its glucoregulatory functions in the periphery [48–50]. In the present study, we compared molecular pathways involved in memory loss in mice as a result of a HFD treatment, with animals which lose memory as a consequence of an APP/PS1 genotype. It was reported previously that the continuous exposure of juvenile (3-weeks-old) mice to a HFD causes relational memory loss (hippocampus-dependent) in a significant number of animals by 5 months of age [51]. We have chosen a similar experimental approach, but have focused on 6-months-old animals, with our data indicating that virtually all HFD-fed WT mice demonstrate pronounced memory loss at this age, as measured by NOR test. Importantly, 6-months-old APP/PS1 control animals present with similar memory deficits when compared to WH group. This has permitted us to make direct comparisons between the treated and untreated WT and APP/PS1 animals. One of the intriguing findings in our study is that the HFD-induced memory loss appears to be independent of the levels of cerebral β-amyloid peptide in mice not predisposed to abnormal βA processing (even though we detected a significant increase in insoluble βA_{1-42} levels in cortical homogenates in TH vs. TC group).

In our experiments, HFD treatment produces metabolic syndrome which includes peripheral hyperinsulinemia, peripheral insulin resistance, peripheral and central hyperglycemia and dyslipidemia among others. So, what are the components of metabolic syndrome that result in memory loss in HFD-fed WT animals? Assuming that the insulin is involved, we need to consider that one of the key differences in brain insulin signaling between AD and diet-induced obesity is the availability/quantity of insulin hormone in the brain. Diet-induced obesity results in the disruption of the Blood-Brain-Barrier (BBB), which limits receptor-mediated insulin transport to the CNS. Therefore, somewhat counterintuitively, peripheral hyperinsulinemia actually provokes insulin deficiency in the brain [52–56]. On the other hand, in AD, cerebral insulin resistance has been widely implicated in disease pathogenesis (reviewed in [48]). While the distinction between insulin deficiency and insulin resistance may seem trivial to some authors in the neuroscience field (to the point that a large number of studies use both terms interchangeably), it may have a significant impact on potential treatment strategies. For example, in an insulin-resistant (at the CNS level) non-obese AD patient, it may actually be beneficial to choose an oral anti-diabetic drug treatment as a means for improving AD symptoms. Conversely, the same treatment in an obese patient may not have the desired effects due to the inability of the extra insulin to cross BBB, in which case the intranasal administration may provide better outcomes. Regardless of the root cause, if we consider insulin signaling perturbations to be one of the contributors to memory loss and AD-like symptoms in our model, then both the proximal and distal insulin signaling pathways should be altered in the hippocampal region. Proximal insulin signaling consists of an insulin-IR/IGF-1R-IRS axis, whereby the binding of the insulin to the extracellular alpha subunit of the IR or IGF-1R initiates a series of Tyr autophosphorylations in the beta subunit, disinhibiting intracellular Tyr kinase activity towards IRS, thereby allowing these adaptor molecules to interact with a large number of targets. Once activated, IRS1 and 2 are further regulated via a highly complex mechanism involving multiple Ser/Thr kinases, which can phosphorylate the tail regions of IRS molecules at over 50 Ser/Thr residues. Unlike Tyr phosphorylation by the IR, which activates IRS, modifications at Ser/Thr residues are capable of both promoting and inhibiting downstream IRS-mediated signaling [57]. As previously mentioned, cerebral insulin resistance has been implicated in AD pathogenesis. Such a resistance may stem from defects in IR itself [58], or may be mediated via negative regulation of the IRS. Our data suggest that the IR is functional in the hippocampi of 6 months-old HFD-treated WT and APP/PS1 mice. Furthermore, we have also detected a significant increase in *igf1* transcripts in WH vs. WC animals, possibly in response to cerebral insulin deficiency. We then considered the possibility that the downregulation of the IRS pathway, via inhibitory Ser/Thr phosphorylation, may attenuate downstream insulin signaling. As it is nearly impossible to determine the phosphorylation state of all of the 50+ Ser/Thr residues of the IRS molecules within the scope of a single paper, we have performed immunoblotting analysis of mouse pSer^{612}-IRS1 and pSer^{723}-IRS2 (corresponding to human pSer^{616}-IRS1 and pSer^{731}-IRS2). Elevated levels of pSer^{616}-IRS1 were previously detected in neurons of AD patients and were associated with insulin resistance [59], and an increase in pSer^{723}-IRS2 protein levels was observed in dorsal root ganglia neurons of diabetic mice [60]. As indicated in our findings, there were no differences in pSer^{612}-IRS1 for any groups. However, we detected a significant increase in hippocampal pSer^{723}-IRS2 in WH, TC and TH mice. While our analysis is by no means exhaustive, it does support the hypothesis that the hippocampus-dependent memory loss is, at least in part, dependent on insulin signaling deficiencies.

Ser/Thr phosphorylation of the IRS molecules is mediated by both insulin-dependent (autologous) and insulin-independent (heterologous) kinases. Autologous regulation is thought to be the predominant form of downstream insulin-mediated signaling under physiological conditions. In a disease state, however, preferential activation of the heterologous pathway may contribute to the underlying pathology. Cellular stress and/or proinflammatory phenotype may cause inappropriate Ser/Thr modifications of IRS, which may result in the "hijacking" of the normal physiological route. In the current study, we measured hippocampal expression of IRS-regulating Ser/Thr kinases, which are also known to play a role in neurodegenerative diseases. These include autologous (Akt, ERK 1/2), heterologous capable of phosphorylating IRS in the basal cellular state (AMPK, GSK3β), as well as heterologous kinases activated in response to cellular stress and sympathetic activation (JNK1) [57].

4.1. Insulin-IRS-Akt-GSK-3β pathway

Apart from the involvement of insulin, insulin-related molecules and their receptors in the cognitive loss observed in HFD-fed animals, we would like to further discuss key pathways and molecules related to insulin signaling. This pathway is one of the major regulators of distal insulin signaling. Activated IRS recruits PI3K, thus activating downstream signaling cascade involving 3-phosphoinositide-dependent protein kinase-1 (PDK1), Akt and GSK-3β. In the context of glucose regulation, GSK-3β promotes glycogen synthesis, but it is also one of the principal kinases responsible for Tau phosphorylation [61]. Successful activation of Akt requires not only phosphorylation at Thr308 by PDK1, but also additional "priming" at Ser473 residue by mammalian target of rapamycin (mTOR) complex [62]. The activity of GSK-3β is, in turn, negatively regulated by phosphorylation at Ser9 residue by activated Akt, as well as positively regulated by autophosphorylation at Tyr216 [63]. Our data demonstrate both HFD- and transgene-dependent decrease in hippocampal pSer^{473}-Akt levels, accompanied by an increase in activated pTyr^{216}-GSK-3β protein levels in 6-months-old mice. Furthermore, we have also observed an upregulation in pSer^{404}-Tau, which suggests abnormalities in IRS-Akt-GSK-3β signaling. These results are in line with previously published postmortem examinations of the brains of human patients suffering from AD and diabetes [64].

4.2. AMPK, PPAR and ERK

ERK has been implicated as a key molecule involved in hippocampal memory consolidation. While directly stimulated by insulin, it may also be partially activated via IRS-independent mechanisms [57], thus offering a potentially attractive modulatory target for the pharmaceutical intervention. ERK acts via ERK/CREB/CBP pathway, activation of which ultimately results in the transcription of genes required for neuronal plasticity and long-term potentiation (LTP), in particular. In the brain, ERK was shown to be positively regulated by AMPK [65], a key energy sensor in the peripheral tissues, the role of which in the CNS remains controversial [66]. In addition, activated ERK was shown to be recruited (thus activating) to PPARγ, a nuclear receptor and a transcription factor which activates a number of genes related to insulin sensitivity and cognition [67]. We did not detect any changes in the α subunit of the AMPK, nor in PPARα and γ levels, but we did observe an increase in pThr^{202}/pTyr^{204}-ERK1/2 in response to HFD treatment. Considering that ERK expression is positively correlated with memory consolidation our results may seem contradictory, however this is not the case. Feld *et al* [68] have recently demonstrated that a minimum threshold of ERK expression is required to maintain memory function, and that the aberrant overexpression of ERK protein leads to memory impairment in a similar manner as ERK deficiency.

4.3. JNK1

This stress activated pro-inflammatory and pro-apoptotic MAPK has been previously linked to neurodegeneration and to AD, albeit the existing evidence for its importance is rather strenuous (reviewed in [69]). There is much more support for the involvement of JNK1 in diet-induced obesity and the peripheral insulin resistance (reviewed

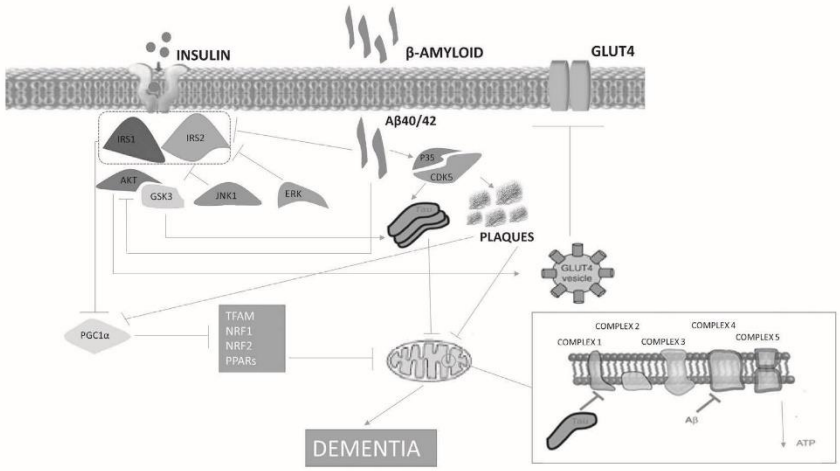

Fig. 8. A proposed mechanism whereby insulin signaling and mitochondrial dysregulation contribute to hippocampal phenotype in HFD-fed and AD-like mice. Increased circulating triglyceride levels in response to HFD treatment initially provoke cerebral insulin deficiency, which results in the downregulation of the canonical insulin signaling pathway in the hippocampus. At the same time, the free fatty acid-mediated increased metabolic stress subverts autologous IR-IRS-Ser/Thr kinase axis signaling, thus favoring heterologous regulation. This route, once activated, initiates a series of self-propagating events which ultimately lead to insulin resistance in a manner similar to that observed in response to elevated β-amyloid levels. As functional IR signaling in the hippocampus is, at least partially, regulating neuronal glucose entry [74], the inhibition of this pathway may result in the reduced supply of readily available energy to the mitochondria, affecting neuroplasticity. Mitochondrial OXPHOS metabolism deficiencies may thus be explained by two possible mechanisms: (a) as a direct result of reduced glucose availability, and (b) as a consequence of decreased mitochondrial biogenesis (PGC1) and/or disruptions to mitochondrial function due to hyperphosphorylated Tau protein and increased β-amyloid levels.

in [70]). The fact that we detected a significant increase in $pThr^{183}$/$pTyr^{185}$-JNK1 in the hippocampi of HFD-fed mice, irrespective of the β-amyloid load, is curious for two reasons. One: it suggests that hippocampal insulin signaling may in fact be strikingly similar to the peripheral phenotype. Two: very high levels of β amyloid, as observed in 6-months-old APP/PS1 control animals, are not sufficient to trigger elevated JNK1 expression.

4.4. CDK5

Cyclin-dependent kinase 5 (co-activated by p35) does not phosphorylate IRS directly, however increased activity of this kinase (and GSK-3β) contributes to Tau hyperphoshorylation, resulting in neurofibrillary tangle formation (reviewed in [71]). We have detected a significant upregulation of both the CDK5 and GSK-3β kinases in the hippocampi of HFD-fed WT mice. These data correlate well with the observed increase in $pSer^{404}$-Tau.

4.5. PGC-1α

Our data demonstrate a significant decrease in insulin signaling and impaired glucose homeostasis in the hippocampus. As hippocampal neurons require copious amounts of energy in order to be able to form and retain memories, such deficiencies will likely have an effect on mitochondrial energy metabolism. Therefore, in the latter part of this study, we have focused on the pathways related to mitochondrial biogenesis and OXPHOS pathway.

The peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a transcriptional co-activator which, apart from other functions, plays a critical role in mitochondrial biogenesis. PGC-1α was previously shown to regulate the expression, either directly or indirectly, of the nuclear respiratory factors 1 (NRF1) and 2 (NRF 2), estrogen-related receptor α (ESRRa) and mitochondrial transcription factor A (TFAM) (reviewed in [72]). While we have observed a significant reduction in PGC-1α protein levels in response to HFD treatment, and various statistically significant changes in the above mentioned molecules, most of the differences were rather modest in numerical values, and may or may not represent the behavior of individual targets under similar experimental conditions. Taken together, however, our data suggest clear impairments in mitochondrial function. Regarding the expression of OXPHOS proteins, we detected significant decreases in mitochondrial OXPHOS metabolism in both the HFD-fed WT mice and in APP/PS1 animals. This finding is especially significant as OXPHOS deregulation has been previously linked to both the βA and Tau pathologies in AD brains (reviewed by [73]).

In conclusion, we have demonstrated some parallels between the hippocampus-dependent HFD-induced memory loss vs. the memory loss occurring in a mouse model of Alzheimer disease. Our results indicate that the brain β-amyloid levels seem not to be the primary cause of the HFD-induced memory perturbations. It appears that the reductions in brain insulin signaling and the resulting mitochondrial dysfunction are among the key culprits leading to cognitive decline in early-stage AD-like rodent models. A summarized view of our hypothesis is provided in Fig. 8.

Disclosure statement

The authors declare no competing financial interests.

Conflict of interest

All authors don't have any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations. All authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data.

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DISCUSSION

1. OVERVIEW

As mentioned in the introductory sections, AD is a complex pathology which is highly resistant to pharmaceutical interventions. Apart from the genetic component and old age, seven primary preventable environmental risk factors contributing to AD have been identified: diabetes mellitus, midlife hypertension, midlife obesity, depression, physical inactivity, smoking and cognitive inactivity (Barnes & Yaffe, 2011; Norton, Matthews, Barnes, Yaffe, & Brayne, 2014). Thus, it is becoming increasingly evident that most of the prognostic preventable AD risk factors may also be linked to obesity and resulting comorbidities, including metabolic syndrome and diabetes. Even though the epidemiological data suggest an existing relationship between AD and energy metabolism, molecular mechanisms behind this relationship are poorly understood. In the work described in this thesis we wanted to address the role of metabolism in AD. While it is true that an APP/PS1 mouse model in which the experiments were undertaken is a rodent model of FAD, and FAD represents only 2% of all diagnosed cases of AD, we believe that this experimental approach is nevertheless capable of identifying certain molecular aspects of the disease which could be useful for understanding the bigger picture.

In the planning stages, as a first step, we wanted to establish whether insulin signaling is affected in our model at the relatively early disease stages. The reason for choosing younger animals is that at the age of 3 months, the typical pathological hallmarks of the disease (senile plaques and neurofibrillary tangles) are not yet present in the brains of these mice. By the age of 6 months, the plaques are already evident and hippocampal-dependent memory deficits are observed. However, even at the age of 6 months, the disease has presumably not yet taken an irreversible aspect. That is to say that the phenotype is not severe enough to affect gross cerebral morphology and that the changes observed at this age are likely to be a direct result of elevated levels of Aβ peptide. Having determined that hippocampal insulin signaling is indeed disturbed in transgenic animals, and that by the age of 6 months APP/PS1 mice develop impaired glucose and insulin tolerance (Publication 1: (Pedrós et al., 2014)) we proceeded with the second major part of this work which is reflected in the title of the current thesis – “The role of high-fat diet in an APP/PS1 model of familial Alzheimer disease”.

Our initial approach was to analyze the changes which occur in response to a HFD treatment in 3- and 6-month-old wild-type and transgenic mice. Unfortunately, the first data which we have collected in 3-month-old animals were rather ambiguous and demonstrated a very significant biological variability. Due to these limitations, a decision was made to focus on 6-month-old animals only. As described in Publication 2 (Petrov et al., 2015), continuous HFD treatment is sufficient to cause hippocampus-dependent memory deficits in wild-type mice. The scientific rationale for studying the phenotypes of mice in which cognition was likely to be altered *a priori*, was to determine the biological relationship between the HFD- and AD-

related memory loss. In addition, we wanted to see if AD-like pathology could lead to the exacerbation of HFD-caused metabolic and CNS phenotypes. The next sections of the discussion are organized in the following manner:

- Changes which occur in the periphery
    - 3-month-old animals on normal chow
    - 6-month-old mice on control and HF diets

- CNS phenotype
    - 3-month-old animals on normal chow
    - 6-month-old mice on control and HF diets

DISCLAIMER: In order to avoid simply copying large portions of the Discussion sections of the papers presented in the Results section, the analysis described here concerns some of the issues that were not fully addressed in Publications 1 and 2. In addition, I will be focusing on the entire pathways as opposed to the role of individual molecules, as the role of each one was described in some detail in the presented research papers.

2. PERIPHERAL PHENOTYPE

2.1 3 months of age – normal diet

Fasting levels of serum insulin remained unchanged between the wild-type and the transgenic animals. We did observe a slight but not a significant increase in glucose levels as a result of intraperitoneal glucose administration in the IPGTT in APP/PS1 mice. The data strongly suggest that the peripheral metabolic phenotype is unaffected at this age.

2.2 6 months of age

By the time the mice are 6 months old, peripheral metabolism differences start to become apparent. Even on a control diet, APP/PS1 mice show impairments in the glucose and insulin tolerance. HFD results in diet-induced obesity, with differences in body weight starting to become evident at around 2 months of age. At the end of the experiment, an approximately 50% weight gain is recorded. The classical features of the metabolic syndrome are present in both the wild-type and the transgenic animals at this age. We detected peripheral hyperinsulinemia, dyslipidemia, significantly impaired glucose and insulin tolerance and obesity. The most important finding in our work is that all of these metabolic parameters were significantly worse in transgenic mice. In fact, HFD alone was not sufficient to raise basal fasting blood glucose levels after an overnight fast in wild-type mice, but in APP/PS1 animals hypercaloric diet treatment produced an over 50% increase in basal glucose. It is quite striking that increases in triglyceride (TG) levels and the extent of impairments detected in IPGTT and ITT demonstrated an almost additive effect as a result of a HFD and the transgenic status. When looking at the graphs of TG, IPGTT and ITT (**Fig. 1**; (Petrov et al., 2015), one can see that the wild-type mice on a HFD present a nearly identical profile as that seen with APP/PS1 mice on a control diet. When transgenic animals were fed a hypercaloric diet, all three of these parameters moved to a nearly diabetic range. Thus, it appears that elevated cerebral secretion of Aβ peptide is sufficient to produce changes in peripheral carbohydrate metabolism which are comparable to those observed with chronic HFD administration. Crucially, in transgenic control animals the body weight is similar to wild-type control mice. Therefore, obesity by itself can-not explain this peripheral metabolic phenotype. The exact mechanisms responsible for this observation are unclear. I can only speculate that excess amyloid, while initially produced in the brain, eventually enters into the peripheral circulation (see introductory section 3.3.4). Once in the periphery, it might be subject to degradation by IDE or other insulin-degrading enzymes. Assuming that under normal conditions endogenous peripheral insulin is indeed degraded by IDE in a physiologically relevant manner (which is controversial (Authier, Posner, & Bergeron, 1996; Deprez-Poulain et al., 2015; Farris et al., 2004)), then the presence of beta amyloid may result in a competitive inhibition of the enzyme. This could then theoretically lead to increased plasma

insulin and the development of peripheral insulin resistance which would be reflected by impaired glucose and insulin tolerance. It is important to note, that our results do not show significant increases in serum insulin levels on a control diet (**Fig. 2** (Pedrós et al., 2014); **Fig. 1** (Petrov et al., 2015)). However, the measurements were taken after a 5-hour morning fast, which may be enough time for any possible differences to disappear. The reason why we do see clear differences in insulin levels in response to HFD treatment, is a direct result of obesity, which would cause chronically elevated insulin levels even in fasted mice. In any case, these data are very interesting and warrant additional investigation in further detail.

3. CNS PHENOTYPE

3.1 3 months of age – normal diet

Hippocampus-dependent memory loss is not present at this age. Therefore, all of the observed changes can be considered as events which occur in the prodromal stages of AD.

3.1.1 Amyloidogenesis and Tau

Elevated levels of APP mRNA were present in the hippocampi of transgenic mice. We had also detected significant amounts of insoluble Aβ (1-42) in the cortical homogenates of 3-month-old APP/PS1 animals. At this age, no visible senile plaques nor neurofibrillary tangles are detectable by microscopy. These data suggest that at this age, the endogenous defense mechanisms which protect against amyloid aggregation are capable of coping with the excess of amyloid peptides.

With regards to tau, we have observed tau hyperphosphorylation at multiple residues (S199; T205; S404) in transgenic mice, indicating that tau pathology may begin to manifest itself as early as 3 months of age. We did not detect elevated expression in some of the kinases which are known to lead to tau hyperphosphorylation (see section 3.5.1 of the introduction). Protein levels of activated forms of MAPK and GSK3β remained unchanged. We did detect a slight but significant increase in CDK5, however this increase was accompanied by elevated expression of p35, suggesting that this kinase may not necessarily be involved (section 3.5.1 as well). Further studies may be necessary to identify the kinase/s responsible for tau hyperphosphorylation at such an early age.

3.1.2 Mitochondrial metabolism and biogenesis

Hippocampal mitochondrial metabolism is clearly impaired in 3 month old transgenic mice as evidenced by significant reductions in OXPHOS complexes I, II, II and IV. Furthermore, we detected reductions in molecules involved in mitochondrial biogenesis including PGC-1α, NRF1 and NRF2. Taken together, our results do not contradict neither the “amyloid cascade” (due to the presence of insoluble amyloid) nor the “mitochondrial cascade” hypotheses (see pages 16 and 20).

3.1.3 Hippocampal insulin signaling

We did not detect major changes in the insulin- INS-R -IRS axis in 3-month-old APP/PS1 mice. However, small but significant differences were observed in the mRNA levels of the INS-R and IRS2, suggesting that at this age the very early signs of hippocampal insulin signaling defects may just be starting to appear. In addition, we observed a significant reduction in genes related to memory, ARC and FOS, both of which are known to be regulated by insulin signaling (Guillod-Maximin, Lorsignol, Alquier, & Pénicaud, 2004;

Kremerskothen, Wendholt, Teber, & Barnekow, 2002). Please note that in the **Fig. 3** of the Publication 1, the statistical significance marking was erroneously transposed to IRS1, instead of IRS2. The data presented in the discussion of the above mentioned paper correctly identifies IRS2 as the molecule mRNA levels of which were significantly reduced.

3.1.4 Synaptic proteins

According to the "synaptic dysfunction" hypothesis (page 24), synaptic loss may occur as a result of Aβ oligomer accumulation, at a stage prior to the senile plaque formation. As we have detected elevated levels of Aβ in the hippocampi of 3-month-old APP/PS1 mice, we have measured the levels of SYP and PSD-95, both of which are the biomarkers of the healthy synapses. Protein expression levels of neither of the molecules were affected by transgenic status.

3.1.5. Summary

On the whole, the data described here is indicative of the presence of the early stages of amyloidogenesis, tau dysfunction, mitochondrial metabolism and biogenesis defects and the beginnings of hippocampal insulin signaling perturbations in the hippocampi of 3 month-old transgenic mice. Once again, all these changes occur in the prodromal disease stages, prior to measurable cognitive deficits.

