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BIOMOLECULAR STUDIES IN ALZHEIMER'S DISEASE MODELS: INVESTIGATIONS *IN VITRO* AND *IN VIVO*

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ABSTRACT

The Alzheimer's disease (AD), the most prevalent form of age-related dementia, is a multifactorial and heterogeneous neurodegenerative disease. The molecular mechanisms underlying the pathogenesis of AD are yet largely unknown. However, the etiopathogenesis of AD likely resides in the interaction between genetic and environmental risk factors. Among the different factors that contribute to the pathogenesis of AD, amyloid-beta peptides and the genetic risk factor apoE4 are prominent on the basis of genetic evidence and experimental data. ApoE4 transgenic mice have deficits in spatial learning and memory associated with inflammation and brain atrophy. Evidences suggest that apoE4 is implicated in amyloid-beta accumulation, imbalance of cellular antioxidant system and in apoptotic phenomena. The mechanisms by which apoE4 interacts with other AD risk factors leading to an increased susceptibility to the dementia are still unknown. The aim of this research was to provide new insights into molecular mechanisms of AD neurodegeneration, investigating the effect of amyloid-beta peptides and apoE4 genotype on the modulation of genes and proteins differently involved in cellular processes related to aging and oxidative balance such as PIN1, SIRT1, PSEN1, BDNF, TRX1 and GRX1. In particular, we used human neuroblastoma cells exposed to amyloid-beta or apoE3 and apoE4 proteins at different time-points, and selected brain regions of human apoE3 and apoE4 targeted replacement mice, as *in vitro* and *in vivo* models, respectively. All genes and proteins studied in the present investigation are modulated by amyloid-beta and apoE4 in different ways, suggesting their involvement in the neurodegenerative mechanisms underlying the AD. Finally, these proteins might represent novel potential diagnostic and therapeutic targets in AD.

1. INTRODUCTION

1.1 ALZHEIMER'S DISEASE

The Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by a progressive mental decline; it is the most prevalent form of age-related dementia in the modern society (*Duff and Suleman, 2004*). Current estimates indicate that there are about 25-30 million people suffering from AD in the World, and the number of cases will double during the next twenty years (*Ferri et al., 2005*). Therefore, with increasing life expectancy, dementia is a growing socio-economic and medical problem, and AD represents an important clinical challenge in terms of diagnosis and treatment. Up to now, since first clinical symptoms of AD overlap with other diseases of the central nervous system (CNS), a definitive diagnosis is uncertain and it can only be done with *post-mortem* histopathological examination of the brain. However, a relative clinical diagnosis based on physical, neurological and psychological evaluations, laboratory tests and neuroimaging, can be made with a noteworthy accuracy.

A recent systematic review of epidemiological studies estimates that AD is the fifth cause of death in elderly population, leading to physical disability more than cardiovascular diseases, stroke and cancer (*WHO report, 2003*). Between 2000 and 2008, deaths attributed to AD increased around 66%, and 5% to 15% of all deaths in people aged ≥65 years can be ascribed to AD (*Miniño et al., 2011*).

The first clinical phase of the AD is characterized by an impairment of the episodic memory that compromises the ability to recall past experiences: the patients develop symptoms such as difficulty to learn new informations or to remember previously learned ones (*Ballard et al., 2011*). This occurs since the functional neuronal degeneration usually begins in brain regions involved in forming new memories, especially hippocampus and entorhinal cortex, and in areas of cerebral cortex in the frontal lobe implicated in thinking and planning. Apathy and depression are also often early symptoms of AD. Later

symptoms include impaired judgment, disorientation, confusion, behavior changes, and difficulty speaking, swallowing, and walking. (**Figure 1**).

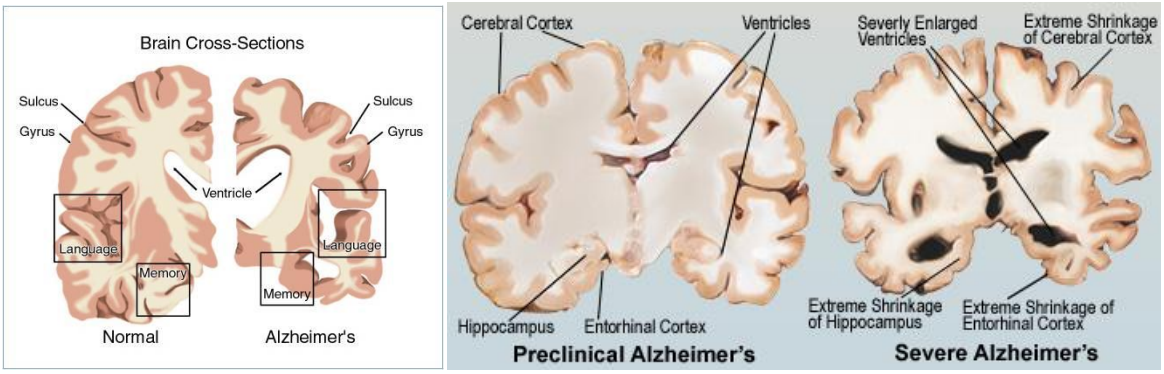


Figure 1. Pathological brain modification during stages of AD progression.

The progression of AD is slow, insidious and implacable, leading the patient to a mental and physical disability. Several studies indicate that people survive an average of 4 to 8 years after a diagnosis of AD, but others can live as long as 20 years with this neurodegenerative disease. In fact, severe dementia frequently causes complications such as immobility, swallowing disorders and malnutrition, increasing the risk of developing pneumonia which is the most common cause of death among people with AD (*Brunnstrom et al., 2009*).