3.2 6 months of age

As mentioned in the overview, we had reason to suspect that hippocampal-dependent memory loss will be present in the HFD-treated wild-type mice. That did turn out to be the case. At the age of 6 months, cognition was affected in HFD-treated wild-type, as well as in control and HFD-treated APP/PS1 animals.

3.2.1 Amyloidogenesis and Tau

Cortical Aβ peptide levels were negligible in wild-type mice. In transgenic animals only the insoluble Aβ (1-42) was significantly increased in response to a HFD. Furthermore, the plaque numbers and size were unchanged in response to treatment. These data indicate that hypercaloric diet is unlikely to significantly affect amyloid pathology at this age. Therefore, the observed worsening of the peripheral and central phenotypes may be attributed to factors outside of the "amyloid cascade hypothesis" (discussed in more detail in the following sections).

*CORRIGENDUM: Aβ ELISA data presented in **Fig. 1D** of the Publication 1 depicts Aβ values on Y axis as picograms/mg of protein. This is incorrect and should read picograms/ml of extract. In **Fig. 2C** of the publication 2 the graphs representing soluble and insoluble levels of Aβ are identical, and it is in fact the same graph of insoluble Aβ (white bar- control chow; black bar – HFD). The complete and correct data is*

presented in Table 3 of the same publication. Note that the first 6 columns of that Table depict soluble peptide, and the columns 7-12 the values of insoluble Aβ.

Tau hyperphosphorylation was detected on multiple residues. In addition to S199, T205, S404 (which were elevated in 3-month-old APP/PS1 animals), tau S396 was also significantly upregulated in 6-month-old hippocampal extracts (**Fig. 6** of Publication 1) of transgenic mice on a control diet. HFD treatment has caused increases in the phosphorylation state of tau S404 in wild-type mice along with a slight (statistically insignificant) increase in HFD- versus control diet-fed transgenic mice (**Fig. 4** of Publication 2). The reason why only the data for S404 (and not any other phosphorylated taus) is presented in Publication 2, is that we had observed significant biological variability in S199, T205 and S396 immunoblots in response to a HFD treatment. As a result, we made a decision to only publish the data which was statistically sound.

Tau-phosphorylating enzymes – control diet: On a control chow, MAPK, GSK3β and CDK5 expression was comparable to that observed at 3 months (see section 3.1.1 of the discussion). We did evaluate the expression values of additional Tau-phosphorylating kinases ERK 1/2 and JNK 1/2 in Publication 2 (**Fig. 4** of that publication). No changes were observed for these kinases in response to normal diet.

Tau-phosphorylating kinases – HFD: MAPK levels remained unchanged, with CDK5 increase observed concomitantly with p35 increases in HFD-fed wild-type versus control mice (3.1.1 of the discussion). Phosphorylation levels of GSK3β were slightly but significantly increased in hypercaloric wild-type group. The results for ERK 1/2 and JNK 1/2 are much more interesting. These kinases were very significantly upregulated in HFD-fed mice of both groups (wild-type and APP/PS1). These data alone may already be sufficient to explain observed increases in tau S404 hyperphosphorylation in HFD-fed wild-type mice. But then, why didn't we observe a significant increase in tau phosphorylation on the same epitope in transgenic mice? A possible explanation is that in this particular case, both JNK and ERK may serve a totally different biological role as is expected on the basis of their relationship to tau. In fact, JNK and ERK are key players in insulin-signaling regulation (see sections 4.2 and 4.3 of Publication 2).

3.2.2 Mitochondrial metabolism and biogenesis

OXPHOS: All 5 of the OXPHOS complexes were significantly downregulated in chow-fed APP/PS1 mice compared to wild-type controls (**Fig. 7** of the Publication 2). Even though we reported no changes in complexes IV and V in 6-month-old animals in Publication 1 (**Fig. 5**), these are the results of independent immunoblotting experiments. In case of Publication 1, representative images of Complex V actually appear to show downregulation of this Complex in transgenic mice. However, when normalized to GAPDH, these differences disappeared. These variations in the reported data are a result of experimental variability.

Diet-induced obesity resulted in the significant reduction of all 5 of the OXPHOS proteins in the hippocampi of wild-type mice. In transgenic animals, hypercaloric diet did not further reduce the expression of the complexes. These data indicate that mitochondrial metabolism disruptions are severe enough in transgenic brains in the basal state, to the point that the presence of metabolic syndrome does not significantly worsen oxidative phosphorylation.

Related molecules: In contrast to the data obtained in the hippocampi of 3-month-old animals, we have only observed a slight reduction in PGC-1α, but not in NRF1 and NRF2 in 6-month-old chow-fed transgenic mice. In addition, it appears that these molecules are downregulated in wild-type in response to normal ageing (compare the results between 3 and 6 month old mice in **Fig. 4** of Publication 1). Furthermore, because PGC-1α, NRF1 and NRF2 were reduced in 3-month-old transgenic group, it seems likely that APP/PS1 status accelerates normal age-related physiological decline of these factors. By the age of 6 months, a physiological steady-state is reached and therefore no further declines are observed in comparison to the control group. HFD treatment did induce slight, but significant reduction of PGC-1α both in the wild-type and transgenic brains. NRF1 and NRF2 were further downregulated in APP/PS1 group only.

Taken together, our data clearly demonstrate that metabolic syndrome significantly affects hippocampal mitochondrial homeostasis and biogenesis in wild-type mice. In a transgenic group, deficiencies in this signaling pathway are already present even before the additional insult of the hypercaloric diet. Because of that, HFD treatment has only a minimally worsening effect on these pathways in APP/PS1 mice.

3.2.3 Hippocampal insulin signaling

Control diet: Our data suggest that hippocampal insulin signaling is impaired in APP/PS1 mice. We did not detect changes in the hippocampal expression of INS-R itself, suggesting that the receptor is fully functional at this age. However, we did detect significant differences in the molecules downstream of the insulin receptor. Most importantly, we observed an over 2-fold increase in the pIRS2 (S723), a key negative regulator of downstream insulin signaling. Phosphorylation at this epitope, results in the desensitization of downstream signaling. In line with these observations, we observed changes in the phosphorylation state of AKT and GSK3β, both kinases regulated by IRS (described in more detail in Section 4.1 and **Fig. 8** of Publication 2). Thus we can conclude that downstream insulin signaling is disturbed in transgenic mice at the basal state at the age of 6 months.

Diet-induced obesity: HFD treatment resulted in much more pronounced changes in hippocampal insulin signaling when compared to normal diet both in the wild-type and transgenic mice. Just like in response to chow, neither the mRNA nor the protein levels of the INS-R were majorly affected by the treatment in any of the groups (there was a slight, but significant reduction of INS-R in HFD- versus chow-treated transgenic

mice). pIRS2 (S723) and pGSK3β (Y216) protein levels were significantly increased in treated wild-type mice. In transgenic animals, the expression of both molecules was already elevated in the basal state and hypercaloric diet did not result in additional increases. pAKT (S473) was very significantly downregulated in the wild-type, with further reductions occurring in HFD-fed APP/PS1 hippocampi. Importantly, we had detected significant increase in the mRNA levels of INS1 in both treated groups. INS1 is one of the two genes which code for insulin in mice (in humans there is only one gene). As mentioned in the discussion section of Publication 2, diet-induced obesity may provoke disruptions in the peripherally-expressed insulin transport across the BBB. The fact that we could detect elevated expression of insulin transcripts in the hippocampus may signify 2 things: A) Not enough insulin is reaching the brain, which could result in the compensatory mechanisms activating endogenous insulin expression directly in the hippocampus; B) diet-induced obesity produces hippocampal insulin resistance, which causes excess production of endogenous brain insulin. Put in simple terms, in the first case there is not enough available insulin, while in the second case there is too much. Additional studies are necessary to determine the exact mechanism behind elevated insulin production in the hippocampal circuits. Another very interesting finding is the behavior of IGF1 and its receptor IGF1-R. IGF1 expression was increased in HFD-fed wild-type mice only (and slightly reduced in transgenic mice), while IGF1-R expression was reduced in only the transgenic mice exposed to the hypercaloric diet. Lower IGF1 expression was previously correlated with cognitive decline in AD (Kimoto et al., 2015), so our data are in agreement with these observations. The reasons for increased expression in HFD-treated wild-type mice are less clear, but may involve similar compensatory mechanisms as described above for insulin. In case of IGF1-R, its reduced expression was demonstrated in the post-mortem analysis of the brains of AD patients (Freude, Schilbach, & Schubert, 2009), so it is quite possible that the reason why we observe its downregulation in HFD-fed APP/PS1 mice is that diet-induced obesity accelerates AD-like pathology in these mice. In order to be sure, however, we would need to study the expression levels of IGF1-R in much older APP/PS1 mice (possibly at the ages of 9 or even 12 months).

JNK1 is another kinase whose protein expression levels demonstrated over 200% increase in the hippocampi of both the wild-type and transgenic groups in response to hypercaloric diet. This is very important, as the increased expression of JNK1 in insulin-responsive peripheral tissues including fat, muscle and liver, was previously linked to diet-induced obesity. JNK1 is thought to be one of the contributors to peripheral insulin resistance. Furthermore, brain-specific deletion of JNK1 prevented diet-induced obesity in rodent models. However, the main site of action in the brain is thought to be the hypothalamic-pituitary-thyroid axis which regulates food intake and energy expenditure (Sabio & Davis, 2010). The fact that we had detected JNK1 upregulation in the hippocampus, suggests that the hippocampal circuits may also be involved.

The big picture: Our data demonstrate a clear involvement of hippocampal insulin signaling in diet-induced obesity as well as in AD pathology. Very recently (it will appear in print in the December 2015 issue of Current Alzheimer Research) an Italian group has published research which explored the relationship between HFD and the peripheral and central metabolic phenotypes. Even though the researchers have used total brain extracts as opposed to hippocampal extracts described in our papers, the conclusions were strikingly similar. That is that hypercaloric diet significantly affects mitochondrial metabolism and both the peripheral and central insulin homeostasis (Nuzzo et al., 2015). A general overview of how exactly that may happen is presented in the **Fig. 4**.

3.2.4 Synaptic proteins

Similar to the data described for 3 month old animals (section 3.1.4 of the discussion), neither SYP nor PSD-95 expression was affected in the hippocampus. HFD treatment did not result in any additional changes to these proteins (data not shown).

3.2.5 Amyloid-degrading enzymes

We had measured hippocampal expression of IDE and neprilysin. On a normal chow, no changes were observed for any of the molecules. HFD administration caused significant downregulation of IDE both in the wild-type and transgenic brains. Because the expression of IDE is regulated by the insulin pathway (overactivation of IR signaling by insulin ultimately results in increased transcription of IDE, which presumably shuts the pathway down), and we had established that insulin signaling is defective in response to diet-induced obesity, these results are not surprising. In addition, we detected significant increases in neprilysin protein in HFD-exposed APP/PS1 mice only. One possible explanation for these data is that neprilysin overexpression is triggered by the Aβ itself as a compensatory mechanism in response to the decreased expression of IDE. In such scenario, amyloid clearance is performed by both IDE and neprilysin: in the wild-type mice which do not express significantly elevated levels of amyloid, there will not be a physiological need for increased expression of another amyloid-degrading enzyme, which is clearly not the case in transgenic animals. In APP/PS1 mice, the physiological defense mechanisms aimed at removing excessive amyloid will be in the overdrive, which would explain high neprilysin levels.

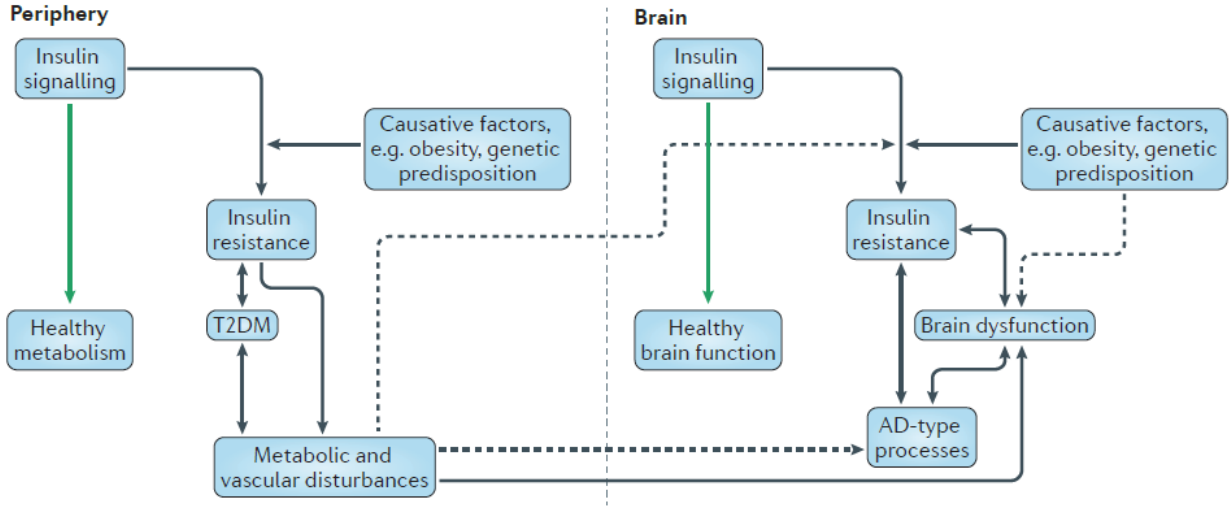

Figure 4. Insulin is an essential hormone which has multiple roles both in the periphery and in the CNS. Deficient insulin signaling in the brain may contribute to neurodegeneration observed in AD. However, peripheral insulin resistance may also be a direct result of AD-like pathology, even in cases when overt obesity is not present (see section 2.2 of the discussion). Once both the metabolic syndrome and AD-like pathology are combined, it is quite possible that disease pathogenesis is further accelerated, thus forming a positive feedback loop. (Solid arrows indicate established disease processes, dashed arrows represent possible disease processes and green arrows represent the effects of normal insulin signaling). *Reproduced from (Biessels & Reagan, 2015)

4. SUMMARY

Research described in the preceding sections has revealed a number of hitherto unknown/insufficiently explored molecular mechanisms which may contribute to AD development in an APP/PS1 mouse model of FAD. In addition, hippocampal and peripheral phenotypes of HFD-induced memory loss were described in some detail. The main focus of the current thesis was to identify signaling networks which contribute to AD pathology in the relatively early-disease stages, presumably prior to the "point of no return", when the pathology becomes irreversible. In the first part of the present work we have determined that mitochondrial homeostasis, insulin signaling and tau processing are all affected by AD-like status of APP/PS1 mice. We have also determined that at the age of 6 months, peripheral carbohydrate metabolism is significantly altered in transgenic animals. Chronic exposure to a HFD resulted in cognitive deficits even in the control animals. However, the exact mechanisms for this hippocampal-dependent memory loss were distinct. Our data suggest that elevated cerebral secretion of amyloid peptides is sufficient to produce similar phenotype as to that observed in response to diet-induced obesity. In transgenic animals predisposed to AD development, the introduction of hypercaloric diet significantly worsened existing phenotype both at the periphery and in the hippocampal networks. Overall, our results suggest that metabolic syndrome, diabetes and related comorbidities clearly do have a potential to significantly worsen the symptoms of AD disease. We identified specific molecules and the associated pathways which may play a role in disease progression. An integrated view of the proposed mechanistic changes which occur in the hippocampus is presented in **Fig. 5** (reproduced from Publication 2).

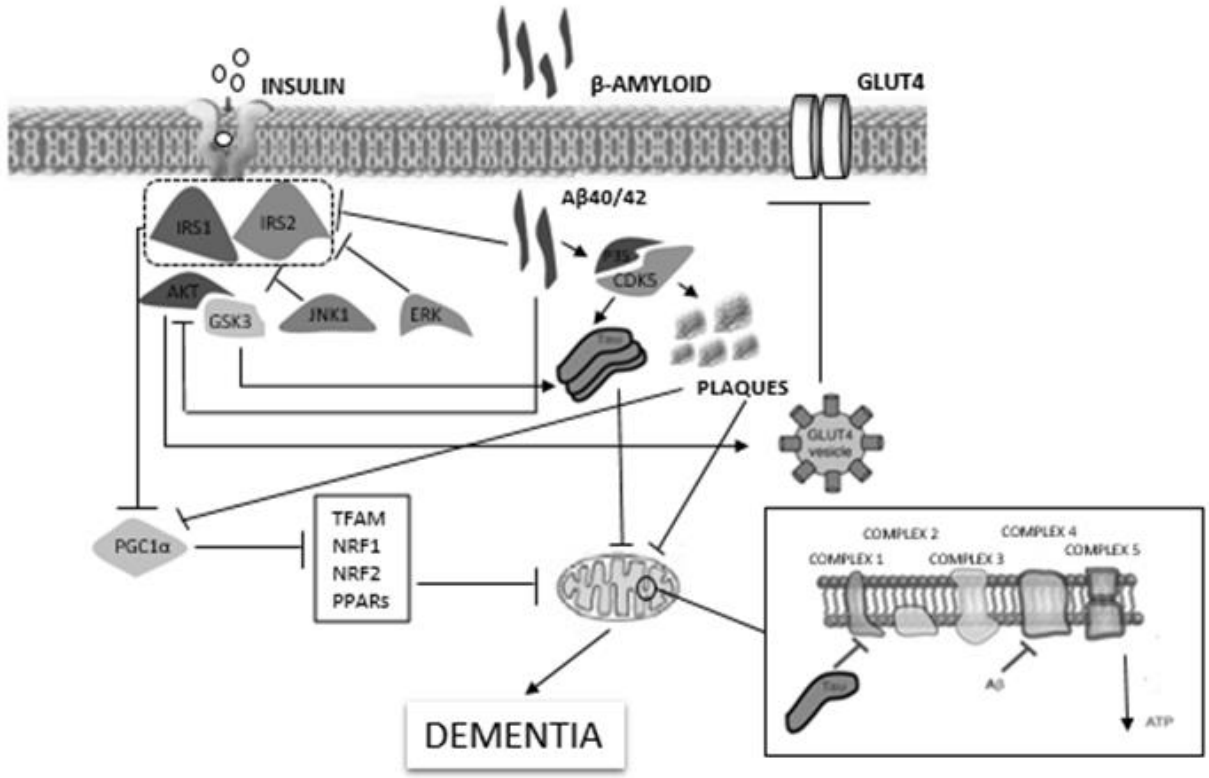

Figure 5. A proposed mechanism whereby insulin signaling and mitochondrial dysregulation contribute to hippocampal phenotype in HFD-fed and AD-like mice. Increased circulating triglyceride levels in response to HFD treatment initially provoke cerebral insulin deficiency, which results in the downregulation of the canonical insulin signaling pathway in the hippocampus. At the same time, the free fatty acid-mediated increased metabolic stress subverts autologous IR-IRS-Ser/Thr kinase axis signaling, thus favoring heterologous regulation. This route, once activated, initiates a series of self-propagating events which ultimately lead to insulin resistance in a manner similar to that observed in response to elevated β-amyloid levels. As functional IR signaling in the hippocampus is, at least partially, regulating neuronal glucose entry, the inhibition of this pathway may result in the reduced supply of readily available energy to the mitochondria, affecting neuroplasticity. Mitochondrial OXPHOS metabolism deficiencies may thus be explained by two possible mechanisms: (a) as a direct result of reduced glucose availability, and (b) as a consequence of decreased mitochondrial biogenesis (PGC1) and/or disruptions to mitochondrial function due to hyperphosphorylated Tau protein and increased β-amyloid levels. *Reproduced from (Petrov et al., 2015)

CONCLUSION

CONCLUSION

In my opinion, the work described in Publications 1 and 2 adequately answers the questions posed in the "objectives" section. Specifically:

1. *Characterization of the peripheral and central metabolic phenotypes at 3 and 6 months of age in APPswe/PS1dE9 versus the wild-type mice on a <u>standard chow</u> in order to:*

    A) Determine if transgenic status has an effect on peripheral carbohydrate metabolism

- Peripheral metabolic phenotype is unaffected in 3-month-old APP/PS1 mice. At 6 months of age, data indicate impaired glucose and insulin tolerance which is suggestive of peripheral insulin resistance

    B) Determine if hippocampal insulin signaling is altered in mice predisposed to Alzheimer disease development

- At an early age, our results suggest the presence of initial indicators pointing toward impaired hippocampal insulin signaling in transgenic animals. In older mice, hippocampal insulin signaling is clearly impaired

    C) Identify molecular pathways which are altered in early stages of AD-like pathology, prior to amyloid plaque development and memory loss in hippocampal circuits

- Mitochondrial homeostasis, the state of tau phosphorylation and amyloidogenesis are all affected in 3-month-old APP/PS1 mice. Furthermore, by the time the cognitive deficits do appear (at 6 months), the phenotype is exacerbated

2. *Characterization of the peripheral and central metabolic phenotypes in 6-month-old APPswe/PS1dE9 versus the wild-type mice on a high-fat diet. In addition to the 1A and 1B described above, the following objectives are considered:*

A) Determine if high-fat diet treatment differentially affects wild-type and transgenic mice

- On the peripheral level, transgenic status significantly worsens the present pathology. HFD-fed APP/PS1 animals present with a nearly diabetic phenotype. Significant differences are also observed at the hippocampal level, discussed in detail in the "discussion" section

B) Determine if chronic hypercaloric diet administration affects memory

- The results of a 2-object Novel Object Recognition test show that HFD-fed wild type mice demonstrate memory loss. The same could be said about transgenic animals both on the control and the hypercaloric diets

C) Determine if high-fat diet accelerates AD-like pathology

- Our data, described in detail in the "discussion" section, suggest that this is indeed the case

D) Determine if hypercaloric diet affects hippocampal signaling molecules traditionally associated with AD

- Insoluble levels of Aβ (1-42) were elevated in cortical extracts of HFD-fed transgenic mice. Some of the kinases known to promote tau phosphorylation were upregulated in response to diet-induced obesity. Hippocampal levels of pTau (S404) were increased in response to a HFD.

SUPPLEMENTARY PUBLICATIONS

Publication 1

CURRENT RESEARCH THERAPEUTIC STRATEGIES FOR ALZHEIMER'S DISEASE TREATMENT

Jaume Folch, **Dmitry Petrov**, Miren Ettcheto, Sonia Abad, Elena Sánchez-López, M. Luisa García, Jordi Olloquequi, Carlos Beas-Zarate, Carme Auladell, Antoni Camins

Neural Plasticity (Accepted)

Summary

This Review paper describes past and possible future pharmacotherapies aimed at Alzheimer disease treatment. The reason for inclusion of this manuscript to this doctoral thesis presentation is that large portions of the Sections 3.3-3.8 of the Introduction are derived from that article.

Current research therapeutic strategies for Alzheimer's disease treatment

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RUNNING TITLE: **Current therapy of Alzheimer's disease**

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Abstract

Alzheimer's disease (AD) currently presents one of the biggest healthcare issues in the developed countries. There is no effective treatment capable of slowing down disease progression. In recent years the main focus of research into novel pharmacotherapies was based on the amyloidogenic hypothesis of AD, which posits that the beta amyloid (Aβ) peptide is chiefly responsible for cognitive impairment and neuronal death. The goal of such treatments is (a) to reduce Aβ production through the inhibition of β and γ secretase enzymes, and (b) to promote dissolution of existing cerebral Aβ plaques. However, this approach has proven to be only modestly effective. Recent studies suggest an alternative strategy centred on the inhibition of the downstream Aβ signalling, particularly at the synapse. Aβ oligomers may cause aberrant N-methyl-D-aspartate receptor (NMDAR) activation post-synaptically by forming complexes with the cell-surface prion protein (PrPC). PrPC is enriched at the neuronal post-synaptic density, where it interacts with Fyn tyrosine kinase. Fyn activation occurs when Aβ is bound to PrPC-Fyn complex. Activated in this manner, Fyn can cause tyrosine phosphorylation of the NR2B subunit of metabotropic glutamate receptor 5 (mGluR5). This results in an initial increase and then a loss of these cell-surface NMDARs. Fyn kinase blockers masitinib and saracatinib have proven to be efficacious in treating AD symptoms in experimental mouse models of the disease. Saracatinib is currently in Phase II and masitinib in Phase III clinical trials for mild-to-moderate AD.

1.- Introduction

Alzheimer's disease (AD) is currently incurable neurodegenerative condition which is highly prevalent in old age [1-3]. It was first described in 1906 by Alois Alzheimer, who analysed brain tissue from a patient who had died from an unknown mental illness. According to the Alzheimer's Association, 13% of people over 65 suffer from this disease in developed countries, where it is the fifth leading cause of death in patients at this age. According to the World Health Organization (WHO) estimates, the overall projected prevalence in global population will quadruple in the next decades, reaching 114 million patients by 2050 [2]. Apart from having a great social impact, this would clearly lead to increased economic burden to healthcare systems worldwide [1-3].

AD is classified according to the age of onset and whether it is developed spontaneously or as a result of genetic mutations. Familial AD (FAD) is an early-onset (sometimes as early as 40 years of age) disease, which is caused by hereditary mutations and represents approximately 2% of diagnosed cases. The vast majority of patients suffer from the sporadic AD, which is subdivided into early- and late-onset forms. If identified in individuals under 65 years of age, early-onset diagnosis is given (3-5% prevalence), with the rest of the cases referred to as a late-onset AD (95-97% prevalence) [3-7]. In FAD, mutations in genes coding for amyloid precursor protein (APP; chromosome 21), presenilin 1 (PS1; chromosome 14) and presenilin 2 (PS2; chromosome 1) serve as triggers for beta amyloid (Aβ) formation, particularly of the long form of the peptide (Aβ1-42). In case of sporadic AD, a significant number of patients (approximately 25%) are carriers of the e4 allele of the ApoE gene (apolipoprotein E; chromosome 19), a lipid transport protein. The exact mechanism whereby ApoE contributes to increased Aβ levels is currently unknown [6-9].

Aging is considered the principal risk factor for sporadic AD development. Other potential risk factors including hypertension, dyslipidemia, metabolic syndrome and diabetes have also been identified [10-12].

In the present manuscript, we discuss treatment strategies structured according to a number of existing hypotheses aimed at explaining the origins of AD:

a) Amyloid cascade hypothesis
b) Cholinergic hypothesis
c) Dendritic hypothesis
d) Mitochondrial cascade hypothesis
e) Metabolic hypothesis

f) Other hypotheses (oxidative stress, neuroinflammation)

The principal targets and clinical trials of the compounds aimed at reducing Aβ formation and plaques are summarized in Table 1. Relevant data for the molecules developed in the context of cholinergic, dendritic, mitochondrial cascade, metabolic and other hypotheses are presented in Table 2.

2.- The amyloid cascade hypothesis

Aβ peptide is derived from proteolysis of APP, an integral transmembrane protein found in different cell types, including neurons and glial cells [1-4]. In humans, alternative splicing produces multiple isoforms of the molecule, with APP695 being the most abundant in the brain [3]. APP is processed into smaller peptide fragments, one of which is Aβ, via cleavage by α-, β- and γ-secretase enzyme protein complexes, which include presenilin and nicastrin molecules [8]. Under physiological conditions, APP is catabolized by the α-secretase to produce a soluble sAPPα fragment, which remains in the extracellular space, and a carboxy-terminal 83-amino acid (C83) fragment, which is anchored in the plasma membrane [8-10]. sAPPα is involved in the regulation of neuronal excitability, improves synaptic plasticity, learning, memory and increases neuronal resistance to oxidative and metabolic stresses [8]. In a neuropathological situation, APP is first preferentially cleaved by β-secretase 1 (BACE), which fragments APP into sAPPβ and a 99-amino acid membrane-bound fraction (C99). Additional processing of the C99 fragment by γ-secretase results in the generation of either Aβ(1-40) or Aβ(1-42) peptides, thought to be responsible for senile plaque formation [8-12]. Whilst sAPPα is beneficial to the organism, Aβ peptides may cause synaptic loss, decrease neuronal plasticity, alter energy metabolism, induce oxidative stress and mitochondrial dysfunction, and may provoke disruptions in cellular calcium homeostasis [8,9].

The amyloid cascade hypothesis suggests that the formation, aggregation and deposition of Aβ peptides, and especially Aβ(1-42), is a primary event in AD pathogenesis which triggers neurotoxicity and neurodegeneration [6-8]. Excessive extracellular Aβ may also presumably lead to increased Tau phosphorylation and the formation of neurofibrillary tangles. Molecular genetics studies into the mechanisms of FAD gave credence to this hypothesis, suggesting potential novel therapeutics, such as inhibitors of β- and γ-secretase or enhancers of α-secretase activity. However, in cases of sporadic AD, where Aβ generation does not appear to have a clear genetic basis, amyloid cascade hypothesis cannot fully explain the root causes of the disease [11-13].

2.a.- Imbalance in the generation / removal of β-amyloid in Alzheimer's disease. Role of neuroinflammation

It is believed that Aβ is generated continuously and its aggregation and subsequent plaque deposition in AD is concentration-dependent. Excessive accumulation of both soluble and insoluble Aβ may occur not only as a result of aberrant APP processing by β- and γ-secretase enzymes, but may also be caused by inefficient removal of newly generated Aβ. Reduced activity of Aβ-degrading enzymes, such as neprilysin, insulin degrading enzyme (IDE) and angiotensin converting enzyme I (ACE I) may provoke an imbalance between the amyloid generation and clearance [13-19]. Additional predisposing factors, including the ApoE status and the presence of comorbidities such as metabolic syndrome and diabetes likely contribute to sporadic AD in a manner, which is still poorly understood.

A lack of direct correlation between amyloid plaque burden and memory loss in AD patients demonstrates that neurotoxicity is not solely dependent on the insoluble Aβ [14-16]. In fact, biochemical studies have demonstrated a good correlation between the levels of soluble Aβ oligomers in the brains of patients with AD and the degree of cognitive impairment [19]. It has been suggested that soluble Aβ-driven synaptic loss may be responsible for neurodegeneration observed in AD. If that turns out to be the case, then central nervous system (CNS) inflammatory processes will likely be implicated [20,21]. Neuroinflammation is a blanket term used to describe immune response in neurodegenerative diseases. It involves the activation of glial cells, especially microglia and astroglia. Under physiological conditions, microglial cells have a phagocytic function. In AD, activated microglia secrete a large number of molecules [21-23]. Such substances, among which are proinflammatory cytokines, prostaglandins, reactive oxygen species (ROS) and nitric oxide synthase (NOS), contribute to a chronic state of perpetual stress. A prolonged release of all these factors can eventually cause neuronal death [22,23].

2.b.- Anti-amyloidogenic pathway and amyloidogenic route as strategies for development of therapeutic treatments modifying the course of Alzheimer`s disease

In the last two decades, the pharmaceutical industry has focused primarily on the amyloidocentric approach, devoting substantial resources to develop effective AD drugs. However, multiple failures of drug candidates in clinical trials have led researchers to question the feasibility of this strategy [10-12]. One possible reason for failure is a lack of biomarkers which could reliably identify AD in relatively early stages. It is entirely

possible that the patients currently recruited for Phase III trials are in such advanced stages of AD that any attempted intervention is probably useless. Therefore, new diagnostic tools capable of early detection are sorely needed. In the meantime, there is still a number of novel treatments under development, which target the amyloidogenic route. In order to reduce Aβ generation from the APP, γ- and β-secretase inhibition and the potentiation of α-secretase activity have been considered.

- Inhibitors and modulators of β-secretase

β-secretase enzyme complex participates in the initial stages of the amyloidogenic APP-processing pathway. The development of β-secretase inhibitors is a challenge because besides the APP, this complex has many more substrates. To give just one example, neuregulin-1, which is involved in the myelination of CNS axons and synaptic plasticity, is a target of β-secretase [3]. Broad range of substrates can lead to significant side-effects, even if the specific inhibition of the enzyme is achieved. Nevertheless, E2609 (clinical trial ID# NCT01600859), MK-8931 (NCT01739348) and LY2886721 (NCT01807026 and NCT01561430) have all shown efficacy in reducing Aβ production by up to 80-90% in the cerebrospinal fluid (CSF) in humans. None of β-secretase inhibitors have reached the market so far [24-28].

- Inhibitors and modulators of γ-secretase

The γ-secretase complex is responsible for the final stage of amyloidogenesis, leading to the generation of Aβ(1-40) and Aβ(1-42). γ-secretase inhibition was initially considered a promising disease-modifying strategy. However, substrate promiscuity presents similar issues facing β-secretase inhibitors [29-31]. Notch protein, responsible for regulating cell proliferation, development, differentiation, and cellular communication is one of the targets of the γ-secretase [29]. Just as with the β-secretase inhibitors, off-target secondary effects are a major concern [31].

Semagacestat (LY450139) is a γ-secretase inhibitor that decreased Aβ levels in blood and CSF in humans [32]. The clinical study results (NCT00762411, NCT01035138, and NCT00762411) which recruited over 3000 patients, are an example of the worst possible outcomes. It was reported that semagacestat treatment was associated with the worsening of cognition and the abilities to carry out the activities of daily living (ADAS-cog scale) in AD patients. Additional side effects included weight loss, increased incidence of skin cancer, and a higher risk of infection. Avagacestat is another γ-secretase inhibitor the development of which was discontinued as a result of a lack of efficacy (NCT00810147, NCT00890890, NCT00810147, NCT01079819) [33-35].

Selective γ-secretase modulators (SGSM) may, in theory, be developed in such a way as to avoid the adverse events associated with general enzyme inhibition. The goal of such treatments is to block APP processing without interfering with other signaling pathways like Notch [36].

SGSM development began with the observation that several nonsteroidal anti-inflammatory drugs (NSAIDs) decreased Aβ (1-42) peptide levels *in vitro* and *in vivo*. Examples of these drugs are ibuprofen, sulindac, indomethacin and flurbiprofen [37]. The accepted mechanism of action (MOA) of NSAIDs is the inhibition of cyclooxygenase (COX) enzymes. While ibuprofen is a COX inhibitor, R-flurbiprofen (Tarenflurbil) is not, and its effects on the reduction of Aβ levels cannot be attributed to COX inhibition. Unfortunately, Tarenflurbil and Ibuprofen did not show efficacy for AD treatment in their respective clinical trials [37,38]. CHF5074, just like R-flurbiprofen, is an NSAID devoid of COX inhibitory activity. *In vitro*, CHF5074 inhibited Aβ(1-42) production presumably by blocking γ-secretase complexes [39-42]. Recent studies have reclassified this compound as a microglial modulator based on its ability to reduce both amyloid burden and microglial activation [40]. Results from a Phase II trial in patients with Mild Cognitive Impairment (MCI) indicate that CHF5074 treatment leads to improvements of several cognitive measures and reduces inflammatory marker levels in the CSF [39-41].

The idea that the long-term use of NSAIDs could confer some protection against AD generated some interest in NSAIDs as a treatment potentially useful for reducing Aβ (1-42) levels. However, negative results reported in clinical trials with NSAIDs suggest that this hypothesis requires further refinement [38].

Another example of a possible SGSM is NIC5-15, which is a naturally occurring molecule. NIC5-15, also known as pinitol, is a natural cyclic sugar alcohol [42]. This compound supposedly modulates γ-secretase and is reportedly capable of reducing Aβ production, while not affecting the substrate cleavage of Notch. No peer-reviewed data are available for this compound, so any reported results should be considered as a forward-looking statements requiring rigorous scientific proof. However, it is claimed that the compound improves cognitive function and memory in preclinical models of AD neuropathology. If true, these data suggest that NIC5-15 may be a suitable therapeutic agent for the treatment of AD for two reasons: a) it preserves Notch activity and b) also it is potentially an insulin sensitizer. Moreover, it is supposedly being investigated as an anti-inflammatory inhibitor because it may prevent microglia activation. Once again, independent researchers have not yet confirmed these results.

2.c.-Inhibition of β-amyloid peptide aggregation

Aβ peptide aggregates give rise to amyloid plaques. The following compounds were developed in order to prevent senile plaque formation.

The only inhibitor of Aβ aggregation that reached Phase III trials is the 3-amino-1-propaneosulfonic acid (3-APS, Alzhemed, tramiprosate) [43,44]. This medication was designed to interfere with or antagonize the interaction of soluble Aβ with endogenous glycosaminoglycans. Glycosaminoglycans have been shown to promote aggregation of Aβ amyloid fibril formation and deposition [44]. However, the disappointing results of the Phase III clinical trial in 2007 have led to the suspension of this compound in Europe [45].

Colostrinin, a complex of proline-rich polypeptides present in ovine, bovine and human colostrum inhibits aggregation of Aβ and its neurotoxicity in cell assays, and improves cognitive performance in mice models [46]. Although a phase II trial showed slight improvements in Mini Mental State Evaluation in patients with mild AD in a treatment period of 15 months, this beneficial effect was not maintained after another 15 months of continuous treatment [46].

Scyllo-inositol (ELND005) is an oral amyloid anti-aggregation agent capable of reducing Aβ toxicity in the mouse hippocampus. 18-month long Phase II clinical trial with ELND005 was conducted in participants with mild-to-moderate AD. This dose-finding, safety and efficacy trial did not meet its primary clinical efficiency outcomes. [47].

Clinical trials for AD treatment were also performed with metal chelating 8-hydroxiquinolines (8-HQ) compounds clioquinol and PBT2 [48]. While their mechanism of action is not completely understood, it is thought that these molecules block the interaction between the base metals and brain Aβ peptide. It was suggested that increased levels of oxidative stress in the brain of AD patients might be partially due to copper ions binding to Aβ, leading to metal-mediated generation of Reactive Oxygen Species (ROS). [49-51]. It was also hypothesized that 8-HQs may prevent Aβ aggregation while simultaneously restoring homeostasis in cellular levels of copper and zinc ions [50,51]. Unfortunately, these molecules failed in the phases II and III of clinical development due to lack of efficacy.

2.d.- Compounds which promote the removal of amyloid deposits and aggregates

Another potential treatment option which is centered on the amyloidogenic pathway is to promote the clearance of existing amyloid aggregates and deposits. To achieve this, three different strategies have been evaluated:

2.d.1. Activation of enzymes that degrade amyloid plaques

Aggregates and amyloid plaques are degraded by multiple proteases including neprilysin, IDE, plasmin, endothelin converting enzyme, angiotensin converting enzyme and metalloproteinases. Protein levels of these enzymes decrease in AD, which contributes to the formation and accumulation of Aβ [13-16]. Despite being an attractive strategy for developing disease-modifying drugs, no compounds with this MOA have ever reached advanced clinical development due to the lack of specificity.

2.d.2. Modulation of β-amyloid transport between the brain and the peripheral circulation

Aβ transport between the CNS and the peripheral circulation is regulated by: 1) apolipoproteins, for example Aβ may be transported from the blood to the brain when it is bound to APOE; 2) low-density lipoprotein receptor-related protein (LRP-1), which increases Aβ outflow from the brain to the blood; 3) receptor for advanced glycation end products (RAGE), which facilitates the transport of Aβ across the blood-brain barrier (BBB) [15,52,53].

The goal of any treatment, which is focused on this mechanism, is to reduce cerebral amyloid load by attempting to restrict Aβ to the peripheral circulation. To this end, a number of different strategies have been proposed, notably the peripheral administration of LRP-1. However, the only drug candidates that have reached clinical stage are the inhibitors/modulators of RAGE. These include PF-0449470052, which failed in phase II trials, and TTP4000, with the Phase I trial completed in February 2013 (NCT01548430). The results of this trial have not been published.

2.d.3. Anti-amyloid immunotherapy

Active immunotherapy: Immunotherapy strategy aimed to promote Aβ clearance with the objective of reducing the amyloid load in AD. Active immunization (vaccination) with either Aβ(1-42) (predominant form found in senile plaques) or other synthetic fragments has been successfully evaluated in transgenic mouse models of AD. Assays are generally based on the stimulation of B cells, T cells and immune responses through activation of the phagocytic capacity of microglia. Human tests were initially promising, however treatment with the first-generation vaccine (AN1792) has produced serious adverse events that led to the discontinuation of the Phase II trial. AN1792 consisted of

a synthetic full-length Aβ(1-42) peptide with a QS-21 adjuvant. As a result of a T-cell-mediated autoimmune response, 6% of patients have developed cerebral inflammation which turned out to be aseptic meningoencephalitis [54].

Second-generation vaccines were designed using a shorter Aβ(1- 6) peptide segment in an attempt to prevent nonspecific immune response seen with the full-length vaccine. CAD 106, designed by Novartis, was the first second-generation vaccine that reached the clinical phases of development [55]. A recently completed Phase II clinical trial have shown a Aβ-specific antibody response in 75% of treated patients, without causing adverse inflammatory reactions. ACC-001, developed by Janssen, has recently completed two-phase II trials (NCT01284387 and NCT00479557) with an additional phase II trial still ongoing (NCT01227564). However, the pharmaceutical company has abandoned the plans for further development of this vaccine. Other vaccines, including tetra-palmitoylated Aβ(1-15) reconstituted in a liposome (ACI-24), MER5101 and AF205 are currently in various stages of preclinical development [56-59].

Passive immunization: Passive administration of monoclonal or polyclonal antibodies directed against Aβ. This therapy consists of the intravenous administration of anti-Aβ antibodies to the patient. An advantage of this strategy compared to active immunization is that the pro-inflammatory T cell-mediated immune reactions should not occur. Studies in transgenic animals have shown that passive immunization reduces cerebral amyloid load and improves cognition, even when the amyloid plaque numbers are not significantly reduced. This could be attributed to the neutralization of soluble amyloid oligomers, which are increasingly recognized to play a fundamental role in the pathophysiologic cascade of AD.

Bapineuzumab and solanezumab are two monoclonal antibodies that have reached advanced stages of clinical development [60]. However, in 2012, two Phase III clinical trials had failed because of a lack of efficacy in patients with mild-to-moderate AD. Both bapineuzumab and solanezumab are humanized monoclonal antibodies against Aβ(1-6) and Aβ(12-28) respectively [61,62]. In case of bapineuzumab, significant reduction in brain amyloid plaques and phosphorylated tau in cerebrospinal fluid was reported. However, the treatment failed to produce significant improvements of cognitive function. In a solanezumab trial, infusions of 400 mg of solanezumab or placebo were administered once a month for 80 weeks in patients with mild-to-moderate AD. The results suggested that solanezumab may improve cognition in mild AD, however statistical significance was not achieved in that study [62]. Currently solanezumab is in Phase III trials in patients with AD (NCT01127633 and NCT01900665) and in older

individuals who have normal thinking and memory function but who may be at risk of developing AD in the future (NCT02008357).

Another monoclonal antibody, gantenerumab, is being investigated in people at risk of developing presenile AD due to genetic mutations. NCT01760005 trial is still recruiting participants and will determine the efficacy of both gantenerumab and solanezumab in the prodromal disease stages. [63-65]. In parallel, two additional Phase III trials of gantenerumab in patients with mild AD (NCT02051608) and prodromal AD (NCT01224106) are ongoing. Gantenerumab is a fully human IgG1 antibody designed to bind with high affinity to a conformational epitope on the β-amyloid fibres. Microglia recruitment and ensuing phagocytosis will presumably lead to amyloid plaque degradation. Experimental studies in transgenic mice support this hypothesis.

Crenezumab (MABT5102A) is a humanized monoclonal antibody which uses IgG4 backbone [66]. A Phase II clinical trial to assess the safety and efficacy in patients with mild-to-moderate AD (NCT01343966) was completed in April 2014, although the results are not yet publicly available. The most recent Phase II trial aiming to evaluate the safety and efficacy of crenezumab in asymptomatic carriers of E280A autosomal-dominant mutation of PSEN1 commenced in November 2013 (NCT01998841).

Other monoclonal antibodies against Aβ developed so far include PF-04360365 (ponezumab) which targets the free carboxy terminal amino acids 33-40 of the Aβ peptide; MABT5102A, which binds to Aβ monomers, oligomers and fibrils with equally high affinity; GSK933776A, which is similar to bapineuzumab in that it binds to the N-terminal Aβ(1-5). In addition, other passive immunotherapies mostly in Phase I clinical development include NI-101, SAR-228810 and BAN-2401 [58,59,62-66].

Gammagard is a preparation of antibodies from human plasma. Its safety for human use had been previously demonstrated in certain autoimmune conditions. Gammagard effects were evaluated in a small number of AD patients (NCT00818662). It is believed that this mixture contains a small fraction of polyclonal antibodies against the Aβ peptide. In addition, this preparation may possess immunomodulatory properties that could potentially enhance microglial phagocytosis [67-69].

3.- Strategies focused on tau proteins

Tau proteins are highly soluble and abundant in the neurons where they play a critical role in microtubule stabilization, particularly in axons [70-72]. Tau hyperphosphorylation leads to the formation of insoluble paired helical filaments (PHF) which form neurofibrillary tangles. The loss of microtubule-binding capacity provokes cytoskeleton

destabilization, which eventually causes neurodegeneration and neuronal death [71]. As an alternative to amyloidocentric approaches, Tau-centered treatments aim to inhibit the phosphorylation and/or aggregation of tau protein. In addition, microtubule-stabilizing drugs could be used as a disease-modifying strategy in AD [72]. In recent years, immunomodulation was suggested as a viable option for promoting effective clearance of tau aggregates.

3.a.- Inhibitors of tau hyperphosphorylation

All tau proteins are a product of alternative splicing of a microtubule-associated protein tau (MAPT) gene. Phosphorylation is the primary mechanism which regulates tau binding to microtubules. Under physiological conditions the protein remains soluble, however in AD, pathological hyperphosphorylation of tau compromises its normal functions [73,74]. Hyperphosphorylation occurs as a result of an imbalance between the catalytic activity of kinases and phosphatases. Increased expression of active forms of various kinases in the areas proximal to neurofibrillary tangles has been described in AD, including CDK5, GSK3β, Fyn, stress-activated protein kinases JNK and p38, and mitogen-activated protein kinases ERK1 and ERK2 [90]. Some or all of these kinases contribute to the perpetuation of the phosphorylation of tau in neurofibrillary tangles [74-81]. As a result, significant research efforts have been devoted to the development of kinase inhibitors as a possible treatment strategy for AD. For example SP600125, a widely used pan-JNK inhibitor, exerts beneficial effects on cognition and reduces neurodegeneration in an APP/PS1 transgenic mouse model of AD [80]. It has been proposed that specific inhibition of JNK3 could be sufficient to bring similar benefits as seen with SP600125 in rodent models [78-81]. Human data in AD patients indicate a positive correlation between the levels of JNK3 and Aβ(1-42) in the brain [77]. Furthermore, JNK3 upregulation was detected in the CSF and was associated with memory loss. Thus, JNK3 inhibition remains a promising target for future therapies [81].