Although some palliative treatments alleviating the AD symptoms are available, no effective strategies currently exist to inhibit the progression of the disease. The standard medical treatments used in the clinical practice act modulating neurotransmitters such as acetylcholine or glutamate; they include acetylcholinesterase inhibitors and *N*-methyl-D-aspartate (NMDA) antagonists (*Winslow et al, 2011*). Antipsychotic drugs are used to treat secondary symptoms of AD, like depression, agitation, irritability and sleep disorders (*Madhusoodanan et al., 2007*).

Currently, the stages of AD are often described as early, moderate, or severe stage. In 2011, the National Institute on Aging (NIA) and the Alzheimer's Association recommended new diagnostic criteria for AD (*Jack CR et al., 2011*). These new criteria propose that the disease begins as preclinical AD, before the early stage. In this preclinical stage, individuals have measurable earliest signs of the disease in brain, cerebrospinal fluid, and/or blood (biomarkers), but without symptoms such as memory loss. This preclinical stage suggests that AD begins several years before symptoms develop. The second stage is mild cognitive impairment (MCI), in which individuals have mild, but measurable, changes in thinking performances without affect the ability to carry out everyday activities. The last stage is the dementia due to AD, characterized by memory, thinking, and behavioral symptoms, encompassing all AD-related processes.

In particular, MCI is defined as a transitional stage between normal aging and dementia. MCI has a complex etiology and, even if may present several symptoms, clinically reflects memory and cognitive impairment with preservation of functional abilities and no evidence of dementia (*Morris JC et al., 2001*). When memory loss is predominant, MCI is termed "amnestic MCI" and it is commonly considered as a prodromal state of AD. MCI is recognized as potential risk factor of AD development, and, since MCI links and overlaps normal aging with AD, the clinical diagnosis is a challenge. Moreover, in some cases MCI can be reverted to normal cognition, it can remain stable or can be actually considered as an early stage of AD or another dementia.

AD is divided into two subtypes based on the age of onset of the disease: early onset AD (EOAD) and late-onset AD (LOAD). EOAD accounts for about 1-6% of all clinical cases and ranges approximately with onset from 30-60 or 65 years; but the most common form of AD is LOAD, with an age of onset over 60 or 65 years. Both types may occur in people with a positive family history of AD. Approximately 13% of AD cases are an autosomal dominant heritage with at least three generations affected (*Bekris et al, 2010*). With the exception of few familial autosomal dominant forms of AD resulting from a single-gene disorder, most AD cases belong to a heterogeneous sporadic disease that involves the

complex interaction of aging, multiple gene susceptibility and environmental risk factors (*Alonso Vilatela et al., 2012).* Age represent the main risk factors for developing AD, along with the poor education, low mental ability, traumatic brain injury, stroke and cardiovascular disease risk factors (eg. physical inactivity, high cholesterol, diabetes, smoking, and obesity); a history of depression may also predispose to AD. However, the combination of genetic profile with several environmental factors seems to have the major role in the increased risk of AD onset (*Alzheimer's Association, 2012*).

The neuropathogenic process of AD probably starts decades before the clinical onset of the disease becomes apparent; during this period a gradual but inexorable neuronal loss occurs, with brain atrophy and synaptic detriment. The principal pathological hallmarks of AD are abundant extracellular senile plaques of beta-amyloid peptide (SP) in cerebral blood vessels and brain parenchyma, deriving from the cleavage of amyloid precursor protein (APP), and intraneuronal neurofibrillary tangles (NFTs), resulting from aggregation of tau microtubule-associated protein. (**Figure 2**). Although SP and NFT deposition in the brain parenchyma is characteristic, the presence of these lesions is not sufficient to support the diagnosis of AD since these features also occur in brains of cognitively intact people (*Ballard et al., 2011*). Moreover, the elucidation of AD pathological mechanism and the identification of additional specific biomarkers are needed to improve the accuracy for an early diagnosis, to distinguish AD from MCI and other dementia forms, and to allow the discovery of new pharmacological targets and effective therapies for this disabling disease.

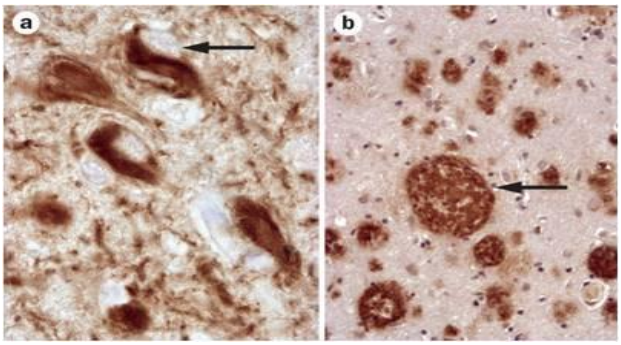


Figure 2. Neurofibrillary tangles (**a**) and senile plaques (**b**) in the AD brain parenchyma.

1.2 ALZHEIMER'S DISEASE NEUROPATHOLOGY

Although the etiology of AD is not yet completely known, it is accepted that the disease, like other chronic diseases, is the result of multiple factors. However, two pathological hallmarks characterize the earlier stages of the disease, the deposit of the protein beta-amyloid (Aβ) outside neurons to form the SP, and the abnormal accumulation of the protein tau inside neurons to constitute the NTFs. The Aβ and NTFs deposition is supposed to interfere with the neuron-to-neuron communication at synaptic level and to impair the transport of nutrients and other essential molecules throughout the cells, contributing to neuronal death. Further neuropathological features include a massive synaptic neuronal loss, leading to cortical and hippocampal atrophy, the degeneration of cholinergic basal forebrain neurons and enlarged ventricles. The SP and the NTFs have a different distribution through the brain; the deposition of NTFs first starts in the medial temporal lobe at level of hippocampus and entorhinal cortex, which is near the hippocampus and directly connected to it, then spanning through other brains regions such as limbic areas and at last to the cortical association areas and the primary cortex (*Braak and Braak, 1991*).

The develop of SP begins in the orbitofrontal and temporal cortices, and the spread of this neuronal damage continues to parietal cortex and throughout the neocortex, usually with the exclusion of the cerebellum. The first clinical symptoms of these lesions are short-term memory problems, which reflect the early involvement of the hippocampus, the structure essential to the formation of short-term and long-term memory and involved in processing of sensory information. The memory deficit later develops into difficulties with executive functions mainly controlled by the prefrontal cortex, the connected cortical and subcortical brain structures, which include planning and initiation of actions, as well as emotional disturbances and apathy. Moreover, during the AD progression, there is a selective loss of cholinergic neurons with the reduction of acetylcholine levels in brain areas involved in Aβ deposition, particularly in the cortex and hippocampus, resulting in the impairment of daily living activities, behaviour and cognition. Although the mechanism underling the degeneration of these neurons is still unknown, evidences suggest also that the excessive

stimulation of glutamate receptors, and in particular the NMDA receptor, associated to a chronic neuroinflammation, may contribute to massive neuronal death (*Wenk, 2003*).

1.2.1 ALZHEIMER'S DISEASE PATHOGENESIS: AMYLOID *VERSUS* TAU HYPOTHESIS

The presence of extracellular beta-amyloid plaques in the brain is a central event in the etiology of AD. Beta-amyloid (Aβ) protein is a peptide of 39-43 amino acids able to form β-sheets structures and fibrillar aggregates. It derives from the sequential proteolytic cleavage of the large transmembrane polypeptide APP involving multiple enzymes (*Perl, 2010*). The primary function of APP is unknown, but it is believed to have a role during neuronal development and trafficking, to be implicated in synaptic formation and repair, transmembrane signal transduction and cell adhesion (*Walsh et al., 2007*). The human *APP* gene is located on chromosome 21 with three main isoforms generated by alternative splicing of exons 7, 8 and 15: *APP*770, *APP*751, and *APP*695, reflecting the number of amino acids encoded and all including the full-length Aβ peptide (*Goate et al. 1991*). *APP*751 and *APP*770 contain the Kunitz Protease Inhibitor (KPI) domain, a 57 amino acid insert within their extracellular region and they are expressed in several tissues; *APP*695, instead, lacks of the KPI domain and it is predominantly expressed in neurons (*Rohan de Silva et al., 1997*). It has been shown that adult rat brains display higher relative amounts of KPI-encoding *APP* isoforms than early post-natal rats, suggesting that specific age-associated regulation pattern of *APP* gene is implicated in the AD development (*Sandbrink et al, 1994*). Moreover, AD brain presents elevated levels of the protein and mRNA of KPI-containing *APP* isoforms and, conversely, reduced levels of KPI lacking *APP* isoform (*Menendez-Gonzalez et al., 2005*). A prolonged activation of NMDA receptors in neuronal cells also seems to increase the expression of KPI containing *APP* isoforms, suggesting that alterations in the *APP* mRNA splicing can be associated with an increased Aβ deposition and contribute to AD pathogenesis (*Bordji et al., 2010*). However, the

mechanism of the direct association between different splicing forms of APP and AD pathology is still to be elucidated.

APP is a type 1 integral cell surface membrane protein that resembles a signal transduction receptor. It is synthesized in the endoplasmic reticulum, modified in the Golgi apparatus, and transported to the cell surface through the secretory pathway. The APP is also endocytosed from the cell surface and metabolized in the endosomal/lysosomal pathway. APP can undergo two distinct cleavage pathways by the enzymes α-secretase and β-secretase (also called β-site amyloid precursor protein-cleaving enzyme, BACE), both active in normal cellular metabolism (*Selkoe, 2001*). The predominant cleavage of APP is in the N-terminal portion close to the plasma membrane and within the Aβ peptide region, and is mediated by α-secretase. This proteolytic process is also called non-amyloidogenic since prevents Aβ formation and produces the neuroprotective soluble APP α fragment (sAPPα) released in the extracellular space. The sAPPα, in fact, plays a role in adult neurogenesis, has neurotrophic effects and is involved in early memory processes (*Wang et al., 2004; Bour et al., 2004*). The α-Secretase activity is mediated by one or more enzymes from the family of disintegrin and metalloproteinase domain proteins (ADAM).

The cleavage of APP leading to Aβ generation, also called amyloidogenic pathway, is sequentially performed by β-secretase and γ-secretase, an enzymatic complex made up of presenilin 1 (PSEN1), presenilin 2 (PSEN2) and nicastrin. Firstly, β-secretase cleaves APP extracellularly producing the soluble β APP (sAPPβ) N-terminal fragment, that lacks most of the neuroprotective effects associated with sAPPα, and a membrane bound C-terminal fragment termed CTF99. The C-terminal fragment deriving from α-secretase cleavage is called CTF83. γ-Secretase proteolysis of CTF83 and CTF99 will result in the generation of p3 and Aβ, respectively, as well as the APP intracellular domain (AICD). The AICD fragment has been implicated in the modulation of several cellular processes such as intracellular trafficking, cytoskeletal dynamics, calcium and ATP levels, and also in the regulation of Aβ levels through the regulation of neprilysin, one of the main Aβ degrading enzymes (*Wang et al., 2010*). (**Figure 3**).