CDK5 belongs to the family of serine/threonine cyclin-dependent kinases and is responsible for a number of physiological functions within the CNS, including neurite outgrowth and the regulation of axonal development [82]. CDK5 catalytic activity is dependent on its direct association with p35, key regulator of CDK5 signaling. This cofactor is cleaved by a non-lysosomal protease calpain in a calcium-dependent manner [83]. Conversion of p35 to p25 results in prolonged activation and mislocalization of CDK5. Due to the increases in intracellular calcium levels observed in the brains of AD patients, pathological activation of CDK5 occurs, resulting in hyperphosphorylation of tau and neuronal cell death [83,84]. CDK5 inhibition may thus also be potentially considered

as a possible drug target. Currently existing CDK5 inhibitors roscovitine and flavopiridol have demonstrated neuroprotective properties in *in vitro* and *in vivo* models of excitotoxicity, ischemia and neurodegeneration [84, 85].

GSK3β inhibitors are arguably in the most advanced stages of clinical development for AD. Among the various drugs that are currently being studied, tideglusib, an irreversible inhibitor of GSK3β, has recently completed Phase II trials (NCT01350362). Tideglusib administration for a period of 26 weeks to patients with mild-to-moderate AD did not show clinical efficacy, and the compound has since been discontinued for this indication [86]. Another study (NCT00948259) evaluated the safety and tolerability of a 20-week administration of NP031112 compared with placebo in patients with AD. No data has been reported for this study.

Phosphatase activation has also been considered as a possible drug target. Currently, there is only one protein phosphatase 2 (PP2A) agonist in development. Sodium selenite (VEL015) is undergoing Phase II trials in Australia (ACTRN12611001200976). Experimental studies have shown that sodium selenate reduces tau phosphorylation, both in cell culture and in mouse models of the disease [86-88]. VEL015 administration to rodents have resulted in significant cognitive improvements and substantial reduction of neurodegenerative phenotype.

3.b.- Inhibitors of tau aggregation

Hyperphosphorylated tau aggregates contribute to neurotoxicity observed in AD brain. Methylene blue dye derivatives have shown some promise in inhibiting the formation of tau aggregates. Methylene blue disrupts the aggregation of tau, has the ability to inhibit amyloid aggregation, improves the efficiency of mitochondrial electron transport chain, reduces oxidative stress, prevents mitochondrial damage and is also a modulator of autophagy [89-90]. The first-generation molecule derived from methylene blue (Rember) appeared to stabilize AD progression in a clinical trial which lasted 50 weeks. These results motivated researchers to develop a next-generation version of methylene blue, TRx 0237. This compound is a purified derivative of methylene blue which not only inhibits tau protein aggregation but also dissolves brain aggregates of tau [90]. Several clinical trials are currently underway (NCT01626391, NCT01689233, NCT01689246, NCT01626378) to evaluate the potential efficacy of this drug in AD.

3.c.- Microtubule stabilizers

Microtubule stabilization may potentially achieve a similar end-result as that seen with the inhibitors of tau hyperphoshorylation and aggregation. Paclitaxel is a microtubule-

stabilizying drug currently in use in the oncology field. Unfortunately, this compound is incapable of crossing the BBB and its use is associated with serious adverse events, which limits its utility in AD [91,92]. In addition to paclitaxel, other microtubule-stabilizing compounds such as TPI 287 have been considered as a possible AD therapy. TPI 287 is a derivative of taxane, also used in cancer treatment. TPI 287 stabilizes the microtubules by binding to tubulin. NCT01966666 clinical trial will evaluate TPI-287 safety, pharmacokinetic properties and tolerability by intravenous infusion in mild-to-moderate AD.

Epothilone D is a microtubule-stabilizing compound which improved axonal transport, reduced axonal dystrophy, decreased tau neuropathology, and reduced hippocampal neuron loss, however drug development for AD was discontinued in 2013 after a failed clinical trial [92].

With respect to tau, additional studies are necessary in order to better understand the exact molecular mechanisms involved in tau neurotoxicity. Recent studies comparing the neurotoxic profiles of various forms of tau suggest that a soluble form is likely the most toxic [70]. This has been corroborated by a recent report specifically identifying oligomeric tau as toxic [93]. Therefore, future therapeutic strategies should be focused on targeting soluble forms of tau.

3.d.- Anti-tau immunotherapy

Just as with the immunotherapies targeting Aβ, both passive and active immunization approaches against tau have been considered. In fact, it was demonstrated that reductions in tau aggregate formation and improved clearance of tau oligomers and insoluble aggregates could all be achieved with either active or passive immunotherapies [94]. In rodents, treatment with monoclonal antibodies directed against hyperphosphorylated tau has led to improvements in cognition and was not associated with significant adverse effects [94].

In 2013 Axon Neuroscience began a Phase I trial to evaluate the safety and tolerability of AADvac-1, an active immunotherapy which consists of a synthetic peptide derived from the tau sequence coupled to keyhole limpet hemocyanin, the precise molecular nature of the antigen has not been disclosed (NCT01850238 and NCT02031198) [95]. AADvac-1 uses aluminum hydroxide as an adjuvant. At the 2014 Alzheimer's Association International Conference (AAIC) in Copenhagen, good preclinical safety profile was reported for the treatment period of up to 6 months in rats, rabbits, and dogs. These early results are encouraging and it remains to be seen whether AADvac-1 will demonstrate acceptable safety and efficacy in human patients.

4.- The cholinergic hypothesis

AD is a neurodegenerative disease characterized by a progressive loss of learning and memory as well as neuronal death. The hippocampus, the main brain region involved in memory processing, is influenced by cholinergic modulation [96]. One of the well characterized anomalies associated with neurotransmitter alterations is the degeneration of cholinergic neurons in the nucleus basalis of Meynert and the loss of cholinergic inputs to the neocortex and hippocampus. Several studies reported decreases in choline acetyltransferase (ChAT), acetylcholine (ACh) release, as well as reductions in nicotinic and muscarinic receptors in the cerebral cortex and hippocampus of postmortem AD brains [97]. Acetylcholinesterase inhibitors (AChEI), one of the only 2 classes of drugs currently approved for AD treatment, act by increasing ACh bioavailability at the synapse. Unfortunately, none of these drugs are capable of reversing the course of AD nor of even appreciably slowing down the rate of disease progression [98]. Their clinical effect is largely palliative, however, their potential use in combination therapy with other disease-modifying compounds should not be excluded.

Lagostigil (TV3326) is both a reversible inhibitor of AChE and is a selective and irreversible inhibitor of brain monoamine oxidase A and B, the use of which improves extrapyramidal symptoms and provides an anti-depressant effect [99,100]. It also appears to be a potent anti-apoptotic, antioxidant, anti-inflammatory and neuroprotective agent. NCT01429623 and NCT01354691 Phase II clinical trials with lagostigil are currently underway.

5.- Dendritic Hypothesis (Aβ-PrPC–mGluR5–Fyn signaling)

Dendritic abnormalities appear in the relatively early stages of AD. While dystrophic neurites, reduced dendritic complexity and dendritic spine loss are all documented features of AD, it is only recently that we are beginning to understand the underlying molecular changes that occur on the postsynaptic side, in the dendrite [101-103]. Some data suggest that soluble Aβ oligomers are the principal neurotoxic species responsible for dendritic pathology. Aβ oligomers may cause aberrant N-methyl-D-aspartate receptor (NMDAR) activation post-synaptically by forming complexes with the cell-surface prion protein (PrPC). PrPC is enriched at the neuronal post-synaptic density, where it interacts with Fyn tyrosine kinase-metabotropic glutamate receptor 5 complex (Fyn-mGluR5). Fyn activation occurs when Aβ is bound to PrPC-Fyn-mGluR5 complex. Activated in this manner, Fyn can cause tyrosine phosphorylation of the NR2B subunit of this NMDAR. This results in an initial increase and then a loss of cell-surface NMDARs [104]. Fyn overexpression accelerated synapse loss and the onset of cognitive impairment in the

J9 (APPswe/Ind) transgenic AD mouse model, while its inhibition produced an opposite effect [101]. In addition, as mentioned earlier, Fyn can also contribute to tau hyperphosphorylation. Previous studies had reported elevated levels of Fyn in AD brain. Furthermore, Fyn was shown to phosphorylate tau at Tyr18 residue [102]. Thus, Fyn appears to be a viable target in the treatment of AD pathology. Saracatinib (AZD0530) and masitinib (AB1010) are Fyn kinase inhibitors currently in Phase II and III clinical trials for mild-to-moderate AD (NCT01864655, NCT02167256, NCT00976118, NCT01872598) [104-106]. Both compounds are capable of blocking Fyn in a nanomolar range.

In a NCT00976118 clinical trial, oral masitinib was administered for a period of 24 weeks, concomitantly with one of the AChEIs (donepezil, rivastigmine, or galantamine) and/or memantine. In that study a significant improvement in the ADAS-Cog test response was reported. These results are encouraging, however the very small patient pool (n=26) on memantine in this Phase II trial is clearly not sufficient to draw conclusions on the potential efficacy of this compound. MOA of masitinib in AD is twofold. Apart from blocking Fyn, masitinib is also a stem cell factor (SCF) receptor (c-KIT) inhibitor. By inhibiting SCF/c-Kit signaling on mast cells (MCs), this compound may prevent neuroinflammation by blocking the activated MCs-microglia interactions [103-106].

6.- 5-HT6 receptors in Alzheimer's disease

5-HT6 receptors are expressed in areas of the CNS involved in learning and memory. Their inhibition was shown to promote acetylcholine release. In AD, 5-HT6 antagonism may lead to the restoration of acetylcholine levels [107]. This hypothesis is supported by evidence that the 5-HT6 receptor antisense oligonucleotides improve spatial learning and memory in the Morris water maze test in normal rats [108]. 5-HT6 inhibitors may be useful in combination therapy, together with AChEIs. For example, Lu-AE-58054 (SGS-518) and PF-05212365 (SAM-531) are being considered as possible treatments for mild-to-moderate AD. Other compounds that are in various stages of clinical research are SUVN-502, AVN-322 and PRX-07034 [109].

7.- Changing the concept: AD as a metabolic disorder

Clinical studies suggest that diabetes is a major risk factor that contributes to AD pathology. Results from published research indicate that there is a close link between insulin-deficient diabetes and cerebral amyloidosis [110]. Peripheral and central insulin signaling impairments are likely present in both diseases. As a result, "type-3 diabetes" hypothesis of AD was developed, which attempts to bridge the observed metabolic

phenotypes present in diabetes and AD into a coherent framework. Insulin hormone is at a centerpiece of this hypothesis [111].

Observations made in the "Hisayama Study" indicate that altered expression of genes related to diabetes mellitus in AD brains may be a result of AD pathology, and suggest that peripheral insulin resistance, metabolic syndrome and/or full-blown diabetes may lead to worsening of cognitive symptoms [112]. Impaired central insulin signaling in the hippocampal circuits, a key region involved in learning and memory, is likely present in AD [113]. Glucose toxicity, insulin resistance, oxidative stress, elevated levels of advanced glycation end products and cytokine-mediated neuroinflammation are among the proposed mechanisms by which diabetes could increase the risk of AD development. In a recent study, Clarke and colleagues demonstrated that hypothalamic administration of soluble Aβ oligomers initiates neuroinflammatory cascades which eventually cause disturbances in peripheral glucose homeostasis [114]. Tumor necrosis factor α (TNFα) quite possibly plays an important role in this process [115,116].

As molecular mechanisms causing AD and T2DM pathologies are possibly related, it is logical to assume that drugs used in T2DM treatment may have a neuroprotective effect in AD [117]. Thiazolidinediones (TZDs) are an example of anti-diabetic compounds whose possible role in AD was investigated. TZDs are agonists of peroxisome proliferator-activated receptor γ (PPAR-γ), which act by promoting PPAR-γ heterodimerization with the retinoid X receptor (RXR). PPAR-γ/RXR heterodimer is a transcription factor, which regulates expression of genes involved in lipid and glucose metabolism. TZDs improve insulin sensitivity and reduce cytokine-dependent inflammation [118-119]. Rosiglitazone and pioglitazone are used as anti-diabetic drugs, which regulate glucose homeostasis by increasing insulin sensitivity, reducing blood glucose levels and improving lipid metabolism. Both compounds have also been studied as potential therapeutics for AD treatment, with reported improvements in mitochondrial oxidative metabolism. In animal models, pioglitazone modified various indices of brain aging but did not slow down the cognitive decline [119]. Rosiglitazone and pioglitazone also induce the expression of peroxisome proliferator-activated receptor-γ co-activator 1 alpha (PGC-1α), a molecule that plays multiple roles in mitochondrial biogenesis, energy metabolism and mitochondrial antioxidants expression. Previous studies have demonstrated that in the human brain tissues, the expression of PGC-1α decreases with progression of AD dementia [120]. Thus, PGC-1α upregulation may improve the mitochondrial energy metabolism and AD pathology [121].

Pioglitazone treatment improved memory and cognition in patients with mild-to-moderate AD in a small clinical trial [122]. A larger phase II trial demonstrated improvements in memory retention and attention with rosiglitazone treatment (6 months) in patients who did not possess an ApoE4 allele [123]. However, phase III trial using rosiglitazone failed to show efficacy in AD (NCT00550420) [124]. It is important to note that in these trials rosiglitazone was administered alone at dosages that were much lower than those needed to exert a beneficial effect on AD pathophysiology in rodent models of the disease. NCT00348140 is a recently completed clinical trial in which rosiglitazone was administrated in combination with AChEIs in patients with mild-to-moderate AD. No results have yet been reported.

Intranasal insulin had also been considered as a treatment option for AD. This particular route of administration is attractive as it bypasses the BBB. This is very significant because insulin transport to the brain from the periphery is dependent on active transport mechanisms which may become disrupted in AD. In addition, the probability of possible adverse events in peripheral tissues is minimized. In theory, insulin delivery directly to the brain will activate cerebral insulin signaling leading to improvements in memory processing and will result in neuroprotection [125, 126]. A currently ongoing clinical trial NCT01767909 is evaluating long-term (12 months) efficacy of intranasal insulin (Humulin® R U-100) in mild AD.

Other pancreatic hormones such as amylin may also play a role in AD. Adler and colleagues reported that patients with AD have reduced concentrations of plasma amylin. In transgenic animal models of AD, amylin and pramlintide (amylin analog) decreased brain Aβ levels and improved cognition. Interestingly, amylin also inhibited β-secretase enzyme, while pramlintide did not [128, 129].

8. - Future strategies

AD is a complex multifactorial disorder which may require equally complex approaches to treatment. Early disease detection, combination therapies and lifestyle choices are all likely contributors to the successful eradication of the pathology (Fig 1) [130-134]. A broad range of studies show that inadequate nutrition can increase the risk of disease development [133]. A healthy diet can certainly improve your chances of not developing AD. However, neither the Mediterranean-type diet, caloric restriction nor antioxidant diet alone can prevent or delay AD. We believe that carefully-developed nutrition regimens coupled to combination pharmacotherapies targeting multiple pathways involved in AD are a way forward.

Biomarker identification, indicative of prodromal stages of AD, can lead to early diagnosis and improve prognostic outcomes. Currently existing diagnostic approaches are focused on the detection of Aβ(1-42) and total and phosphorylated tau levels in the CSF and in the brain. Imaging techniques such as brain MRIs are also used [135-137]. As both Aβ and tau increases likely appear when the disease had already taken hold, we would welcome the discovery of diagnostic markers which could predict the likelihood of AD development at earlier stages.

Growth factors (GFs) are yet another set of molecules which can potentially improve AD pathology. Transforming growth factor β family, insulin-derived GFs (insulin-like growth factor 1, IGF-1; and 2, IGF-2), basic fibroblast growth factor (bFGF) and neurotrophins (nerve growth factor, NGF; brain-derived growth factor, BDGF; glial-derived neurotrophic factor, GDNF) all participate in neurogenesis and neurodevelopment and may be considered as potential targets for AD treatment [137,139].

9.- Concluding remarks

In summary, accumulated evidence suggests that AD neuropathology shows a multifactorial nature and involves multiple biological pathways. Amyloid cascade hypothesis has dominated the field for over 20 years, as a result of which a large number of studies have focused on inhibition and removal of Aβ and senile plaques. Unfortunately, the amyloidocentric approaches have failed to demonstrate improvements in cognition in patients. Dendritic spine defects clearly contribute to cognitive decline observed in AD. These defects are considered an early event in memory circuit's destabilization and should be taken into account for future development of investigational drugs. Novel pharmacotherapies should not be limited to the postulates of the amyloid cascade hypothesis. Events occurring at the synapse may prove to be instrumental in understanding the underlying pathology of this devastating disease.

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ABBREVIATIONS

Aβ: β-amyloid

ACE I: angiotensin converting enzyme I

Ach: Acetylcholine

AD: Alzheimer's disease

ApoE: Apolipoprotein E

APP: amyloid precursor protein

AB1010: Masitinib

AZD0530: Saracatinib

BACE1: PPAR and aspartyl protease β-site AβPP-cleaving enzyme

Cdk's: Serine / threonine cyclin dependent

CNS: Central nervous system

CSF: Cerebrospinal fluid

IDE: insulin degrading enzyme

LRP: low-density lipoprotein

RemberTM: Methylene blue

MABT5102A: Crenezumab

MAPT: Microtubule-associated protein Tau

MOA: mechanism of action

MCs: Mast cells

NOS: Nitric Oxide Synthase

NSAIDs: nonsteroidal anti-inflammatory drugs

PBT1: Clioquinol

PGC-1α: peroxisome proliferator-activated receptor-γ co-activator 1 alpha

PrP: Prion protein

PS1/2: Presenilin 1/2

RAGE: of advanced glycation end products

ROS: Reactive Oxygen Species

SGSM: Selective γ-secretase modulators of the enzyme

TV3326: Lagostigil

VEL015: Sodium selenite

WHO: World Health Organization

Figure 1. Future strategies in the treatment of late-onset and sporadic forms of AD could be centered on three main points: avoidance of habits and lifestyle leading to obesity and diabetes; early detection of AD biomarkers or structural alterations in pre-symptomatic individuals; and combined therapies in early phases of cognitive loss.

Table 1.

Activity	Compound	Clinical trial
Inhibitors of β-secretase	-E2609 - MK-8931 - LY2886721	-NCT01600859 -NCT01739348 -NCT01807026 and NCT01561430
Inhibitors and modulators of γ-secretase	-Semagacestat (LY450139) -Avagacestat	-NCT00762411, NCT01035138, and NCT00762411 -NCT00810147, NCT00890890, NCT00810147, NCT01079819
Selective γ-secretase modulators (SGSM)	-Ibuprofen, sulindac, indomethacin and R-flurbiprofen (Tarenflurbil) - NIC5-15	NCT00322036, NCT00105547
Non-steroidal inhibitory of cyclooxygenase activity (NSAIDs)	- CHF5074	NCT01203384, NCT01303744, NCT00954252
Inhibitors of Aβ aggregation	-glycosaminoglycans 3-amino acid, 1-propaneosulfonic synthetic (3APS, Alzhemed, tramiprosate) -Colostrinina -Scyllo-inositol compound (ELND005) - PBT1 (clioquinol) and PBT2	-Phase III in 2007
Modulation of β-amyloid transport from the brain to the peripheral circulation	- PF-0449470052 - TTP4000 (NCT01548430)	-Phase II -Phase I (February 2013)
Active immunotherapy	- Anti-Aβ42 vaccine (AN1792) - CAD 106 - ACC-001 -ACI-24, MER5101 and AF205 - Bapineuzumab, solanezumab - Gantenerumab - Crenezumab (MABT5102A) - **Ponezumab (PF-04360365)** **- MABT5102A, GSK933776A, NI-101, SAR-228810 and BAN-2401** **- Gammagard ™**	 -Phase II -NCT01284387, NCT00479557 and Phase II NCT01227564 (rejected) -Pre-clinical -NCT01127633, NCT02008357 and NCT01900665 Phase III (2012) -NCT01760005, NCT02051608 and NCT01224106 Phase III -NCT01343966, **NCT01998841** Phase II (April 2013) - Phase I - **NCT00818662**

Table 2.

Activity	Compound	Clinical trial
Inhibitors of tau hyperphosphorylation: Glycogen synthase kinase 3 inhibitors (GSK3β)	-Tideglusib - NP031112 - Sodium selenite (VEL015)	-NCT01350362 Phase II - NCT00948259 - ACTRN12611001200976 Phase II
Inhibitors of Tau aggregation	- RemberTM, TRx 0237	- NCT01626391, NCT01689233, NCT01689246 and NCT01626378
Microtubule stabilizers	- Paclitaxel - Epothilone D - TPI 287 (taxane)	-Clinical trial 2013 (interrupted) - NCT01966666
Tau-specific Immunotherapy	- AADvac1 vaccine	- NCT01850238 and NCT02031198 Phase I trial (2013)
Anti-cholinesterase inhibitors	-Donepezil, rivastigmine, galantamine, - Lagostigil (TV3326)	
PrPC–mGluR5–Fyn signaling	- Masitinib - Saracatinib (AZD0530)	- NCT00976118 - NCT01864655 and NCT02167256
5-HT6 receptor blockage	- Lu-AE-58054 (SGS-518), PF-05212365 (SAM-531), SUVN-502, AVN-322, PRX-07034	-Different phases of clinical trials
Antidiabetic drugs	- Rosiglitazone and pioglitazone -Intranasal insulin (Humulin® R U-100) - Amylin and pramlintide (amylin analog)	- NCT00550420, NCT00348140 Phase III - NCT01767909 - NCT01429623 and NCT01354691 Phase II
Cdk5 inhibitors	- Roscovitine and flavopiridol	

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Publication 2

EVALUATION OF HYPOXIA INDUCIBLE FACTOR EXPRESSION IN INFLAMMATORY AND NEURODEGENERATIVE BRAIN MODELS

Maria Luisa de Lemos, Aurelio Vazquez de la Torre, **Dimitry Petrov**, Susana Brox, Jaume Folch, Mercè Pallàs, Alberto Lazarowski, Carlos Beas-Zarate, Carme Auladell, Antoni Camins

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Summary

This paper is included because the data presented here indicate that the inflammatory aspect of AD pathology is not yet manifested in 3-month-old APP/PS1 mouse brain. At the age of 6 months, first signs of cerebral inflammation are evident. This research had helped us to make an informed decision as to the time points which are likely relevant to the investigations described in the current thesis. As mentioned in the Discussion sections of the thesis presentation, we wanted to focus at an age when AD phenotype is still in the prodromal stages and to compare hippocampal and peripheral phenotypes in mice in which classical disease symptoms are already present, but presumably have not yet taken an irreversible aspect.

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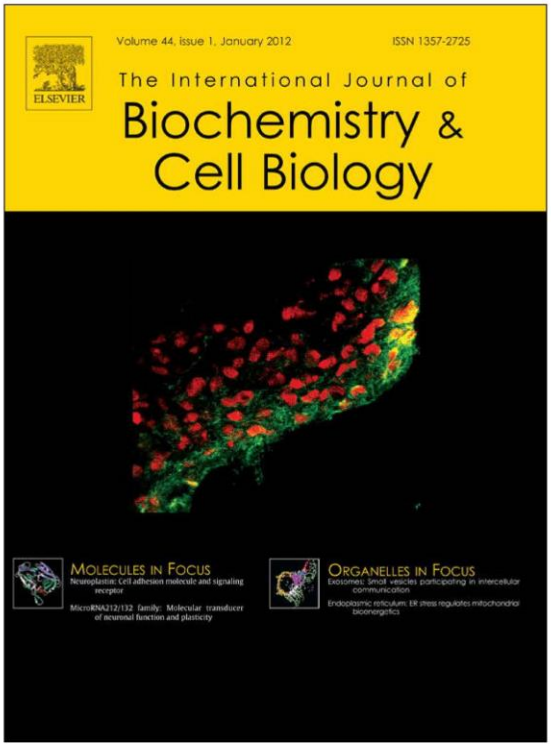

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Evaluation of hypoxia inducible factor expression in inflammatory and neurodegenerative brain models

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ABSTRACT

The neuroinflammatory process is thought to contribute to the progression of neurological disorders and brain pathologies. The release of pro-inflammatory cytokines and chemokines by activated glial cells, astrocytes and microglia plays an important role in this process. However, the role of hypoxia-inducible factor-1α (HIF-1α), the key transcription factor regulating the expression of hypoxia-inducible genes, during glial activation is less known. Thus, we examined the significance of HIF-1α in three experimental models: first in an acute model of inflammation induced by pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ; secondly, in a chronic model of inflammation using an APPswe/PS1dE9 (APP/PS1) transgenic mouse model of Alzheimer's disease and thirdly via the inhibition of the PI3K/AKT pathway in a model of neuronal apoptosis. During acute glial inflammation induced by in vitro administration of TNF-α, IL-1β and IFN-γ, mRNA expression levels of HIF-1α were significantly upregulated; however, this effect was blocked by SP600126, a pharmacological inhibitor of mitogen-activated protein kinases (MAPKs). These data suggest that MAPKs could be involved in HIF-1α regulation. In addition, we observed that HIF-1α is not involved in the neuronal apoptotic process mediated by PI3-kinase inhibition, which is regulated by c-Jun. Finally, we did not detect significant differences in the expression of HIF-1α mRNA in APP/PS1 mice during the course of the study (3–12 months of age). Thus, we demonstrated that HIF-1α has a prominent role in acute but not in chronic inflammatory processes, such as the one which occurs in the APP/PS1 experimental model of AD. Moreover, HIF-1α is not involved in neuronal apoptosis after PI3K/AKT inhibition.

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1. Introduction

The regulation of oxygen homeostasis and the organism adaptation to an environment with oxygen deprivation are considered two of the main physiological functions of the transcriptional activator hypoxia-inducible factor (HIF-1) (Powell et al., 2009; Correia and Moreira, 2010; Harten et al., 2010). For example, under ischaemic conditions, activation of HIF-1 in cells results in protection against future lethal insults (Shi, 2009; Jeong et al., 2012). HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β subunits. The difference between the two subunits is that, while the expression and activation of the HIF-1α subunit is inducible and regulated by cellular oxygen, the expression of HIF-1β subunit is constitutive (Powell et al., 2009). Moreover, degradation of HIF-1α in the presence of oxygen is regulated by the tumour suppressor gene von Hippel–Lindau (VHL); thus, the loss of VHL results in HIF-1α accumulation (Correia and Moreira, 2010). HIF-1α and the

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genes regulated by it are currently the focus of extensive research because of their potential as therapeutic targets for the treatment of ischaemia (Shi, 2009). Although it is well known that HIF-1α is a key regulator of cellular response to hypoxia in living cells, it can also be activated under normoxia in response to a variety of non-hypoxic stimuli, including vasoactive peptides, cytokines, β-amyloid, hormones, ionophores and growth factors (Bodles and Barger, 2005; Brault et al., 2007; Vangeison et al., 2008; Imtiyaz and Simon, 2010; Ji et al., 2012; Ogunshola and Antoniou, 2009). Likewise, iron chelators, cobalt chloride, oxidative stress and nitric oxide (NO) have been shown to induce HIF-1α activation via stabilization of HIF-1α (Caltana et al., 2009; Ji et al., 2012; Bae et al., 2006; Kawasaki et al., 2007; Sinke et al., 2008; Yoshioka et al., 2010; Keswani et al., 2011; Kupershmidt et al., 2011; Merelli et al., 2011; Weinreb et al., 2009).

It is known that HIF-1α is a transcriptional activator which mediates the induction of several genes such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), the multidrug resistance mdr1 (P-gp), glycolytic enzymes, heme oxygenase and glucose transporters (Shi, 2009; Correia and Moreira, 2010; Harten et al., 2010). Interestingly, the fact that HIF-1α can be activated in response to inflammatory cytokines indicates that it may play a role in inflammation (Albina et al., 2001; Bae et al., 2006; Brigati et al., 2010; Imtiyaz and Simon, 2010). Previous studies reported that tumour necrosis factor alpha (TNF-α) and IL-1β increases the transcriptional activity of HIF-1α via the NF-κB pathway (Albina et al., 2001; Sandau et al., 2001; Kuo et al., 2009).

Furthermore, it has been reported that HIF-1α serves as a link between inflammation and oncogenesis and that expression of IL-1β by gliomas may regulate HIF-1α (Nardinocchi et al., 2009).

Importantly, HIF-1α signal transduction is known to be mediated by the MAPKs cascade, including ERK1 and ERK2, p38 and JNK, which can augment HIF-1α protein levels under both normoxia and hypoxia (Naruishi et al., 2003; Xanthi et al., 2009; Zhang et al., 2012a,b,c). Likewise, c-Jun, a downstream transcription factor activated by the MAPKs pathways, is also reported to contribute to HIF-1-mediated cellular responses (Yu et al., 2009).

Another signalling pathway causing increased HIF-1α protein levels is the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian Target of Rapamycin (mTOR) pathway. Thus, activation of mTOR-dependent synthesis of HIF-1α has been suggested by some researches as a major determinant of HIF-1 activity in normoxia (Chen et al., 2012). An additional pathway implicated in the regulation of HIF-1 levels is the Signal Transducer and Activator of Transcription (STAT) 3. STAT3 is a transcription factor that is activated by cytokine receptors upon ligand binding (Zhu et al., 2011). Phosphorylation of critical tyrosine residues of the STAT3 molecule by receptor tyrosine kinases triggers STAT3 nuclear translocation, where it participates in the transcriptional regulation of target genes involved in cell proliferation and survival, among them HIF-1α (Demaria et al., 2012).

Furthermore, studies performed in glial cultures demonstrated that HIF-1α is involved in the regulation of glucose metabolism (Hossain et al., 2000; Vangeison et al., 2008; Du et al., 2010, 2011; Shay and Celeste Simon, 2012). It is known that astrocytes play a crucial role in regulating neuronal function and survival as well as the control of synapse formation (Yoo et al., 2005; Wuestefeld et al., 2012). Although glia are generally considered more resistant to stress compared to neuronal cells, recent studies have indicated that astrocytes are also susceptible to a variety of neurotoxic stimuli, including hypoxic injury, cytokine treatment and heavy metals (Zhang et al., 1996; Hossain et al., 2000; Sinke et al., 2008; Vangeison et al., 2008; Yoshioka et al., 2010; Weidemann et al., 2010).

The purpose of the present study is to investigate and compare the effects of HIF-1α in acute and chronic inflammation as well as to examine the role of HIF-1α in neuronal apoptosis. Firstly, we evaluated the expression of HIF-1α in an acute model of inflammation in glial cell cultures treated with pro-inflammatory cytokines TNF-α, IL-1β and IFN-γ. Secondly, we assessed the role of HIF-1α in a well-known neuronal apoptotic model via the inhibition of AKT by LY294002. Thirdly, we studied mRNA HIF-1α expression in a mouse model of familial Alzheimer disease using APPswe/PS1dE9 (APP/PS1) mice, a chronic inflammatory model of neurodegenerative disease. We also investigated the possible involvement of HIF-1α regulated gene signalling by considering mechanisms of inflammation. Additionally, we aimed at assessing whether inflammation and hypoxia present similar phenotypes, both in vitro (glia cultures) and in vivo (APP/PS1).

2. Materials and methods

2.1. Animal models

The study was conducted on three experimental models. First, an acute model of inflammation in mixed glial cultures prepared from the brains of 3-day old C57BL/6 mice (Harlan, IN, USA). Secondly, a model of neuronal apoptosis in primary cultures of cerebellar granule neurons (CGNs) from the cerebellum of 7-day old Sprague-Dawley rats. Lastly, an in vivo model of chronic inflammation in APPswe/PS1dE9 (APP/PS1) transgenic mice of 3–12 months of age.

All research protocols and general animal care were carried out in accordance with the Directive 86/609 EEC of the Council of the European Union and the procedure established by the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya. Every effort was made to minimize animal suffering and reduce the number of animals used.

2.2. In vitro cell culture procedures

Mixed glial cell cultures were prepared from the cerebral cortices of 3-day old C57BL/6 mice. Briefly, cerebral cortices were dissected and meninges removed. The tissue was incubated with trypsin solution (trypsin 0.05% [w/v] trypsin–EDTA 5 mM) for 15 min at 37 °C. After that, the tissue was fragmented with a fire-polished Pasteur pipette. The dissociated cells were plated into 60 mm diameter culture plates (300,000 cells/ml) and grown in Dulbecco's Modified Eagle's/F-12 medium, supplemented with 10% foetal bovine serum (FBS), 2% HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin. Fresh medium was changed the next day and then every 3 days afterwards. All experiments were done 7 days after plating.

Primary cultures of cerebellar granule neurons (CGNs) from Sprague-Dawley rats were prepared from day 7 postnatal animals, as described elsewhere. Briefly, cells were dissociated in the presence of trypsin and DNase I (Sigma–Aldrich) and placed in poly-L-lysine-coated dishes (100 μg/ml) at a density of 6×10^5 cells/ml in basal Eagle's medium supplemented with 10% heat-inactivated FBS, 0.1 mg/ml gentamicin, 2 mM L-glutamine and 25 mM KCl. Cytosine-D-arabinofuranoside (10 μM) was added to the culture medium 24 h after plating to prevent the replication of non-neuronal cells. The cultures were maintained at 37 °C in a humidified incubator with 5% CO_2/95% air for 7 days. One hour before the assays were performed, the medium was changed to 25 mM potassium supplemented with 1% heat-inactivated FBS.

2.3. Cell treatment

Glial cultures were treated with pro-inflammatory cytokines (TNF-α, 20 ng/ml; IL-1β, 20 ng/ml; IFNγ 20 ng/ml) (PeproTech Inc., Rocky Hill, NJ, USA) or with the same volume of phosphate buffer

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Table 1
Primers used in this study for quantitative real-time PCR.

Gene name	Forward	Reverse
Mus musculus primer sequence		
Actina	CAACGAGCGGTTCCGAT	GCCACAGGATTCCATACCCA
CCL2	TCTCTCTTCCTCCACCACCATG	GCGTTAACTGCATCTGGCTGA
COX-2	TGACCCCCAAGGCTCAAATA	CCCAGGTCCTCGCTTATGATC
CXCl2	GGCGGTCAAAAAGTTTGCCT	GCTCCTCCTTTCCAGGTCAGTT
CXCl10	GGCTAGTCCTAATTGCCCTTGG	TTGTCTCAGGACCATGGCTTG
IL-6	ATCCAGTTGCCTTCTTGGGACTGA	TAAGCCTCCGACTTGTGAAGTGGT
TNF-α	AGCCGATGGGTTGTACCTTGTCTA	TGAGATAGCAAATCGGCTGACGGT
Rattus norvegicus primer sequence		
HIF-1α	AAAAGTAAGCCGGGCCGCCG	AAGATGGCGACGTGGACCGC
β-Actin	ATGGATGACGATATCGCTGCG	AGGGTCAGGATGCCTCTCTT

Note: primer sequence given in the 5′–3′ order.

saline (PBS) as the control. As indicated, cells were pre-treated with 10 μM SP600125 (Enzo, New York, USA) 1 h prior to cytokine treatments or with SP600125 alone as a control.

CGNs were treated with 30 μM LY294002 hydrochloride (Sigma, St. Louis, USA) or subjected to serum and potassium deprivation (S/K deprivation) during 8 h for gene expression experiments.

2.4. Detection of condensed nuclei by microscopic cell counting

Propidium Iodide staining was also used to evaluate morphologic evidence of apoptosis (e.g. condensed nuclei). After the corresponding treatment, cells were fixed in 4% paraformaldehyde PBS solution, pH 7.4, for 15 min at room temperature. After washing in PBS, they were incubated for 3 min with a solution of PI (75 μg/ml) in PBS. Stained cells were visualized under UV illumination using the 20× objective and digitalised images were captured. Apoptotic cells appeared as shrunken, brightly fluorescent nuclei showing high fluorescence. Apoptotic cells were scored by counting at least 500 nuclei for each sample in three different experiments. The percentage of apoptotic cells was calculated as follows: total number of cells with condensed nuclei/total number of cells × 100.

2.5. In vivo model

APP/PS1 double transgenic mice were obtained from Jackson Laboratory. The male transgenic mice aged 3–12 months and age-matched non-transgenic littermates were used in the present study. Transgenic and C57/BL6 mice were kept under controlled temperature, humidity and light conditions with food and water provided ad libitum. Mice used for the present study were divided in groups of 3, 6, 9 and 12 months.

2.6. Immunohistochemistry

For detection of Aβ deposits, free-floating coronal sections, 20 μm thick, were rinsed in 0.1 mol/l PB, pH 7.2 and pre-incubated in 88% formic acid. Then, sections were treated with 5 ml/l H_2O_2 and 100 ml/l methanol in PBS and pre-incubated in a blocking solution (100 ml/l of FBS, 2.5 g/l of BSA and 0.2 mol/l of glycine in PBST, PBS with 5 ml/l of Triton X-100). Sections were then incubated overnight (O/N) at 4 °C with primary antibody mouse anti-human beta-amyloid clone 6F/3D (1:100; DakoCytomation, Denmark). They were then incubated with biotinylated antibody (1:200; Sigma–Aldrich) and with the avidin–biotin–peroxidase complex (ABC; 1:200; Vector, Burlingame, CA). Peroxidase reaction was developed with 0.5 g/l diaminobenzidine in 0.1 mol/l PB and 0.1 ml/l H_2O_2, and immunoreacted sections were mounted on gelatinized slides. Stained sections were examined under a light microscope (Olympus BX61).

2.7. Nitric oxide measurement

NO released from glial cells was converted to nitrite in the culture medium, which can be determined using the Griess reaction. Culture medium was collected after 48 h of treatments and an equal volume of Griess reagent was added (sulfanilamide 1%, α-naphthylethylenediamine dihydrochloride 0.1%, 2.5% phosphoric acid). Absorbance was read in a spectrophotometer at 540 nm and sodium nitrite was used as a standard. From the same dishes, protein concentrations were measured using bicinchoninic acid (Pierce Company, Rockford, IL, USA) in order to normalize the nitrite concentrations.

2.8. p38 activity assay

P38 activity assay was performed using the p38 MAP Kinase Assay Kit (Cell Signalling) following the manufacturer's recommendations. Briefly, p38 was immunoprecipitated from 200 μg protein. After that, the samples were washed and centrifuged twice with lysis buffer and kinase buffer discarding supernatants. ATP, protein fusion (ATF), and kinase buffer were added to each sample, which was then incubated at 37 °C for 30 min. After that, samples were used to follow the Western blot protocol described previously. The primary antibody was pATF2 in a 1:1000 dilution. Chemiluminescence measurements and semi-quantitative values were obtained using the corresponding software Imagelab (Bio-Rad) and the results were expressed as a percentage with respect to control.

2.9. Western blotting

Tissue and cells samples were homogenized at 4 °C in lysis buffer containing 150 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris, 1 mM EDTA, 1% triton X-100 and protease inhibitor cocktail (complete, Roche Diagnostics, Germany). Protein concentration was determined spectrophotometrically using Bradford reagent at 595 nm. Cell homogenate containing 15 μg of protein per sample were analyzed by Western blot. Briefly, samples were placed in sample buffer (0.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 10% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured by boiling from 95 to 100 °C for 5 min. Samples were separated by electrophoresis on 10% acrylamide gels. Thereafter, proteins were transferred to PVDF sheets (Millipore Corp., Bedford, MA) using a transblot apparatus. Membranes were blocked overnight with primary monoclonal antibodies against pc-Jun Ser73 (rabbit polyclonal IgG, 1:1000, Cell Signalling), HIF1-α (mouse monoclonal IgG, 1:1000, Abcam, Cambridge, UK). We used β actin (mouse monoclonal IgG, 1:20,000, Sigma, USA) as a protein loading control. Afterwards, blots were washed thoroughly in 1 × TBS–Tween® 0.1% buffer (50 mM Tris; 1.5% NaCl, 0.05% Tween® 20, pH 7.5) and incubated for 1 h with a peroxidase-conjugated IgG secondary antibody

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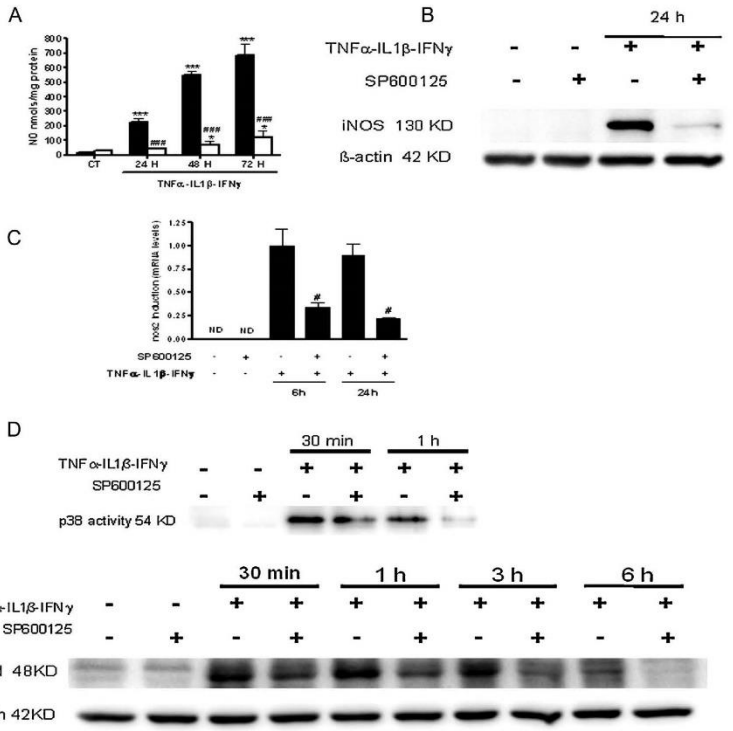

Fig. 1. (A) Nitrite levels in medium of glial cells at 24, 48 and 78 h of treatment with three pro-inflammatory cytokines (TNF-α, IL-1β and IFN-γ) cytokines. Bar graphs summarize the effects of three cytokines in glial cells alone (black) or in presence (glial pre-treated cells) of 10 μM SP600125 (white). Each point is the mean ± SEM of three or four independent experiments. SP600125 significantly decrease nitrite levels in glial cells with respect to cytokine treatment (***$p < 0.001$), (B) representative Western blot against iNOS at 24 h after cytokine treatment which induces iNOS expression increase in glial cells and this increase is reduced by SP600125 pretreatment (10 μM), (C) cytokine treatment increases *nos2* mRNA levels at 6 and 24 h of different of different treatments and this increase is significantly reduced by SP600125 pretreatment (10 μM). Each point is the mean ± SEM of three independent experiments in presence of SP600125 with respect to cytokine treatment (*$p < 0.05$) and (D) representative Western blots against phospho c-Jun and p38 activity at different time points after treatment of glial cells with three cytokines and SP600125 pretreatment (10 μM). All Western blots were normalized with actin.

(1:2000, Biorad, Hercules, USA). Immunoreactive protein was visualized using a chemiluminescence-based detection kit (Pierce® ECL Western blotting substrate, Thermo scientific, USA). Protein levels were determined by densitometry of the bands using ImageLab®. Measurements are expressed as arbitrary units and normalized with β-actin.

2.10. Semiquantitative real-time PCR

Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA was reconstituted in DEPC-treated water, and RNA concentration was determined using a nanodrop ND-1000. First-strand cDNA was reverse-transcribed from 1 μg of mRNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). The same amount of cDNA was subsequently used for semiquantitative RT-PCR, and each sample was analyzed in triplicate for each gene. For TaqMan RT-PCR assays primer/probe set for murine HIF1-α, Pgp, VEGF and GAPDH genes were purchased from Applied Biosystems (Foster City, CA, USA). Tables 1 and 2 shows respectively the primers used in this study to real-time PCR and TaqMan probes. Optical density was measured using a StepOnePlus from Applied Biosystems and normalized against either β-actin or GAPDH. The semiquantitative value of mRNA expression was calculated by means of $2^{-\Delta Ct}$ method.

2.11. Statistical analyses

Results were analyzed by one-way ANOVA followed by Bonferroni multiple comparison tests. Differences of $p < 0.05$ were considered statistically significant.

Table 2
TaqMan probes used for quantitative real-time PCR.

Symbol/gene name	Cat no.
Abcb1a/Pgp1α	Mm_00440761_m1
Gaph	Mm_99999915_g1
HIF-1α	Mn00468869_m1
nos2/iNOS	Mm_00440502_m1
VEGF	Mn01281449_m1

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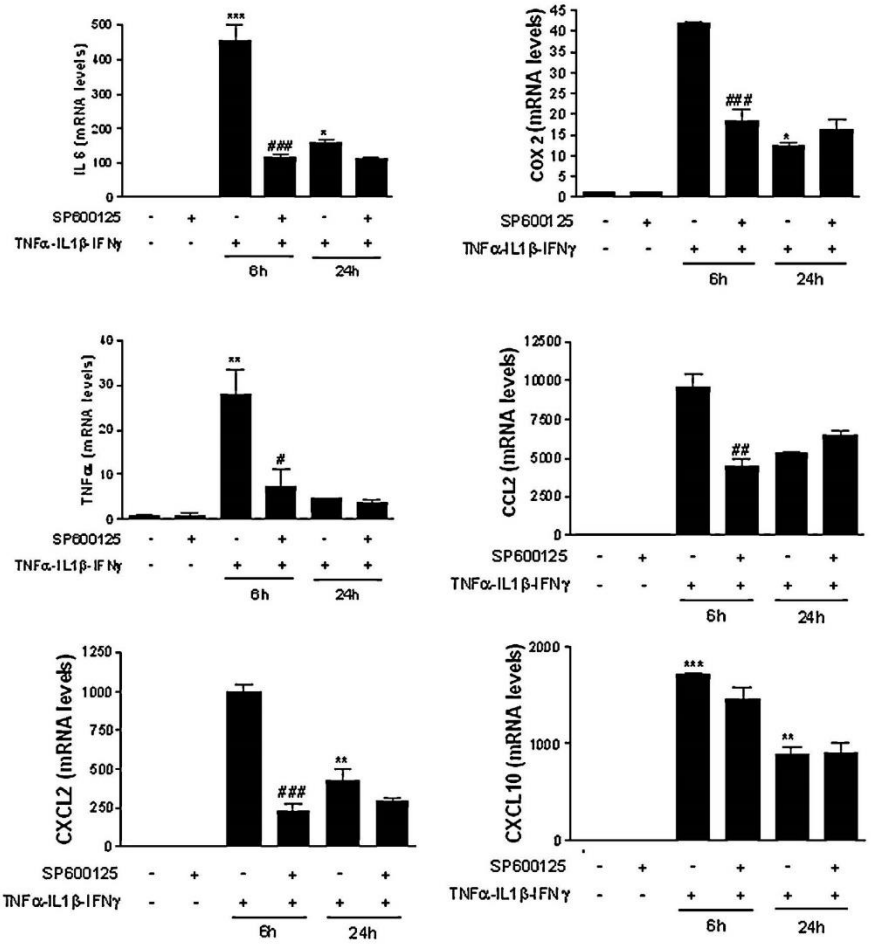

Fig. 2. Cytokine treatment increases mRNA levels of pro-inflammatory target genes *COX-2*, *IL-6*, *CCL2*, *CXCl2*, *CXCl10* at 6 and 24 and this increase is significantly reduced by SP600125 pretreatment (10 μM). Statistical analyses were carried out using one-way ANOVA followed by Bonferroni's post hoc tests. Each point is the mean ± SEM of three independent experiments in presence of SP600125 with respect to cytokine treatment ($^{a}p < 0.05$; $^{aa}p < 0.01$; $^{aaa}p < 0.001$).