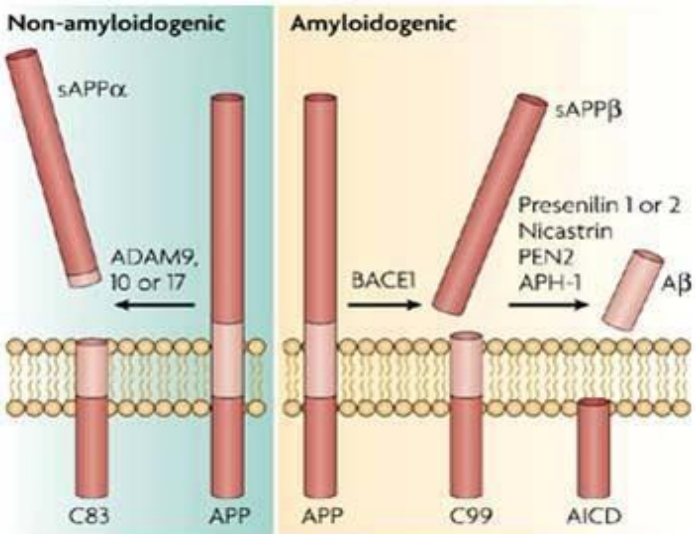


Figure 3. Non-amyloidogenic and amyloidogenic APP cleavage.

It is important to notice that β- and γ-secretases are not only implicated in the cleavage of Aβ peptide but also in the proteolytic processing of a wide range of substrates, involving in several cellular activities.

The Aβ peptide generated from the amyloidogenic pathway can then aggregate and determine neurotoxic effects; alternatively, it can be degraded by different enzymes such as neprilysin, insulin degrading enzyme or endothelin converting enzyme (*Turner et al., 2004*). It still unclear why Aβ aggregates, but the sequence, the levels of the protein and the conditions that can destabilise the Aβ structure are considered important factors in the amyloid plaques deposition (*Ballard et al., 2011*).

Although Aβ has a potential role in the pathogenesis of AD, it has been shown that it also plays a physiologic role in the CNS (*Pearson and Peers, 2006*). Moreover, low levels of Aβ peptide have been shown in the cerebrospinal fluid (CSF) of individuals without signs of dementia (*Selkoe and Schenk, 2003*). It has been demonstrated that low concentrations of Aβ enhance synaptic plasticity and memory, with a positive modulatory role on the neurotransmission (*Puzzo et al., 2012*). This positive role of Aβ in the normal physiological function of cells complicates therapeutic strategies direct to reduce Aβ levels in the AD.

The AD brain displays two forms of amyloid plaques: neuritic or senile plaques, and diffuse plaques. The neuritic plaques are extracellular deposits of fibrillar Aβ containing activated microglia cells within the central amyloid core and they are surrounded by reactive astrocytes. The plaques can also be diffused, without a compacted core and neuritic dystrophy; it has been suggested that these kinds of plaques are immature precursors of the neuritic plaques. The activation of microglia by fibrillar Aβ is a very early phenomenon in the AD pathogenesis, whereas the localization of astrocytes at the neuritic plaques occurs later, when the dementia is already developing. Microglia is involved in the clearance of Aβ by phagocytosis, but can also generate toxic products, such as reactive oxygen species (ROS) and pro-inflammatory cytokines, that contribute to neurodegeneration (*Eikelenboom and van Gool, 2004*). It is still unknown if amyloid-beta plaques themselves cause AD or if they are a by-product of the AD processes.

One of the most accepted theories proposed twenty years ago to elucidate the pathogenesis of AD is the "amyloid hypothesis", stating that Aβ deposition plays a central role in the etiology of the disease. According to this theory, the chronic imbalance between production and clearance of Aβ leads to synaptic dysfunction, tau pathology, glial activation and eventually neuronal loss in selected brain areas (*Hardy and Allsop, 1991*). In fact, several studies demonstrated that the overproduction/aggregation of Aβ in the brain can be one of primary causes of AD features. Two main Aβ toxic species are produced from the amyloidogenic proteolysis of APP: Aβ40 and Aβ42, the Aβ42 being less abundant but more hydrophobic and prone than Aβ40 to the fibrils formation (*Näslund et al., 2000*). It has been demonstrated that the familial AD (FAD) - linked mutations of *PSEN1*, *PSEN2* and *APP* causes AD by increasing the extracellular concentrations of Aβ42, thereby promoting its deposition on diffuse plaques in the earliest stage of the disease (*Iwatsubo et al., 1994; Scheuner et al., 1996*). The amyloid hypothesis sustains that missense mutations in *APP*, *PSEN1* or *PSEN2* genes lead to an increased Aβ42 production and aggregation, forming diffuse plaques widespread in the brain parenchyma. Aβ oligomers induce a toxic effect directly on synapses and participate to the activation of microglia and astrocytes, increasing the release of inflammatory mediators and leading to a

progressive synaptic and neuritic damage, altered neuronal ionic homeostasis and oxidative injury. These cellular imbalances produce alterations on kinase/phosphatase activities, determining an abnormal tau phosphorylation and neurofibrillary tangles deposition, a widespread neuronal/neuritic dysfunction with transmitter deficit and cell death. The amyloid hypothesis is supported not only by genetic evidence in AD familial cases, but also by other observations. In fact, it has been shown that mutations in the gene encoding the tau protein cause frontotemporal dementia with Parkinsonism characterized by severe tau deposition without amyloid plaques (*Spillantini et al., 1998*). Thus, NTFs observed in AD brains are likely deposited after the initial Aβ plaques formation speculating that altered APP processing and amyloid deposition predate tau changes and neuronal injury.