3. Results

3.1. HIF-1α expression in glial cells after treatment with cytokines in acute model of inflammation

Primary glial cells cultures (astrocytes and microglia) treated simultaneously with three pro-inflammatory cytokines (TNF-α, IL-1β and IFN-γ) were used as an acute model of inflammation. As described elsewhere, pro-inflammatory stimuli lead to an increase in NO production measured by nitrite released into the culture medium using the Griess reagent (see Section 2), iNOS protein and *nos2* mRNA expression which was also observed in our model (Fig. 1A and B). Interestingly, the inhibition of JNK by SP600125 prior to cytokine treatment resulted in the reduction of NO levels (Fig. 1A). Moreover, this effect was also observed in the reduction of *nos2* transcription (Fig. 1C), accompanied by a reduction in iNOS protein levels (Fig. 1B). Taken together, our current data show that SP600125 inhibits the inflammatory activation of glial cells in terms of *nos2* induction and NO production.

MAPK family, including JNK and p38 kinases, is known to contribute to glial activation (Naruishi et al., 2003; Pawate and Bhat, 2006). Thus, time-course studies were performed to analyze the protein expression of c-Jun, the transcription factor induced by the MAPK as well as phosphorylated c-Jun (p-c-JUN) and p38. We observed a significant increase of p-c-JUN and p38 activity 30 min after the three cytokine treatment (Fig. 1D). In addition, the expression of pro-inflammatory target genes *COX-2*, *IL-6*, *MCP-1/CCL2*, *CXCl2*, *CXCl10* and *TNF-α* was induced six and 24 h after treatment (Fig. 2). Conversely, pre-incubation with the JNK inhibitor SP600125 diminished the induction of pro-inflammatory genes *IL-6*, *COX-2*, *TNF-α*, *MCP-1/CCL2*, *CXCl2* and *CXCl10* six and 24 h after the onset of stimulation with the three cytokines (Fig. 2).

Next, we determined the effects JNK signalling has on HIF-1α and its target genes. We found that HIF-1α mRNA and protein levels

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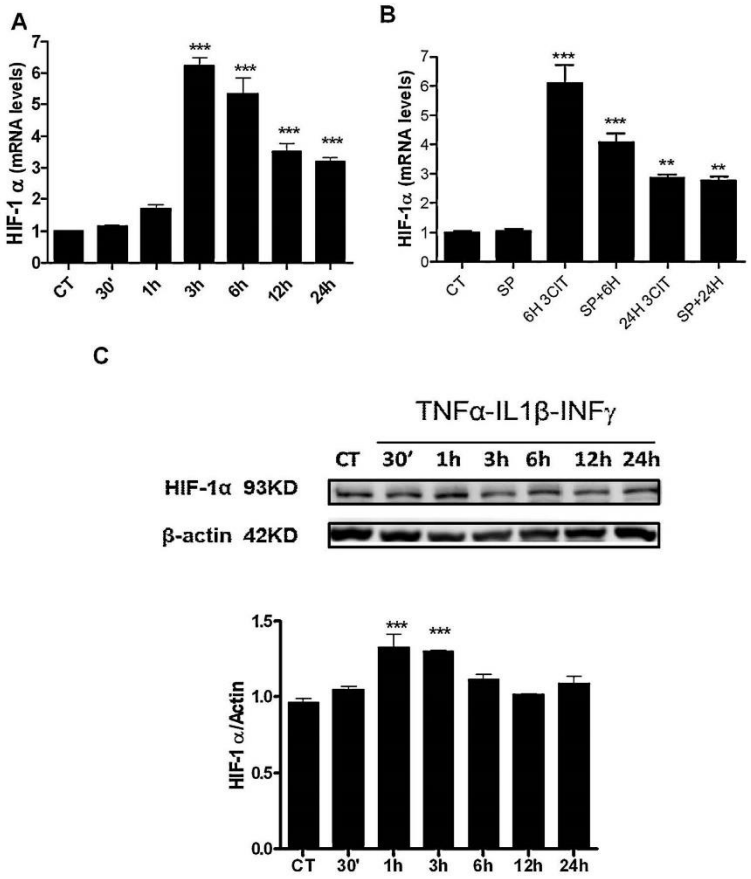

Fig. 3. (A) and (B) Time-dependent cytokine treatment increases glial mRNA levels of *HIF-1α*. SP600125 pretreatment (10 μM) at 6 h significantly decrease mRNA levels of *HIF-1α*. Each point is the mean ± SEM of three independent experiments in presence of SP600125 with respect to cytokine treatment (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) and (C) time dependent Western blot analysis against HIF-1α after cytokine treatment of glial cells. All Western blots were normalized with actin. For each point is presented the mean value of three independent experiments (*$p < 0.05$ with respect to vehicle control).

were up regulated 3 h after cytokines treatment. Thus, cytokines increased HIF-1α mRNA expression in a time-dependent manner and maximal induction was reached between 3 and 6 h (Fig. 3A). Administration of SP600125 prior to the addition of cytokines to glial cell cultures significantly reduced the expression of HIF-1α mRNA (Fig. 3B). Western blot analysis revealed a time-dependent increase in HIF-1α protein levels (at 1–3 h, *$p < 0.05$) in treated glial cells, compared to control. These results indicate that the up regulation of HIF-1α by pro-inflammatory cytokines is likely mediated via the JNK pathway. Surprisingly, cytokine treatment resulted in a significant reduction of VEGF mRNA, which is a direct target of HIF-1α, 6 h after the treatment, suggesting that cytokine treatment could inhibit the HIF-1α dependent VEGF-transcripts up-regulation in glial cells (Fig. 4A). Additionally, SP600125 at 24 h lead to a significant increase in VEGF mRNA expression, whereas Pgp1α, another direct target of HIF-1α, showed a reduction (Fig. 4B).

Taken together, our data demonstrate that SP600125 treatment results in a significant reduction of pro-inflammatory phenotype in glia and implicates mainly the JNK pathway in acute inflammation.

3.2. Role of HIF-1α in a model of neuronal apoptosis

LY294002 is a PI3K inhibitor widely used as a drug for the study of the pathways involved in AKT inhibition that induce apoptosis. Previous studies reported that LY294002-mediated apoptosis occurs via the c-Jun induction and MAPK activation (de la Torre et al., 2012). After the treatment with LY294002 we found a significant increase in the number of condensed nuclei (PI incorporation) in CGCs (up to 35%) and this increase was significantly attenuated by 10 μM SP600125 (Fig. 5A). SP600125, significantly reduced the percentage of apoptotic cells (control; 7 ± 0.57; LY294002 30 μM: 35 ± 0.41; SP600125 10 μM; 11 ± 0.34) (**$p < 0.001$ respect LY294002 treatment). Likewise, we demonstrated that SP600125 prevents LY294002-mediated apoptosis in CGCs (10 μM SP600125) and c-Jun expression (Fig. 5B).

Exposure to LY294002 of the cerebellar granule cell cultures maintained in normoxia conditions did not affect mRNA or protein expression of HIF-1α within the 24 h period (Fig. 5C and D). Moreover, it is well known that serum and potassium in CGNs is a well-established model of apoptosis. In our studies we showed

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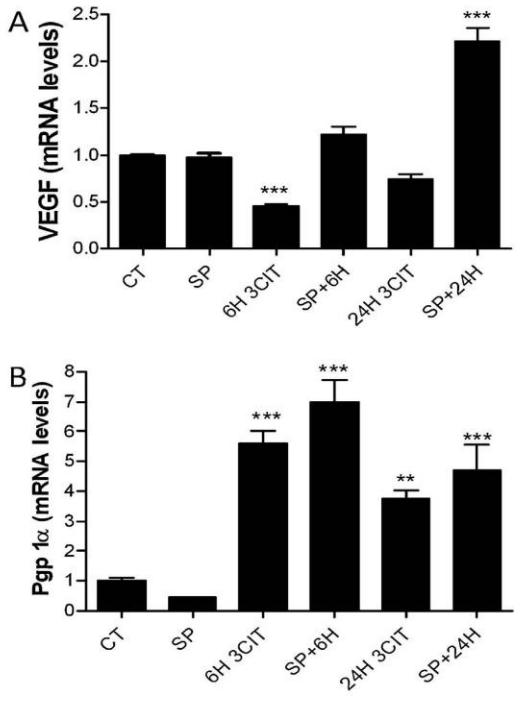

Fig. 4. (A) Effects of SP600125 pretreatment (10 μM) at 6 h and 24 h on *VEGF* and (B) *Pgp1α* mRNA levels. Statistical analyses were carried out using one-way ANOVA followed by Bonferroni's post hoc tests. Each point is the mean ± SEM of three independent experiments in presence of SP600125 with respect to cytokine treatment (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

that after 8 h of deprivation the HIF-1α protein levels were not increased. Thus, contrary to the hypoxia-based experimental models of ischaemia, our data suggest that the transcription factor HIF-1α is not involved in the regulation of neuronal apoptosis in normoxia conditions.

3.3. Role of HIF in an experimental model of Alzheimer disease

Because HIF-1α was induced after the three-cytokine treatment of glial cells, in the next series of experiments, we evaluated HIF-1α expression in a chronic model of inflammation in the APP/PS1 double transgenic mouse model of AD. As mentioned earlier, neuroinflammation is a hallmark of AD brain and is characterized by the presence of activated astrocytes and microglia surrounding amyloid plaques (for review, see Akiyama et al., 2000). Similar to AD, APP/PS1 transgenic mouse models have elevated inflammation markers and gliosis associated with amyloid deposits (Games et al., 1995; Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Ruan et al., 2009).

The analysis of astrocytes along different disease stages in an APP/PS1 animal model was done using an antibody that detects the glial acidic fibrilar protein (GFAP). The results obtained in 3, 6, 9 and 12 month old transgenic mice revealed an accumulation of GFAP-positive activated cells around amyloid deposits/aggregates, when compared to the control group (Fig. 6). Significantly, the activation of astrocytes was only detected in close proximity to amyloid aggregates (Fig. 6) Thus the spatial pattern of gliosis in APP/PS1 brain closely followed the distribution of amyloid deposits (Fig. 6C′–C‴). The same pattern of response was detected using the iba1 (ionized calcium binding adaptor molecule 1) antibody which immunostains microglial cells, meaning that the number of activated microglial cells accumulated around the myeloid aggregates (Fig. 7), consistent with previous data in indicating that APP/PS1 mice display characteristics of neuroinflammation similar to those observed in AD brain.

However, the analysis of the HIF-1α mRNA expression in mouse hippocampus and cortex does not show any changes in mRNA expression from 3 to 12 months (Fig. 8). The same can be said for downstream targets of HIF-1α such as *VEGF* and *Pgp* mRNA (Fig. 8). These findings indicate that HIF-1α is unlikely to play a role in neuroinflammation caused by Alzheimer's disease.

4. Discussion

The mechanisms of inflammation-induced HIF-1α expression in glial cells and mouse models of Alzheimer's disease are not well characterized. In the present study, we investigated HIF-1α signalling using three experimental models:

(i) In glial cells under an acute inflammatory stimulus with three cytokines which lead to a significant increase of HIF-1 mRNA expression. We hypothesize that the mechanism whereby SP600125 inhibits the glial inflammatory response occurs via the inhibition of JNK and the down-regulation of HIF-1α mRNA expression.
(ii) In neuronal cell cultures upon AKT inhibition which promotes the apoptotic process HIF-1α mRNA and protein levels remain unchanged.
(iii) In the APP/PS1 experimental model of familial Alzheimer's disease, HIF-1α mRNA expression was not affected.

These results provide novel insights into various regulatory mechanisms and suggest the physiological role of an acute inflammatory response triggered by HIF-1α activation in glial cells, thereby facilitating our understanding of the regulation of HIF-1α in non-hypoxic conditions. We showed that HIF-1α is involved in inflammatory gene activation following the exposure of glia to pro-inflammatory cytokines. In addition, our data demonstrates the participation of p38 in this process, as evidenced by a significant upregulation of p38 at 30 min after the three cytokine treatment.

Time-course experiments suggest that JNK/p38 and c-Jun contribute to the inflammatory response induced by cytokines in glial cells because at 30 min they are both overexpressed (Fig. 1C). Moreover, cytokine-mediated increase in c-Jun may not only result in elevated expression of HIF-1α mRNA, but may also favour cooperative binding of HIF-1α and c-Jun to the promoters of particular target genes such as iNOS, thus further exacerbating the inflammatory response. However, in the present study we have also demonstrated that SP600125 counteracts the production of nitric oxide in glial cells (Fig. 1A) and the over-expression of iNOS and *nos2* (Fig. 1 B), as well as, *IL-6*, *COX-2*, *TNF-α*, *CCL2*, *CXCl2* and *CXCl10* mRNA (Fig. 2). Interestingly, HIF-1α mRNA expression was only partially inhibited (Fig. 3), suggesting that in addition to MAPKs, HIF-1α gene expression may be regulated via an alternative pathway.

Previous studies have reported that MAPK signalling is involved in HIF-1α activation, thus linking HIF-1α to an oncogenic process (Wang et al., 2009; Brigati et al., 2010; Harten et al., 2010). Additionally, under the hypoxic conditions, c-Jun was demonstrated to be a direct target of HIF-1α (Imitiyaz and Simon, 2010). Likewise, a transcriptionally active JNK binding site was recently identified within a HIF-1α promoter region in neurons (Xanthi et al., 2009).

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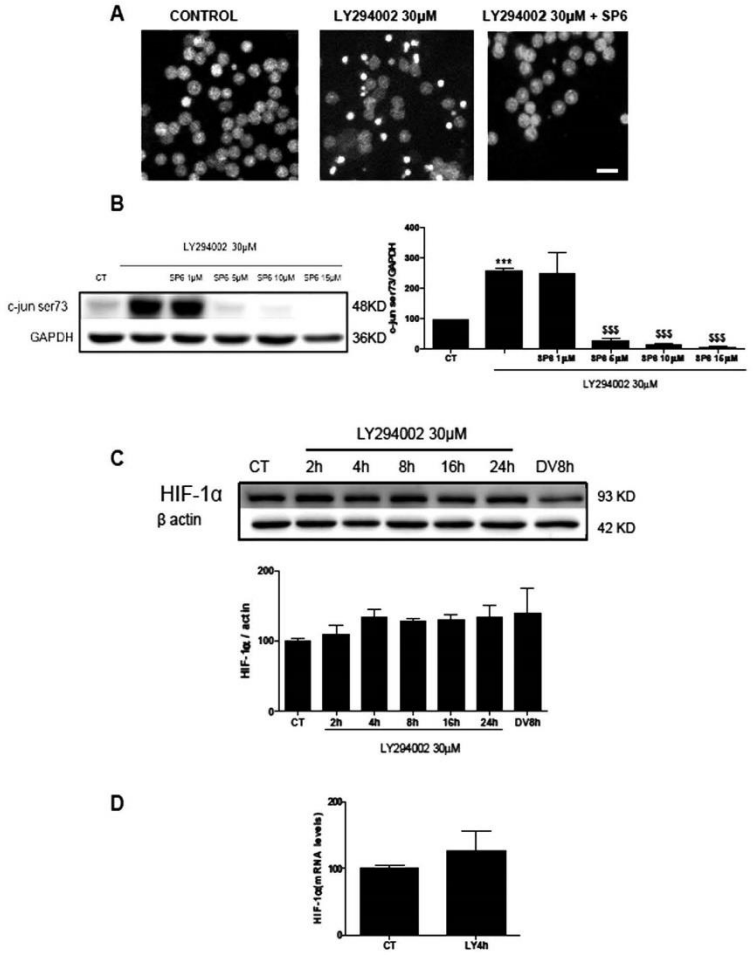

Fig. 5. (A) Representative images of nuclei control samples, 30 μM LY294002 treated CGNs and CGNs treated with 30 μM LY294002 in the presence of 10 μM SP600125 (calibration bar, 10 μm). The nuclei were counted on a fluorescence microscope, distinguishing normal nuclei from the condensed ones following the criteria stated in the material and methods. (B) representative Western blot analysis of the expression levels of c-Jun ser73 in neurons after LY294002 (30 μM) treatment alone, or in presence of SP600125 (1–15 μM treatment) respectively. GAPDH was detected as loading control. Immunoblots were representative from $n = 4$–5 experiments. The proteins were quantified and statistically analyzed. ***$P < 0.001$ LY294002 vs CT. $^{\$\$\$}p < 0.001$ LY294002 vs SP600125 treatments respectively, (C) representative Western blot analysis of the expression levels of HIF-1α in neurons after LY294002 (30 μM) treatment, and (D) LY294002 (30 μM) treatment of CGCs does not induce changes in mRNA levels of HIF-1α gene.

Accordingly, the HIF-1α targets genes analyzed in this study did not follow the same pattern of expression, because VEGF expression appears to be repressed while P-gp appears to be stimulated by the cytokines. However, the inhibition of c-Jun had actually resulted in the recovery of basal levels at 6 h and significant increased at 24 h of VEGF mRNA levels while P-gp had an additional increase of mRNA levels at both times (Fig. 4). This raises an intriguing possibility that transcriptional regulation of HIF-1α and its target genes may differ significantly in hypoxia and in our model of acute inflammation in glial cells. One potential explanation for the unexpected VEGF and P-gp signalling in a three cytokine model may be that the increase in NO could also regulate VEGF and Pgp, thus adding to the model's complexity (Zhang et al., 1996; Kimura and Esumi, 2003; Pawate and Bhat, 2006; Kawasaki et al., 2007; Barouk et al., 2011; Pocivavsek et al., 2009; Yoshioka et al., 2010; Zhang et al., 2012a,b,c).

Furthermore, we assessed the potential role of HIF-1α in a well characterized apoptotic model – the inhibition of AKT by LY294002 in CGNs (de la Torre et al., 2012). Here we demonstrate that in normoxic conditions and after LY294002 treatment HIF-1α mRNA levels were unchanged. Therefore, our data suggest that the regulation of HIF-1α by c-Jun is likely cell-type and stimuli-dependent. Our results are in contrast to those previously published by other authors, who reported a neuroprotective role of HIF-1α in hypoxia-induced injury in neurons, likewise protects sympathetic neurons against neuronal growth factor (NGF) deprivation and also prion

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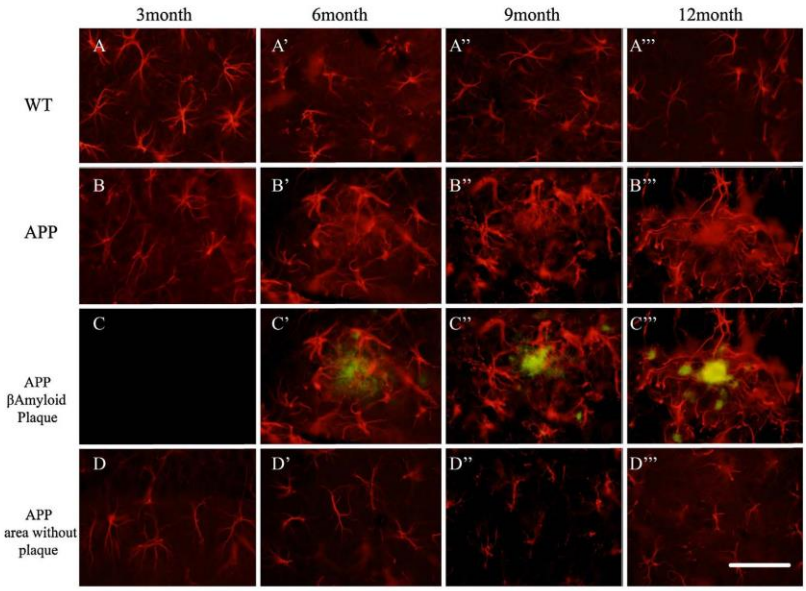

Fig. 6. Glial fibrilar acidic protein immunohistochemistry of coronal brain slices of wild type and APP/PS1 mice of 3, 6, 9 and 12 months old. From panel A to B‴ immunofluorescence against GFAP: A–A‴ immunostainig from wild type animals and B–B‴ from transgenic APP/PS1 mice. Panel C–D‴ double immunostaining of astrocytes and β-amyloid plaque by Thio S flavine: C–C‴ show β-amyloid plaque (in green) colocalized with astrocytes (GFAP in red), D–D‴ are representative GFAP immunoreactive glial cells of an area without β-amyloid plaques. Scale bar: 100 μm for all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

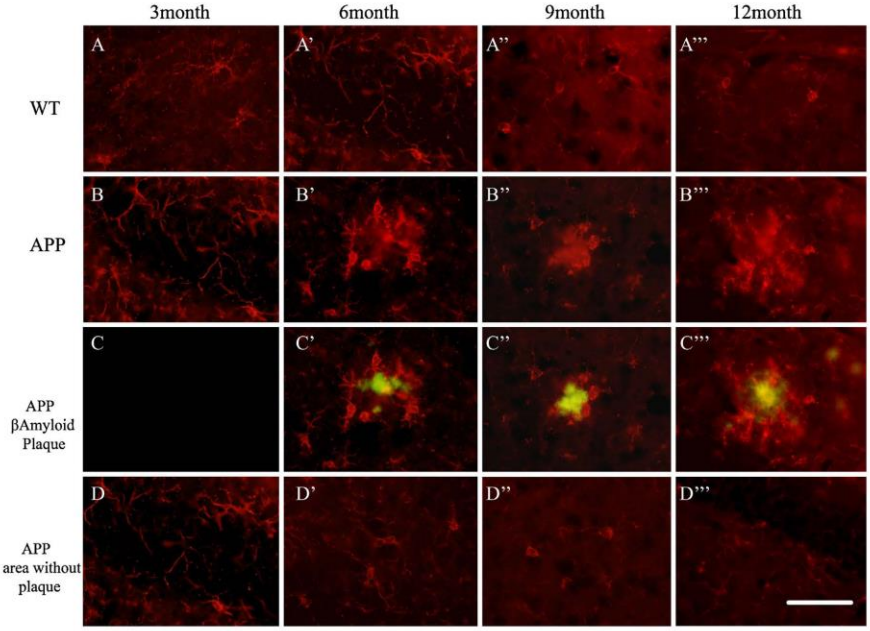

Fig. 7. Microglia immunostaining of brain slices of wild type and APP/PS1 at 3, 6, 9 and 12 months. (A–A‴) wild type and (B–B‴) are showing immunofluorescence to Iba-1 (red), microglial specific marker, (C–C‴) detail of the β-amyloid plaque (green) colocalized with Iba-1 (red), and (D–D‴) representative APP/PS1 microphotographs from an area without β-amyloid plaque. Scale bar: 100 μm for all panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

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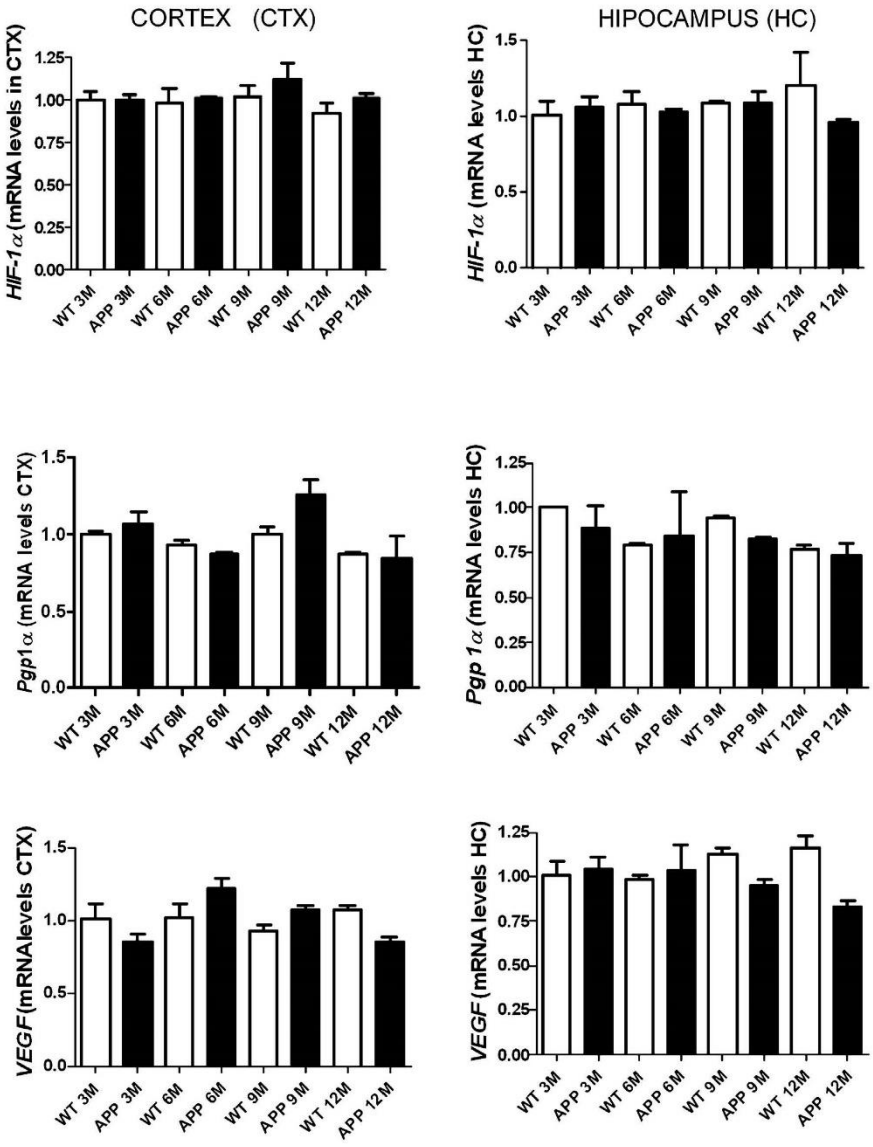

Fig. 8. Time dependent mRNA expression levels of mRNA levels of HIF-1α, VEGF and Pgp1α in the hippocampus and cortex of wild-type and APP/PS1 mice from 3 moths up to 12 months. Each point is the mean ± SEM of three or four mice.

peptide-induced neuronal cell death (Nardinocchi et al., 2009; Shi, 2009; Kirito et al., 2009; Yang et al., 2010; Yu et al., 2011; Song et al., 2012; López-Hernández et al., 2012; Sendoel et al., 2010; Jeong et al., 2012).