However, the "amyloid hypothesis" is not uniformly accepted since it is quite simplistic and is not able to elucidate the whole complex mechanism behind the AD pathogenesis. The biggest concern is that this theory does not explain the increased Aβ production in sporadic AD cases, the form of the disease with the highest incidence, where no mutations in APP or PSEN 1/2 genes are present. Furthermore, on *post-mortem* analysis, amyloid plaques may be undetectable in brains of patients whit severe AD and may be present in brains of elderly patients without signs of dementia (*Davinelli et al., 2011*). It has been shown that the pathological progression of AD and the degree of the cognitive impairment correlate with the number of neurofibrillary tangles much better than the beta-amyloid plaques deposition. However, it has also been suggested that the amyloid pathology correlates with AD progression at the earlier stages of the disease and that subsequent changes in Aβ levels do not affect cognition, especially late in the disease (*Teich and Arancio, 2012*). Moreover, the amyloid cascade hypothesis suggests that changes in tau stability and neurofibrillary tangles formation are induced by toxic concentrations of Aβ; although several mechanisms have been proposed, the linkage between Aβ and tau accumulation is not yet well understood. Despite many efforts to elucidate the deficiencies of the Aβ hypothesis, an alternative theory explaining the cause and the early pathogenesis of AD has not been proposed. (**Figure 4**).

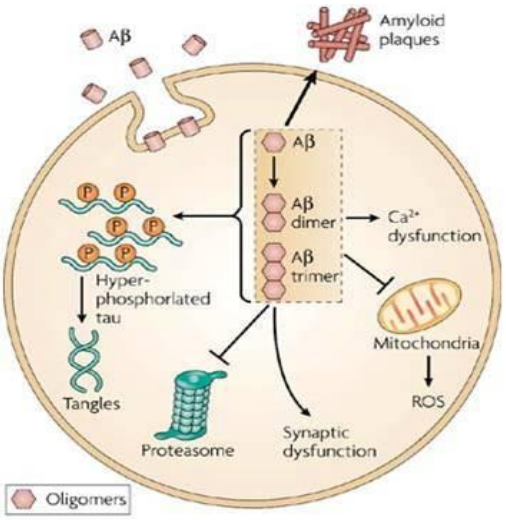


Figure 4. Pathological effects of Aβ on neurons.

Tau is a soluble protein that normally binds to and stabilizes axonal and dendritic microtubules, the essential components of the cytoskeleton, conferring dynamism to the main support structure for transport and neurotransmission. In the CNS, tau protein is present as six isoforms deriving from a single gene by alternative splicing; all isoforms are present in an abnormally hyperphosphorylated state in the NFTs (*Goedert et al., 1989*). The microtubule-binding domain of tau protein is the main region involved in the tau aggregation. When the tau structure is altered by modifications such as an abnormal hyperphosphorylation in the proline-rich region, the protein loses the affinity to bind microtubules and begins to self-assemble. The deriving oligomers aggregates into tangles of hyperphosphorylated tau forming paired helical filaments (PHFs) and straight filaments (*Mandelkow et al., 2007*). These tau polymers are present in several types of dementia-related disorders as well as AD, also known as tauopathies. During NTFs formation, the destabilization of the microtubular system is involved in the structural and functional damage of neurons, contributing to the synaptic loss and cell death. The cytoskeleton alteration seems to be also connected with the disruption of the Golgi apparatus, inducing abnormal protein processing and increased production of Aβ.

Although the mechanism by which the phosphorylation of tau induces its aggregation is still unclear, the final effect is the reduction of tau affinity to bind microtubules (*Meraz-*

Ríos et al., 2010). Several kinases are involved in tau phosphorylation, such as glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (Cdk5), MT-affinity regulatory kinase, cAMP-dependent protein kinase, Tau–tubulin kinase 1, protein kinase A (PKA), calmodulin-dependent protein kinase 2 and extracellular signal-related kinase (ERK) 1/2. In physiological conditions, the phosphorylation is regulated by phosphatases like protein phosphatase 2A (PP2-A) and PP-1 (*Wang et al. 2007 a*). When the balance between phosphorylation and dephosphorylation fails, hyperphosphorylation process triggers to tau aggregation. In fact, it has been reported that the activity of these phosphatases is decreased in AD brains; the inhibition of the abnormal hyperphosphorylation has been investigated as potential therapeutic approach to the disease (*Iqbal and Grundke-Iqbal, 2008*).

1.3 GENETIC AND OTHER RISK FACTORS IN AD

1.3.1 GENETIC RISK

AD can be divided in two subgroups depending on the frequency and on the age of the disease onset. The familial form of AD (FAD) is usually characterized by an early onset in the midlife (age<65 years) and it is associated with heritable mutations involving the *APP*, *PSEN1* and/or *PSEN2* genes; they represent less than 5% of AD cases. The sporadic form of AD (SAD) with late onset (age>65 years) and without heritable gene mutations is the most common type of dementia, responsible for over 95% of all AD cases, and influenced by complex interactions between genetic and environmental risk factors.

The mutations within *APP* gene appear to alter the proteolytic processing of the APP and generate Aβ deposition. Moreover, *APP* has a gene-dosing effect on Aβ production and increased levels of APP seem to enhance the severity of AD pathological features. In fact, Down's syndrome patients that have three copy of *APP* gene on the chromosome 21 usually develop AD over the age of 35 (*Tyrrell et al., 2001*). All the mutations of *APP*,

PSEN1 and *PSEN2* involved in the early-onset familial AD lead to a relative excess in the production of Aβ42 and amyloid plaques deposition, although not all cases of these AD form present these genetic mutations.

Up to now the main risk factor for sporadic AD is advancing age, but other risk factors and potential risk genes involved in the pathology of the disease have been identified. The gene encoding the cholesterol-carrying apolipoprotein E (*apoE*) on chromosome 19 is the strongest and most consistently associated risk gene for the sporadic form of AD, principally the late-onset one, but also some early-onset cases. The gene is inherited as three common alleles, ε2, ε3 and ε4, originating six different phenotypes. The ε2 allele is the least prevalent among the population (frequency of 7-8%) and it is associated with the lowest risk of developing AD. Epidemiological studies demonstrated that apoE2 displays a protective effect by delaying the onset of the disease; the apoE2 is also associated with a reduction of hippocampal atrophy, higher Aβ and lower phosphotau levels in the cerebrospinal fluid (*Caselli and Dueck, 2010; Chiang et al., 2010*). The ε3 allele is the most common (frequency in population of 60-70%) and confers intermediate risk of developing AD, but less than the ε4. The ε4 presents a gene-dosing effect on the disease pathology, correlated with increased risk and earlier onset (*Finch and Morgan, 2007*). In fact, it has been shown that individuals with two copies of ε4 alleles, compared with those carrying ε3, have a significantly increased risk (more than seven times) of AD, associated with an enhanced amyloid deposition, decreased Aβ clearance and cholinergic dysfunction (*Corder et al., 1993*). ApoE ε4 carriers have enhanced AD symptoms, accelerated age-dependent cognitive decline and worse memory performances. Moreover, apoE4 genotype is also associated with several structural and functional brain changes related to AD pathogenesis before that the clinical features become evident. Genome-wide association studies confirmed that the ε4 allele of *APOE* is the strongest genetic risk factor for AD (*Harold et al., 2009*). *APOE* ε4 probably increases the risk of both early-onset and late-onset AD by modulating and accelerating Aβ deposition in the brain, and by directly regulating brain lipid metabolism and synaptic functions through APOE receptors. Although *APOE* ε2 is associated with a reduced risk of dementia, both the ε2 and ε4 alleles of *APOE* increase amyloid burden compared with *APOE* ε3 in oldest individuals,

suggesting that the protective effects of *APOE* ε2 might not be associated with Aβ deposition. However, unlike known genetic mutations, inheriting *APOE* ε4 is not sufficient to develop the disease and many patients with AD are not carrying this allele. ApoE accounts for only 10-20% of late-onset AD risk, suggesting that additional genes are involved in the disease onset.

Several candidate risk genes have been identified, in particular those implicated in the cholesterol metabolism, synaptic function and immune response, although the impact of these genes in the late-onset AD remains to be confirmed (*Karch et al., 2012*). According to the Alzgene website meta-analysis, excepting for *apoE*, the majority of identified candidates genes have a relative risk for AD onset around 1,5%. In addition to apoE, other genes involved in the transport or in the metabolism of cholesterol have been suggested as putative risk factors for AD. Polymorphisms in receptors for the uptake of cholesterol, such as low-density lipoprotein receptor-related protein (LRP) and the very-low-density lipoprotein (VLDL) receptor, as well as polymorphisms in enzymes that regulate the cholesterol catabolism, have been associated with an increased risk for AD (*Zerbinatti et al., 2005*).

Among genetic causes of late-onset AD, the lipoprotein receptor sortilin-related receptor (*SORL1*) gene has also been identified as an important factor involved in the pathogenesis of the disease. It has been reported that SORL1 interacts with apoE, is a substrate of γ-secretase enzyme, affects APP trafficking and it seems able to reduce the interaction between APP and β-secretase resulting in reduced Aβ production. Some studies reported that AD brains show a down-regulation of *SORL1* gene expression and mutations in this gene are associated with the late-onset AD pathology (*Offe et al., 2006; Rogaeva et al., 2007*).

The *GSK3β* gene encoding for one of the main tau kinase, together with Cdk5, is considered as a potential risk gene in AD. It has been proposed that the Aβ peptide, APP cleavage products and PSEN complexes can activate neuronal GSK3β leading to glia

activation, tau increased phosphorylation and tangles deposition. *GSK3* polymorphism has been linked to the sporadic form of AD and it has been reported that apoE4 and Aβ have a higher effect in the activation of GSK3β (*Cedazo-Minguez et al., 2003*) suggesting that potential biochemical interactions between *APOE* and *GSK3B* are worth further investigation. Thus, GSK3β deregulation is suggested to be one of the links between amyloid deposition and tau protein hyperphosphorylation; several GSK3 inhibitors are under investigation as a treatment strategy for AD. However, it has been found that some *PSEN1* mutations may activate GSK3β and promote tau phosphorylation by an alternative pathway from Aβ peptide (*Baki et al., 2004*).

Tau mutations can also affect splicing of tau protein isoforms and microtubule binding efficacy. The tau polymorphism is associated with AD, although its relevance in the pathology of the disease is not completely clear. Polymorphisms of other phosphokinases as well as GSK3β might be associated with an increased risk of AD and have a role in explaining the link between Aβ and tau pathology. The *DYRK1A* is a gene located on the chromosome 21 encoding for a kinase that plays a significant role in the cell proliferation and neuronal development. *DYRK1A* is involved in tau and APP phosphorylation, leading to an increased amyloidogenic processing, and it might considered as a risk gene on AD onset (*Kimura et al., 2007*).

TOMM40 is another gene associated with an increased risk of developing late-onset AD; it is a channel-forming subunit of the translocase of outer mitochondrial membrane (TOM complex), which forms the protein-conducting channel facilitating the translocation of unfolded proteins from the cytosol into the mitochondrial intermembrane space. *TOMM40* gene is located on the chromosome 19, next to the *APOE* gene, and polymorphisms on this gene can affect the onset age of AD (*Roses, 2010*).

A recent identified risk gene for AD is *CLU*, encoding for the chaperone clusterin (also known as ApoJ) thought to bind and remove Aβ from the brain. Another risk gene functionally related is *PICALM*. This gene encodes for the phosphatidylinositol-binding

clathrin assembly protein, an endosomal protein involved in synaptic neurotransmitter release that binds Aβ and may promote its clearance. However, these genes have minimal effects on the development of AD lesions and are not predicting for AD; they could have an important role in the identification of pathways involved in the disease (*Kok et al., 2011*).