On the contrary, when the HIF-1α expression was inhibited in cultured neurons, isoflurane-induced neurotoxicity and hypoxia-induced cell death was prevented (López-Hernández et al., 2012; Jiang et al., 2012). In this way, Helton and colleagues demonstrated that the loss of HIF-1α in the brain is neuroprotective to acute global ischaemic injury and favours the depletion of apoptotic genes (Helton et al., 2005). Likewise, some reports have showed that HIF-1α can also activate pro-apoptotic proteins such as Nip-3 and Nix (Aminova et al., 2008).

Cell-specific effects were also reported in studies of cortical neuron survival due to a hypoxic insult showing that neuronal expression of HIF-1α supported neuronal survival in hypoxia, while astrocytic expression of HIF-1α promoted neuronal death in hypoxia (Vangeison et al., 2008). Therefore the role of HIF-1α in

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neurons can be complex; its dual action is likely dependent on the cell type and the injury stimulus and then may exert a neuroprotective or neurotoxic role in regulating cellular apoptosis (Xie et al., 2005).

In order to identify the cell-specific role of HIF-1α, we measured mRNA levels of HIF-1α in the cortex and the hippocampus of APP/PS1 mice in an experimental model of familial AD. We did not detect significant differences in HIF-1α expression within the 3–12 months of age in our model, while the expression of *VEGF* and *Pgp1*, was not affected. However, as described elsewhere, pharmacological treatment of the APP/PS1 transgenic animals with an M30, which is an iron chelator, resulted in a significant up-regulation of HIF-1α protein expression in the brain and in activation of a wide range of neuroprotective genes (Kupershmidt et al., 2011; Zhang et al., 2011). These data suggest that the brain HIF-1 signal transduction pathways induction or activation could be important in the improvement of AD symptomatology such as memory loss and beta amyloid plaque reduction and may be a suitable strategy for AD treatment (Correia and Moreira, 2010).

One of HIF-1α targets is Pgp which functionally constitutes a major component of the Blood–Brain-Barrier (BBB) which acts by limiting CNS penetration of various therapeutic agents (Cirrito et al., 2005). Furthermore, the lack of Pgp expression at BBB, correlated with an increased amyloid-β deposition in an AD mouse model (Cirrito et al., 2005; Harten et al., 2010). Thus an increase in Pgp expression and transport activity may decrease the risk of β-amyloid deposition and development of AD. Therefore, even although our data indicate that HIF-1α mRNA expression is not altered in neither ageing wild-type, nor APP/PS1 mice it may still be that the administration of iron chelating drugs may have beneficial effects on AD progression (Weinreb et al., 2009; Kupershmidt et al., 2011).

In summary, we have found that the HIF-1α is expressed by activated glial cells in acute inflammation. We reported that JNK/p38/c-Jun is partially involved in HIF-1α regulation, however we did not detect the expected expression pattern in HIF-1α target genes in glial cells. We demonstrated that HIF-1α signalling pathway is not involved in chronic inflammation observed in an APP/PS1 model of AD. Therefore additional studies are warranted in order to elucidate the exact role of HIF-1α in inflammation and neuronal apoptosis in normoxic conditions.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Publication 3

ADIPOKINE PATHWAYS ARE ALTERED IN HIPPOCAMPUS OF AN EXPERIMENTAL MOUSE MODEL OF ALZHEIMER'S DISEASE

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Summary

This paper can be considered as a parallel study to the research described in the current thesis. Rather than focusing on the insulin signaling, we have addressed the issue of hippocampal adipokine receptor and cholesterol regulation. 3- and 6-month old APP/PS1 and wild-type animals on a control chow were chosen. Just like in the results pertaining to insulin signaling presented in the current thesis, we have detected significant perturbations in adipokine and cholesterol pathways at an early age, prior to amyloid plaque and cognitive symptoms development.

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ADIPOKINE PATHWAYS ARE ALTERED IN HIPPOCAMPUS OF AN EXPERIMENTAL MOUSE MODEL OF ALZHEIMER'S DISEASE

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Abstract: A growing body of evidence suggests that β-amyloid peptides (Aβ) are unlikely to be the only factor involved in Alzheimer's disease (AD) aetiology. In fact, a strong correlation has been established between AD patients and patients with type 2 diabetes and/or cholesterol metabolism alterations. In addition, a link between adipose tissue metabolism, leptin signalling in particular, and AD has also been demonstrated. In the present study we analyzed the expression of molecules related to metabolism, with the main focus on leptin and prolactin signalling pathways in an APPswe/PS1dE9 (APP/PS1) transgenic mice model, at 3 and 6 months of age, compared to wild-type controls. We have chosen to study 3 months-old APP/PS1 animals at an age when neither the cognitive deficits nor significant Aβ plaques in the brain are present, and to compare them to the 6 months-old mice, which exhibit elevated levels of Aβ in the hippocampus and memory loss. A significant reduction in both mRNA and protein levels of the prolactin receptor (PRL-R) was detected in the hippocampi of 3 months old APP/PS1 mice, with a decrease in the levels of the leptin receptor (OB-R) first becoming evident at 6 months of age. We proceeded to study the expression of the intracellular signalling molecules downstream of these receptors, including stat (1-5), sos1, kras and socs (1-3). Our data suggest a downregulation in some of these molecules such as stat-5b and socs (1-3), in 3 months-old APP/PS1 brains. Likewise, at the same age, we detected a significant reduction in mRNA levels of lrp1 and cyp46a1, both of which are involved in cholesterol homeostasis. Taken together, these results demonstrate a significative impairment in adipokine receptors signalling and cholesterol regulation pathways in the hippocampus of APP/PS1 mice at an early age, prior to the Aβ plaque formation.

Key words: APP/PS1, leptin, hippocampus, prolactin, Alzheimer.

Introduction

Alzheimer's disease (AD) is the most common cause of senile dementia in the world, followed by Parkinson's disease (1). AD progression is associated with the formation of senile β-amyloid (Aβ) plaques and neurofibrillary tangles composed of hyperphosphorylated tau (1, 2). Currently it is widely accepted that Aβ is generated by a specific proteolytic clavage of the amyloid precursor protein (APP). In this amyloidogenic pathway, the β- and γ-Secretases cleave APP at the N- and C-termini of the Aβ peptide, respectively. The relationship between APP and Aβ caused the formulation of the amyloid cascade hypothesis that states that mutations in APP (or other genes) lead to an increase in Aβ, and that this in turn leads to disease progression (3, 4).

A number of animal models attempting to mimic the progression of the AD have been extensively investigated. APP/PS1 mice, which possess 2 of the more frequent mutations leading to familial AD (FAD) in humans, are commonly used in experimental animal studies of AD. One of the principal features of these mice is the development of memory loss and a significant Aβ plaque deposition in the hippocampus, clearly evident by 6 months of age (5-8). We have chosen to study 3 months-old animals which do not present brain Aβ deposits nor cognitive loss and we have compared them to the 6 months-old mice. The rationale behind this approach is to identify molecular events involved in the early pre-plaque stages of the AD-like pathology in this mouse model. In our opinion, this is especially relevant because despite the genetic and cell biological evidence that supports the amyloid cascade hypothesis, it is becoming increasingly clear that AD aetiology is more complex and that Aβ alone is unable to account for all the aspects of AD (9). Hundreds of genes have been identified as being involved in this neurodegenerative disease (10, 11). Recent studies suggest that metabolic alterations such as diabetes mellitus, cholesterol metabolism dysregulations and metabolic syndrome in general are strongly correlated with AD (11-17). Thus, a continuous effort should be made to identify components of the network involved in the progression of diseases like AD in order to develop more efficient and specific treatments (18-20).

Since the sporadic form of AD is a multifactorial disease influenced by several risk factors such as hypertension, diabetes, hypercholesterolemia, age, neuroinflammation, hypoxia and others, it is difficult to point out a single pathogenetic mechanism leading to the onset and progression

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of this devastating disorder (6-8, 14-20). For example, obesity significantly increases cognitive decline and AD risk, supporting the notion that molecular mechanisms of cellular energy homeostasis are linked to AD pathogenesis (15-20). Additionally, there is evidence of a relationship between adipokines and AD (21-24). The adipokines or adipocytokines are cytokines secreted by adipose tissue (21). These include leptin, adiponectin, tumor necrosis factor (TNF)-alpha, interleukins, including IL-6, and also molecules like prolactin (Prl), a well-known regulator of the lactating mammary gland, recently shown to be produced by human adipose tissue (21-27). Adipokines have come to be recognized for their contribution to the mechanisms by which obesity and related metabolic disorders influence diseases like cancer or AD (24-28). It has been observed that AD patients display increased circulating levels of anorexigenic adipokines that may contribute to the metabolic changes observed in AD patients (21).

Among the adipokine genes associated to AD, an adipostatic hormone leptin, coded by the ob or lep gene, stands out. Leptin is a hormone secreted by adipose tissue that acts to suppress appetite and regulates energy expenditure. In humans, a correlation between elevated leptin levels and reduced incidence of dementia and AD had been reported (28). In rodents, leptin modulates production and clearance of Aβ (29-31). Mice with leptin receptor disruption show impairments in long-term potentiation, synaptic plasticity and spatial learning, whereas treatment with leptin increases Aβ- and tau- clearance as well as ameliorates AD-like pathology (21, 25-31). Thus, in the context of the amyloid cascade hypothesis, leptin may interfere with the pathogenesis of AD in multiple ways: (a) by inhibiting the amyloidogenic process; (b) by decreasing the activity of glycogen synthase kinase-3β (GSK3β), causing a reduction in Tau protein phosphorylation; and (c) by improving cognitive function (25-27).

Beside the roles of adipokines per se, it has been shown that alterations in lipid metabolism can also promote the development of AD. The brain is rich in cholesterol and substantial evidence from in vitro and in vivo studies, as well as from human trials, indicates that cholesterol levels affect the synthesis, clearance, and the toxicity of Aβ (13, 14, 32). For example, elevated cerebral Aβ levels in living humans were found to be correlated with serum cholesterol fractions in a pattern analogous to that found in coronary artery disease (11).

In the current study we have focused on molecular mechanisms related to adipokine signalling, AD progression and memory loss in the hippocampus of an APP/PS1 mouse model of FAD at two time points: 1) at 3 months of age, prior to the plaque formation and memory loss, and 2) at 6 months of age, by which both cognitive decline and hippocampal Aβ deposits are clearly evident.

Materials and methods

Animals

Male APPswe/PS1dE9 and C57BL/6 mice were used in this study. APP/PS1 animals co-express a Swedish (K595M/N596L) mutation of a chimeric mouse/human APP (Mo/HuAPP695swe), together with the human exon-9-deleted variant of PS1 (PS1-dE9), allowing these mice to secrete elevated amounts of human Aβ peptide. Both mutations are associated with familial AD, are under control of the mouse prion protein promoter, directing both mutated proteins mainly to the CNS neurons, and result in age-dependent amyloid plaque depositions in mouse brain. The APPswe-mutated APP is a favourable substrate for β-secretase, whereas the PS1dE9 mutation alters γ-secretase cleavage, thereby promoting overproduction of Aβ42. Animals were kept under controlled temperature, humidity and light conditions with food and water provided ad libitum. Mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya. Every effort was made to minimize animal suffering and to reduce the number of animals used. Forty animals, divided into four groups, were used for the present study, with at least 8 wild-type and 8 APP/PS1 transgenic mice of 3 and 6 months of age, per group. Following in vivo testing, the animals were sacrificed and at least 6 mice in each group were used for RNA and protein extracts isolation.

Blood Cholesterol and Triglyceride measurements

Cholesterol and trygliceride levels were measured in the blood, collected from heart puncture, following a 5-hour morning fast, at the point of sacrifice with the Accutrend Plus meter (Roche; Mannheim, Germany).

Immunohistochemistry

For detection of Aβ deposits, free-floating coronal sections, 20 μm thick, were rinsed with 0.1 mol/L PB, pH 7.2, and pre-incubated in 88% formic acid. Then, sections were treated with 5 ml/L H2O2 and 100 ml/L methanol in PBS and pre-incubated in a blocking solution (100 ml/L of FBS, 2.5 g/L of BSA and 0.2 mol/L of glycine in PBS with 5 ml/L of Triton X-100). After that, sections were incubated for 10 minutes with Thioflavin S (Sigma T1892). Sections were mounted on gelatinized slides. Images were taken with a fluorescence laser and optic microscope (BX41, Olympus, Germany) and stored in tiff format. All images were acquired using the same microscope, laser and software settings.

Western blot analysis

Aliquots of hippocampus homogenate containing 15 mg of protein per sample were analyzed using the Western blot method. In brief, samples were placed in sample buffer (0.5 m Tris–HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2--mercaptoethanol, 0.05% bromophenol blue) and denatured

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by boiling at 95–100°C for 5 min. Samples were separated by electrophoresis on 10% acrylamide gels. Following this, the proteins were transferred to PVDF sheets using transblot apparatus. Membranes were blocked overnight with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris; 1.5% NaCl, 0.05% Tween 20, pH 7.5). They were then incubated with primary antibodies directed against the GAPDH (Mab374, Millipore), leptin (PA1-28843, Thermo Scientific) and prolactin (ab98015, Abcam) receptors. After O/N incubation, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG secondary antibody (1:2000). Immunoreactive protein was detected using a chemiluminescence-based detection kit. Target protein levels were determined by densitometry, using Chemi doc XRS+ Molecular Imager detection system (Bio-Rad), with ImageLab image analysis software. Measurements are expressed as arbitrary units. All results are normalized to GAPDH, unless stated otherwise.

RNA extraction and quantification

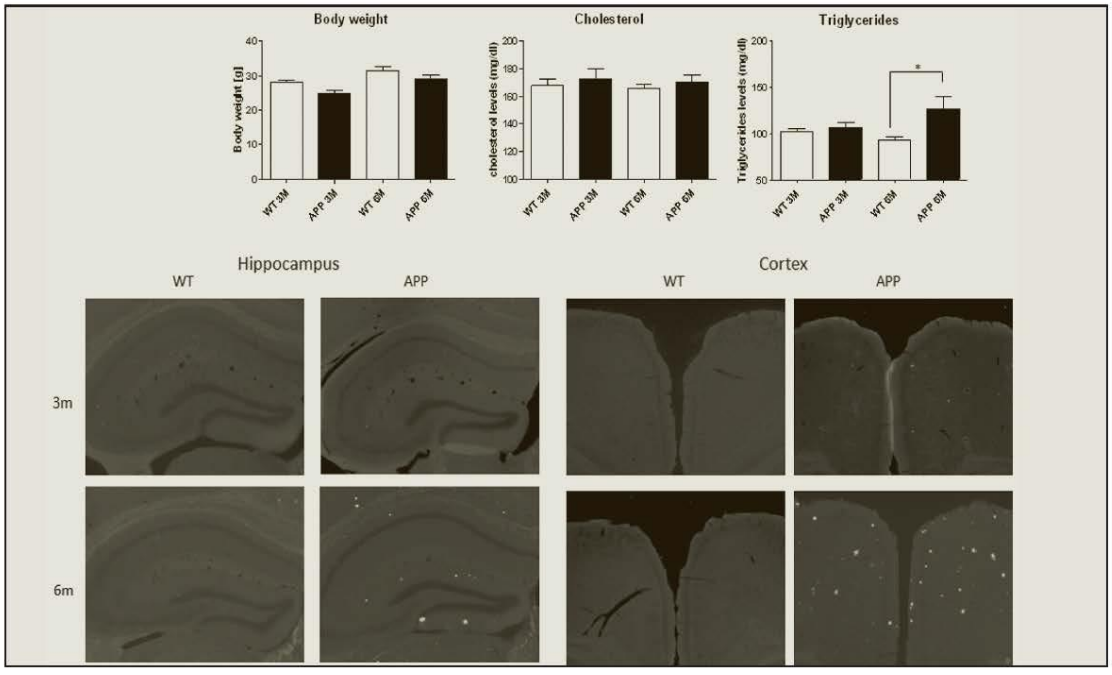
Total RNA was isolated from the hippocampi of wild-type and APP/PS1 transgenic mice, as described previously (Chomczynski and Sacchi, 1987). Briefly, the tissue was homogenized in the presence of Trizol reagent (Life Technologies Corporation). Chloroform was added and the RNA was precipitated from the aqueous phase with isopropanol at 4°C. RNA pellet was reconstituted in RNase-free water, with the RNA integrity determined by Agilent 2100 Bioanalyzer.

Quantitative RT-PCR

First-strand cDNA was reverse transcribed from 2 μg of total RNA from hippocampi of 3 and 6 months-old mice, using the High Capacity cDNA Reverse Transcription kit, according to manufacturer's protocol (Applied Biosystems). Equal amounts of cDNA were subsequently used for qRT-PCR, and each sample was analysed in triplicate for each gene. TaqMan gene expression assays (Applied Biosystems) as detailed in Supplementary Table 1, were used to determine transcription levels of individual genes. qRT-PCR was performed on the StepOnePlus Real Time PCR system (Applied Biosystems) and normalized to the transcription levels of gapdh, actin and/or tbp, using the delta-delta Ct method.

Figure 1

Mean body weight (n=20), whole blood cholesterol and triglycerides levels in 3 and 6 months-old wild-type and APP/PS1 mice (n=7-12). (Statistical analysis was performed with one-way ANOVA, with Tukey's post-hoc test, where * denotes $p < 0.05$). (A). Representative immunofluorescent staining with the Thioflavin S, in 3 and 6 months-old mice, demonstrating Aβ plaque deposits in the hippocampus and cortex of 6 months-old APP/PS1 animals (B)



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Statistical analysis

All data are presented as means ± SEM, and differences are considered significant at $p < 0.05$, $p < 0.01$. Differences between samples/animals were evaluated using either one-way ANOVA, with Tukey's post-hoc test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, and with the student's t-test, where $ denotes $p < 0.05$.

Results

Physiological and metabolic parameters of APP/PS1 mice

No changes in either the body weight or blood cholesterol levels were detected between the 3 and 6 months old wild-type and APP/PS1 animals. A significant increase in blood triglycerides levels ($p < 0.05$) was observed in 6 months-old APP/PS1 mice, compared to respective controls (Fig.1A). As expected, Aβ plaque deposits were present in the hippocampal and cortical regions of 6 months-old APP/PS1 mice (Fig.1B).

Identification of differentially expressed genes related to food intake and obesity in the hippocampus

We did not detect significant differences in the mRNA expression of leptin, however a significant down regulation of the leptin receptor (OB-R), both at the mRNA (Fig. 2a) and protein (Fig. 2b) levels, was observed in the hippocampi of 6 months-old APP/PS1 mice compared to wild-type littermates. In contrast, we detected a significant reduction in the levels of the prolactin receptor (PRL-R), which is involved in the regulation of energy metabolism, already at 3 months of age in APP/PS1 brains (Fig. 2). The changes observed in the above mentioned molecules were related to the APP/PS1 phenotype (rather than age), as we did not detect statistically significant alterations when comparing 3- and 6- months-old APP/PS1 animals.

We determined mRNA expression profiles of neurotrophic factors and related receptors which play a role in food-intake regulation, including brain derived neurotrophic factor (bdnf), glucagon-like peptide 1 receptor (glp1r), insulin-like growth factor 1 (igf1), nerve groqth factor (ngf), and Neuropeptide Y (npy) (33-37) (Fig. 3). Of these, only npy was significantly downregulated in the hippocampi of the APP/PS1 mice, both at 3 and 6 months of age, when compared to wild-type littermates. Interestingly, there was a tendency towards the downregulation of the glp1r in 3 months-old animals, although this reduction did not reach statistical significance. Apart from the regulation of the food intake, GLP1 signalling has been implicated in the regulation of glucose metabolism, memory formation and may have neuroprotective effects against excitotoxic insults (33, 34).

Corticotropin-releasing hormone (CRH) is highly expressed in paraventricular nucleus neurons and is involved in the regulation of food-intake and body weight in rats (38, 39). It may also play a role in cognition and has been linked to neuroprotection in response to stress in the hippocampus (40). Semi-quantitative RT-PCR analysis showed a significant decrease in crh in hippocampi of 6 months-old APP/PS1 mice, compared to controls. We did not find significant changes in

Figure 2

mRNA expression profile (n=5-8) (A) and representative immunoblot images and quantification (n=5-8) (B) of the leptin and prolactin hormones and their respective receptors in hippocampal extracts of 3 and 6 months-old wild-type and APP/PS1 mice. Immunoblot images are normalized to beta actin. (Statistical analysis was performed with one-way ANOVA, with Tukey's post-hoc test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, and with the student's t-test, where $ denotes $p < 0.05$)

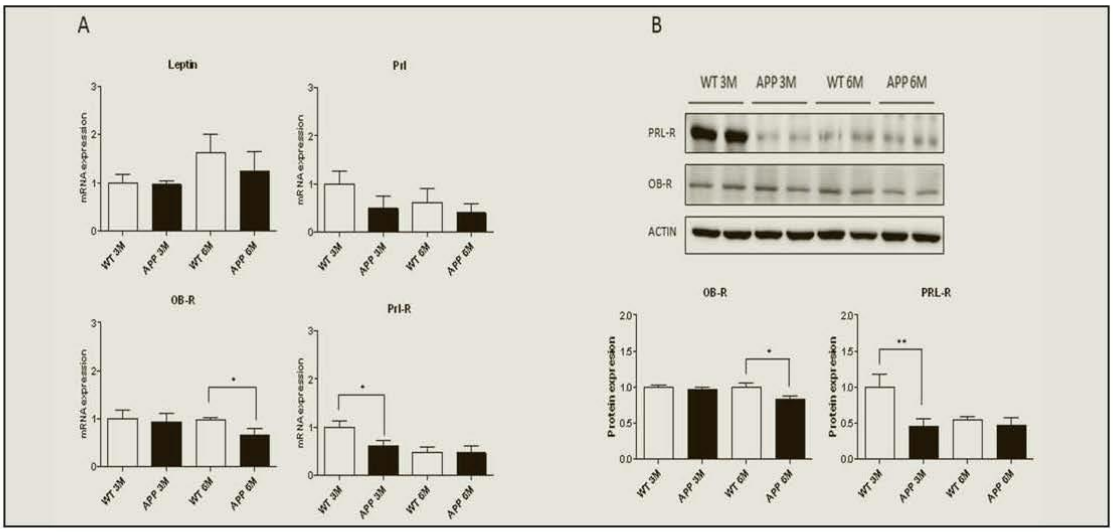

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Table 1
Probes and primers used for semi-quantitative RT-PCR. Gene ID# corresponds to the official classification of the Entrez Gene database (NCBI)

Name	Gene	ID	TaqMan Probe	Primers pairs (5'→3')
Actb	actin, beta	11461	Mm00607939_s1	
Apoc1	apolipoprotein C-I	11812	Mm00431816_m1	
Apoe	Apolipoprotein E	11816	Mm01307193_g1	
Bdnf	brain derived neurotrophic factor	12064	Mm04230607_s1	
Crh	corticotropin releasing hormone	12918	Mm01293920_s1	
Cyp46a1	cytochrome P450, family 46, subfamily a, polypeptide 1	13116	Mm00487306_m1	
Gapdh	glyceraldehyde-3-phosphate dehydrogenase		14433	Mm99999915_g1
Glp1r	glucagon-like peptide 1 receptor	14652	Mm00445292_m1	
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	15357	Mm01282499_m1	
Igf1	insulin-like growth factor 1	16000	Mm01228180_m1	
K-Ras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	16653		F:AGACACGAAACAGGCTCAGGAGT, R:AGGCATCGTCAACACCCTGTCTT
Ldl-R	low density lipoprotein receptor	16835	Mm00440169_m	
Lep	leptin	16846		F:CTCCAAGGTTGTCCAGGGTT, R:AAAACTCCCCACAGAATGGG
Lrp1	low density lipoprotein receptor-related protein 1	16971	Mm00464608_m1	
Ngf	nerve growth factor	18049	Mm00443039_m	
Npy	neuropeptide Y	109648		F:CTCCGCTCTGCGACACTACA, R:AATCAGTGTCTCAGGGCTGGA
OB-R	leptin receptor	16847		F:CTGCACTTAACCTGGCATATCCA, R:GGCTCCAGCAGGTGAGAGAA
Pomc	pro-opiomelanocortin-alpha	18976	Mm00435874_m1	
Prl	prolactin	19109		F:GTATGTGCAAGACCGTGAGT, R:AGGGACTTTCAGGGCTTGTT
Prl-R	prolactin receptor	19116		F:ATCTGTGGGTAAAATGGTTGCC, R:GTTTGATGACCTGTGAAGTGGA
Socs1	suppressor of cytokine signaling 1	12703	Mm00782550_s	
Socs2	suppressor of cytokine signaling 2	216233	Mm00850544_g1	
Socs3	suppressor of cytokine signaling 3	12702	Mm00545913_s1	
Sos1	son of sevenless homolog 1	20662		F:TCCCCTAAAATCTCCTGGTGTTCGT, R:AGATGCTGTGCTTTCCGTCTCACT
Srebf1	sterol regulatory element binding transcription factor 1	20787	Mm00550338_m1	
Stat1	signal transducer and activator of transcription 1	20846	Mm00439531_m1	
Stat3	signal transducer and activator of transcription 3	20848	Mm01219775_m1	
Stat5a	signal transducer and activator of transcription 5A	20850	Mm03053818_s1	
Stat5b	signal transducer and activator of transcription 5B	20851	Mm00839889_m1	
TBP	TATA box binding protein	21374		F: ACCCTTCACCAATGACTCCTATG, R: TGACTGCAGCAAATCGCTTGG

the mRNA expression of a polypeptide hormone precursor pro-opiomelanocortin (pomc), mutations of which had been previously linked to the development of childhood obesity (Fig. 3) (41).

Leptin and prolactin signalling pathways

Because we detected significant alterations in the hippocampal expression of Prl-R at 3 months of age and of OB-R at 6 months of age in APP/PS1 animals, compared to controls, we proceeded to study the expression profiles of the downstream signalling molecules related to the physiological functions of both receptors.

It has been shown that the prolactin receptor activates Signal Transducer and Activator of Transcription (STAT-1), (STAT-3) and (STAT-5) (20). We did not detect any changes in the expression of stat-1 and stat-3, stat5a and k-ras, however mRNA levels of the 2b isoform of STAT-5 (stat5b) were significantly downregulated in the hippocampus of 3 months-

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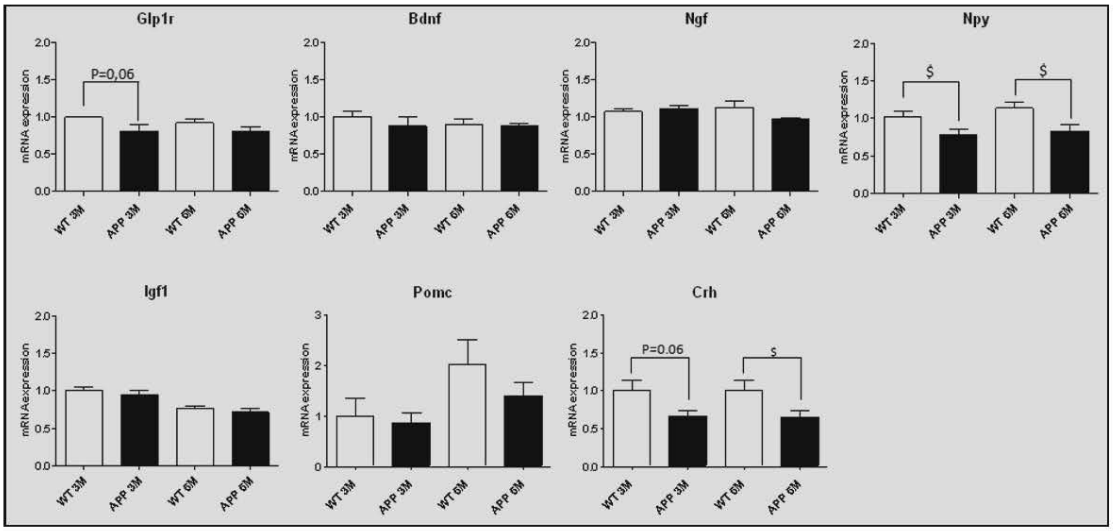
Figure 3

mRNA expression profile (n=5-8) of glp1r, bdnf, ngf, npy, igf1, pomc and crh in hippocampal extracts of 3 and 6 months-old wild-type and APP/PS1 mice. (Statistical analysis was performed with the student's t-test, where $ denotes $p < 0.05$)



old APP/PS1 animals, versus wild-type animals (Fig. 4).

In addition, we identified a significant downregulation of the Son of Sevenless homologue 1 (SOS1) in 6 months-old APP/PS1 mice, compared to wild-type, which is another molecule downstream of the prolactin receptor (23, 24, 42). Moreover, suppressors of cytokine signaling (socs1), (socs2) and (socs3) were also downregulated at 3 months of age in APP/PS1 animals, with the mRNA expression levels of socs2 being just short of reaching statistical significance ($p = 0.06$) (Fig. 4). Taken together, our results clearly show a significative impairment in the adipokine receptors-related signalling pathways.

Changes in the transcripts involved in disorders of lipid metabolism in the early stages of amyloidogenesis

Impairment in cholesterol metabolism and biosynthesis, which may lead to neuronal damage, is thought to be a contributing factor to AD progression (43). We have detected a significant increase in the mRNA levels of the Low Density Lipoprotein receptor (Ldl-r) and 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase (hmgcr) transcripts in the hippocampi of the APP/PS1 mice at 6 months of age, compared to the control group (Fig. 5). In addition, in our study a decrease in lrp1 mRNA levels in 3 months-old APP/PS1 brains was found, which did not correlate to apoe and apoc1 mRNA levels, where no changes were detected (Fig. 5). Both apoE and Apolipoprotein C1 (apoc1) genes have been implicated in the development of sporadic AD (42, 43). Our data also indicate a significant downregulation in the mRNA of the cholesterol 24-hydroxylase enzyme (cyp46a1) that converts cholesterol to 24S-hydroxycholesterol, in the 3 months-old APP/PS1 mice.

Discussion

Recent evidence indicates that metabolic deficiencies may contribute to AD development and progression (43-50). Pathological changes in glucose and cholesterol metabolism, adipose tissue signalling, as well as in food-intake controlling neuropeptides have all been implicated (45, 46). At a peripheral level, we observed a significant increase in trygliceride levels in blood of 6 months-old APP/PS1 mice, when compared to wild-type littermates. Neither the peripheral cholesterol levels, nor the body weight were affected. At the CNS level, the role of feeding-regulatory peptides in the hypothalamus is well known, but their function in the hippocampus is less clear (31-33). As the hippocampus is involved in the processes of learning and memory formation, it has been proposed that these neuropeptides may take part in memory-related processes, and may also participate in neuroprotection (32).

As one of the main purposes of our study was to examine potential changes at the central nervous system (CNS) level, we investigated gene expression profile of molecules related to adipokine, neuropeptide and cholesterol signalling in 3-6 months-old APP/PS1 transgenic animals.

It is becoming apparent that neuroendocrine hormones including oxytocin, progesterone and prolactin, apart from their roles in lactation, may also have neuroprotective effects on hippocampal neurons (45). Neuroprotective properties of PRL

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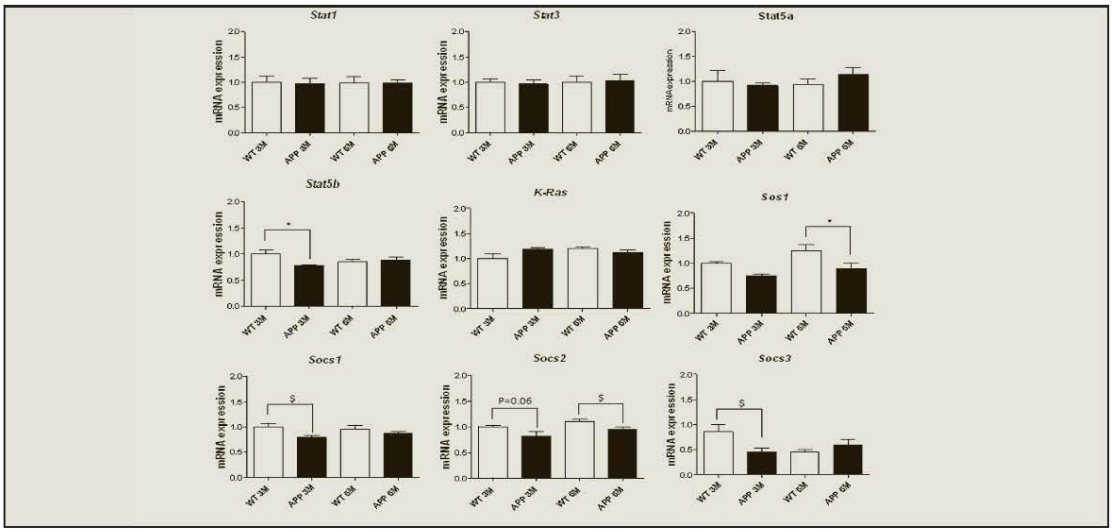
Figure 4

mRNA expression profile (n=5-8) of stat1, stat3, stat5a, stat5b, K-ras, sos1, socs1, socs2 and socs3 in hippocampal extracts of 3 and 6 months-old wild-type and APP/PS1 mice. (Statistical analysis was performed with one-way ANOVA, with Tukey's post-hoc test, where * denotes $p < 0.05$, and with the student's t-test, where $ denotes $p < 0.05$)



are demonstrated in a Kainic acid (KA)-induced rat model of epilepsy, where the administration of PRL to ovariectomised rats significantly reduces seizures and KA-neurotoxicity in the hippocampus (51). Prolactin is also involved in immune regulation (52). In the brain Prl-Rs, which belong to the class I cytokine receptor superfamily, were detected in cortex, hypothalamus and hippocampus, and in astrocytes and glial cells (53, 54). This is noteworthy, as recent data suggest that prolactin may be a possible marker for obesity in humans (54) In the current study we observed a significant downregulation of the PRL-R mRNA and protein in the hippocampi of the 3 months-old APP/PS1 mice, when compared to a wild-type control group, indicating early perturbations in this particular biological route, at an age when both cognitive impairments and Aβ deposits have yet to develop.

Leptin is an adipostatic hormone with a range of effects at the CNS level, and the distribution of the OB-R in the human brain is wide. In the hypothalamus, leptin signalling plays a prominent role in food-intake regulation (22-24). In the hippocampus, leptin has been implicated in the processes of learning and memory, neuroprotection, as well as synaptic plasticity (54). Leptin signalling alterations have recently been described in human patients with AD (55). In that study, authors detected a significant increase in leptin levels both in the cerebro-spinal fluid (CSF) and in the hippocampus of AD patients, compared to age-matched controls. Interestingly, an increase in circulating hormone levels was accompanied by a reduction in OB-R mRNA levels and the localization of the receptors to neurofibrillary tangles, hinting at the possibility of leptin resistance (55). In our study, we did not detect significant changes in the mRNA levels of leptin itself, however we observed a significant reduction in OB-R mRNA and protein in the hippocampus of 6 months-old APP/PS1 mice, compared to wild-type controls. On the other hand, targeted leptin delivery may in fact lead to a reduction in Aβ-load and to an improvement in the symptoms of AD-like pathologies, as described by Pérez-González et al., (2014). Authors demonstrated that the 3 months-long intra-cerebroventricular administration of a lentiviral vector expressing leptin protein resulted in the improvement of memory functions and a reduction in Aβ levels in APP/PS1 mice, compared to untreated controls (29).

Janus kinase (JAK)/STAT signalling pathways are among the principal transcriptional regulators in most cell types (56). Within the CNS, JAK/STAT signalling is activated in response to cytokines, growth factors and hormones, including leptin and prolactin (57-59). Perturbations in JAK/STAT signalling have been implicated in the processes of synaptic plasticity, neuroinflammation and the survival of both glia and neurons (59). Among the eight members of the STAT family of transcription factors, four have been identified as PRL-R transducer proteins: STAT1, STAT3, STAT5a and STAT5b. Upon activation of the PRL-R by JAK (which promotes dimerization, phosphorylation and the activation of the prolactin receptor), STATs are recruited, dimerized and phosphorylated, which results in nuclear translocation

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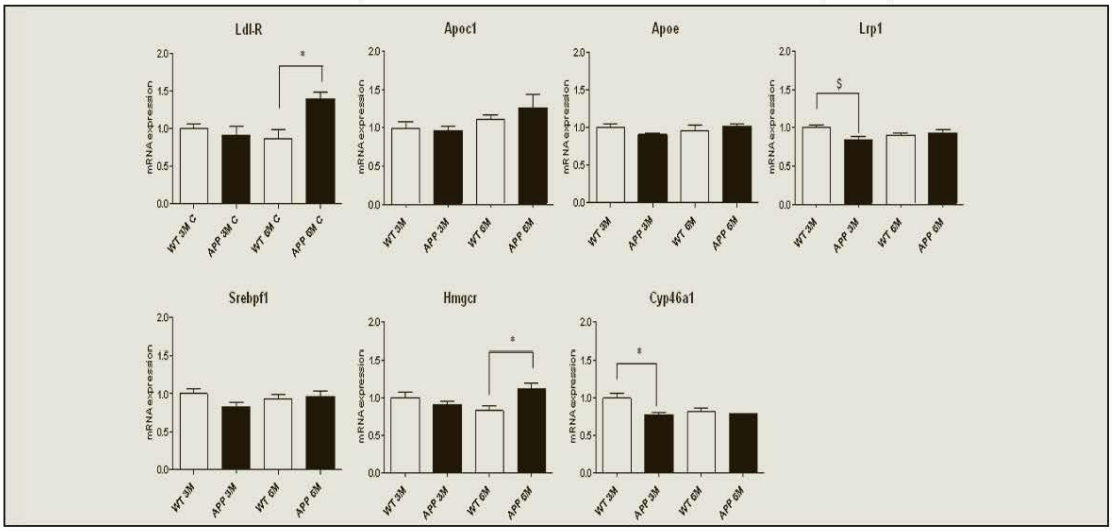
Figure 5

mRNA expression profile (n=5-8) of ldlr, apoc1, apoe, lrp1, srebp1c, hmgcr and cyp46a1 in hippocampal extracts of 3 and 6 months-old wild-type and APP/PS1 mice. (Statistical analysis was performed with one-way ANOVA, with Tukey's post-hoc test, where * denotes $p < 0.05$, and with the student's t-test, where $ denotes $p < 0.05$)



of activated STATs, where they stimulate the transcription of the respective target genes (57). Moreover, three different classes of negative regulators of PRL-R have been identified, including protein tyrosine phosphatases (PTP), such as SH2 domain containing phosphatase-1 (SHP-1), protein inhibitors of activated STATs (PIAS) and suppressors of cytokine signalling (SOCS) proteins (59). Their expression is induced by PRL itself, constituting an auto-negative feedback loop. SOCSs (1 and 3 in particular) bind to the PRL-R or to JAKs and prevent the recruitment of JAKs, and thus the phosphorylation of STATs (57). In our model, we had detected a significant reduction in stat5b, socs1, socs2 (this being just short of reaching statistical significance with a p=0.06) and socs3 mRNA in the hippocampus of 3 months-old APP/PS1 animals, when compared to age-matched wild-type littermates. Interestingly, this reduction occurred at an early age, prior to the Aβ plaque formation. Thus, we demonstrate for the first time to our knowledge, a dysregulation of prolactin signalling, together with the alterations in the JAK/STAT pathways in the hippocampi of 3 months-old APP/PS1 animals.

The processes of learning and memory in the hippocampus are also regulated by NPY signalling (35-37). Several studies have reported reduced levels of NPY and its receptor densities in the brains of AD patients, as well as in rodent models of the disease (37). Moreover, a reduction in NPY levels had been detected in the plasma and the CSF of AD patients, compared to healthy controls (60). In a transgenic mouse model overexpessing APP, a decrease in NPY levels was observed in the hippocampus, while exogenous administration of NPY produced a neuroprotective effect (61, 62). Our data is in agreement with the above mentioned research, as we had detected a significant downregulation of NPY mRNA in the hippocampi of APP/PS1 animals, both at 3 and 6 months of age, when compared to the respective controls.

Moreover, we found a decrease in the mRNA transcripts of the crh in the hippocampus of 6 months-old APP/PS1 mice, compared to the control group. Interestingly, CRH protein has neuroprotective properties and also regulates APP processing, both via the CRH1 receptor activation (39). Previous studies suggest that the reduction of CRH signalling in the brains of AD patients and a decrease in CRH-levels in CSF could be a possible marker of the disease (39-40).

A number of studies have indicated that neurotrophins NGF and BDNF may promote survival and differentiation of neuronal cells in the CNS (62, 63). In the present study we did not find significant differences in either of the transcripts.

Since it is hypothesised that deregulation of cholesterol homeostasis could contribute to neurodegenerative diseases progression by provoking neuronal loss, as well as the formation of neuritic plaques and neurofibrillary tangles, we decided to focus on hippocampal expression of genes related to cholesterol metabolism (60-63. An increase in brain cholesterol levels augments Aβ deposition, which can regulate cholesterol homeostasis by itself, as demonstrated by Barbero-Camps et al., (2013). In a triple transgenic APP/PS1/SREBP-2 mice, which overexpress SREBP-2 in combination with APP/PS1 mutations,

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authors detected an increase in mitochondrial cholesterol levels as well as an accelerated β-secretase activation and Aβ accumulation, when compared to age-matched APP/PS1 double transgenic controls (16). In our study, although we did not observe changes in blood cholesterol levels, we did detect a significant increase in LDLr mRNA in 6 months-old APP/PS1 animals, compared to wild-type littermates. The synthesis of LDLr in the cell is regulated by the levels of free intracellular cholesterol (65). These data is probably correlated with an increase in the levels of HMG-CoA reductase (hmgcr) in the hippocampus of APP/PS1 animals. HMG-CoA reductase is a rate-limiting enzyme involved in the cholesterol biosynthesis and it is affected by statins and cholesterol synthesis-inhibiting drugs, treatment with which correlates negatively with the incidence of AD (46-49). Besides, cholesterol is imported into the neurons by apoE, via LRP1 receptors on the cell surface, mRNA levels of which were significantly reduced in our model already at 3 months of age, compared to wild-type controls (67-69).

Significantly, cholesterol does not readily pass the blood-brain barrier (BBB), necessitating prior metabolism of the cholesterol molecule for the successful elimination of excess brain cholesterol (65). Cholesterol 24S-hydroxylase (CYP46A1) is an enzyme that converts cholesterol into the 24S-hydroxycholesterol (24OHC), one of the principal cholesterol metabolites in the brain, which easily crosses the BBB (65-69). A decrease in the brain 24OHC levels may thus be an indirect marker of elevated brain cholesterol levels. In our study, we have detected a significant reduction in the mRNA of cyp46a1 in the hippocampus of 3 months-old APP/PS1 mice, compared to controls. This is in accordance with previous reports indicating decreased hippocampal and plasma 24OHC levels in AD patients (67-69). Taken together, our results suggest that alterations in brain cholesterol metabolism pathways may be associated with the early signs of AD-like symptoms in an APP/PS1 mouse model.

In summary, we have detected disturbances in intracellular signalling pathways downstream of prolactin and leptin receptors, JAK/STAT signalling, as well as abnormalities in cholesterol metabolism in the hippocampi of APP/PS1 double transgenic mice between the 3 and 6 months of age. Significantly, a number of phenotypic alterations were observed in 3 months-old transgenic mice, at an age prior to the appearance of AD-like symptoms. The data presented here reinforces the hypothesis that AD may indeed be considered as a brain-type metabolic disorder.

Conflicts of interest: Authors declare no conflicts of interest

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