1.3.2 APOLIPOPROTEIN E: BIOLOGICAL AND PATHOLOGICAL ROLE

ApoE is a polymorphic 299-amino acids (~34 kDa) protein responsible for the transport of cholesterol and other lipids. The three corresponding human apoE isoforms differ only in the amino acids at positions 112 and 158. ApoE3 has cysteine-112 and arginine-158, whereas apoE4 only has arginine at both sites and apoE2 only has cysteine. This small amino acid substitution is able to affect the three-dimensional structure and the lipid-binding property of the protein, conferring a specific isoform-function in several biological processes. ApoE contains two independently folded structural domains: the N-terminal, that includes the receptor binding region, and the C-terminal, that contains the major lipid binding region (*Hatters et al., 2006*). ApoE is important for cholesterol and triglycerides metabolism, transport and homeostasis, in an isoform-dependent manner; it has prominent functions also in the cell signal transduction pathways, including regulation of neurotransmission and cell death. ApoE is an integral constituent of many lipid transport lipoproteins complexes, playing a role in assembly, structure and uptake of lipoproteins by binding to the cell surface LDLRs (low density lipoprotein receptors) family with a specific isoform-affinity. These receptors are involved in signal transduction pathways, although their main function is to provide cells with cholesterol and remove lipoproteins from the blood.

ApoE has a different preference to tie to specific lipoproteins, depending on the isoform. In fact, apoE4 preferentially binds to VLDL, whereas apoE3 and apoE2 bind preferentially high density lipoproteins (HDL) (*Strittmatter and Bova, 2002*). In peripheral tissues, apoE is mainly produced by the liver and macrophages. In the CNS it is synthesized

predominantly by astrocytes and to some extent by microglia, although also neurons are able to generate it under physiological and pathological conditions. The main function of apoE in the brain is to transport cholesterol from astrocytes to neurons via LDLR receptors, playing a critical role in the distribution and homeostasis of lipids among neuronal cells. Dysfunctions of LDLR as well as apoE4 are associated with hyperlipidaemia and hypercholesterolaemia, leading to atherosclerosis, coronary heart disease and stroke.

Several evidences show that apoE is involved in the maintenance of neuronal structure and activity, repairing injured neurons through the regulation of lipids homeostasis necessary for the synaptogenesis, cells proliferation and scavenging toxins (*Cedazo-Minguez, 2007*). ApoE4 seems less effective and more detrimental than apoE3 and apoE2 in the normal maintenance and repair of neuronal cells.

ApoE differentially regulates Aβ production, aggregation and clearance in an isoform-dependent manner. However, apoE4 can contribute to risk of AD pathogenesis and cognitive decline also by Aβ-independent mechanisms involving synaptic plasticity, neurovascular functions and neuroinflammation (*Liu et al., 2013*). (**Figure 5**).

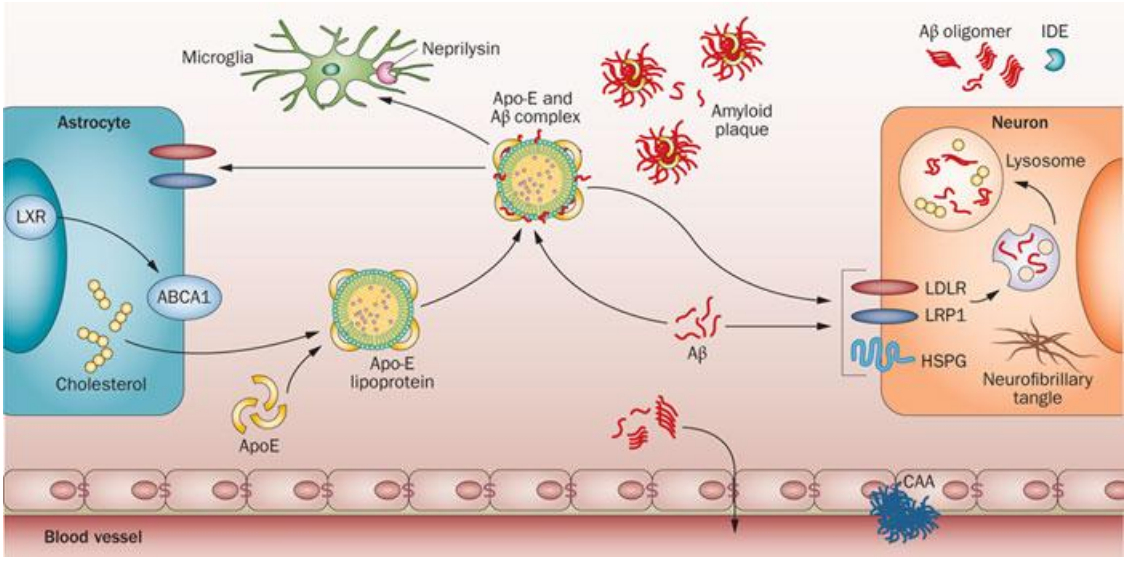


Figure 5. ApoE and Aβ metabolism in the brain.

Independently of Aβ, apoE4 might be less efficient than apoE3 and apoE2 in delivering cholesterol and essential lipids for maintenance of synaptic integrity and plasticity. Immunohistological evidences show that apoE co-localize in senile plaques in the AD brains and Aβ deposition is more abundant in E4 carriers (*Namba et al., 1991*). ApoE4 and Aβ aggregates act synergistically in the induction of neurodegeneration *in vivo*. Although apoE4 has an active role in Aβ and NTF formation, it is difficult to find an hypothesis to explain the mechanism by which apoE4 increases the pathological processes involved in AD. *In vitro* studies suggest that apoE isoforms may differently influence tau pathology and NTF deposition. In particular, it has been shown that apoE3, but not apoE4, forms a stable complex with non-phosphorylated tau. This interaction between apoE3 and tau is inhibited by the tau-phosphorylation, suggesting that apoE3 might be able to prevent abnormal tau hyperphosphorylation and destabilization of the neuronal cytoskeleton (*Strittmatter et al., 1994*).

In vivo studies in apoE transgenic mice showed an increased phosphorylation of tau in mice expressing human apoE4 in neurons, but not in mice expressing apoE4 in astrocytes, indicating that apoE4 induces tau phosphorylation specifically in neurons (*Brecht et al, 2004*). An alternative mechanism by which apoE isoforms would differentially contribute to tau hyperphosphorylation is the modulation of tau kinases and phosphatases.

Intraneuronal accumulation of hyperphosphorylated tau has been also found in apoE knock-out mice fed with a high cholesterol diet, suggesting a synergic interaction between cholesterol and lack of apoE function (*Rahman et al., 2005*). Moreover, apoE4 is less efficient than other isoforms in promoting cholesterol efflux from neurons and astrocytes; this is probably related to the structural differences between apoE isoforms (*Michikawa et al., 2000*). In AD brains a decreased cholesterol level has been described and several evidences indicate that cholesterol is directly involved in AD pathogenesis (*Reid et al, 2007*).

Abnormal lipid metabolism is strongly related to the pathogenesis of AD. Clinical and epidemiological studies showed that patients with elevated plasma cholesterol levels have increased susceptibility to AD; the use of statins to inhibit the synthesis of cholesterol

seems to decrease the frequency and the progression of the disease. However, the therapeutic effect of statins on AD pathology is influenced by several factors, such as the efficiency of blood flow to the brain and the presence of concomitant disease conditions, including hypertension, diabetes and hypercholesterolaemia. Cholesterol is an essential component of membranes and is crucial for synaptic integrity and neuronal functions implicated in learning, memory formation and neuronal repair (*Mauch et al., 2001*). Cholesterol levels in hippocampal and cortical areas in patients with AD are lower than healthy brains. It has been demonstrated that apoE4 is less efficient than apoE3 in transporting brain cholesterol (*Svennerholm and Gottfries, 1994*). Furthermore, a number of studies suggest that cholesterol regulates the Aβ production and increases the activity of β-secretase. Changes in cholesterol levels or distribution within the membrane have been shown to alter the localization of APP and its availability to be cleaved by the secretases; however, the effect of cholesterol on the amyloidogenic processing of APP remains controversial (*Abad-Rodriguez et al., 2004*). On the other hand, Aβ modulates the synthesis and the distribution of cholesterol in neurons. Although the effect of cholesterol on the Aβ production is complex and not completely clarified, the cholesterol/Aβ interactions are probably modulated by the apoE genotype.

In the nervous system, the apoE-mediated distribution of lipids plays a fundamental role in processes such as growth, regeneration and synaptic plasticity. ApoE4 is associated with impaired synaptic plasticity in the hippocampus and age-dependent disruption of synaptic organization in *APOE* knockout transgenic mice (*Buttini et al., 1999*). In AD and healthy aged controls, *APOE* ε4 gene dosage inversely correlates with dendritic spine density in the hippocampus, suggesting that the effect of ε4 genotype on risk of AD might be mediated, at least in part, by direct effects on synaptic function (*Ji, 2003*). ApoE colocalizes with amyloid plaques and microglia, suggesting that apoE has a role in the innate immune response in AD. In fact, ApoE4 seems to have pro-inflammatory and/or reduced anti-inflammatory functions, which could exacerbate AD pathology and cause neurovascular dysfunction.

ApoE isoforms have differential roles in maintaining vascular health, and a recent meta-analysis showed increased risk of vascular dementia in individuals with *APOE* ε4

compared with *APOE* ε3 (*Yin et al., 2012*). The *APOE* ε4 genotype combines synergistically with atherosclerosis, peripheral vascular disease, and type 2 diabetes in contributing to an increased risk of AD.

Cholinergic signal transduction is well known to be impaired in AD. ApoE4 carriers with AD show greater deficits than non-carriers in cholinergic activity in the hippocampus and the cortex, as well as a reduction in the number of cholinergic neurons markers, such as choline acetyltransferase activity and nicotinic receptor binding. A direct negative influence of apoE4 on cholinergic signaling may reduce the effectiveness of cholinergic replacement treatments reported for apoE4-AD patients (*Soininen et al., 1995*).

ApoE receptors mediate cellular signaling by binding to several extracellular and intracellular ligands, some of which are relevant to AD pathology. Several studies suggest that apoE4 is associated with the disruption of multiple signal transduction pathways, loss of cell protection, and alteration of mitochondrial metabolism.

In neurons, apoE isoforms differentially affect the activity of proteins such as the extracellular-signal-regulated-kinase (ERK) and the c-Jun N-terminal Kinases (JNK), principle members of the mitogen-activated protein kinase (MAPK) family, involved in the regulation of processes including cell proliferation, differentiation and survival (*Hoe et al., 2005*). ApoE also has specific isoform-related effects on calcium channels. ApoE4, but not apoE3, significantly increases calcium levels and NMDA stimulation in cultured hippocampal neurons, leading to neurotoxicity (*Qiu et al., 2003*).

Gene expression studies in hippocampus of AD patients demonstrated that apoE4 carriers have higher expression of negative regulators of cell growth that may lead to increased cell senescence and apoptosis, and in contrast decreased expression of genes associated with synaptic plasticity and axonal/neuronal outgrowth. ApoE4 is also associated with the reduction of the neurotransmitter receptors and Ca2+ homeostasis, disruption of multiple signal transduction pathways, loss of cell protection, and mitochondrial dysfunction (*Xu et al., 2007*). However, apoE derived from various cellular sources might exhibit different physiological and pathological activity. In order to elucidate the role of apoE in

neurodegenerative processes is crucial understanding the mechanisms that govern the apoE toxicity as well as protection on neurons.

1.3.3 OTHER RISK FACTORS IN AD

DEPRESSION

The role of depression in AD is debated; several results from population-based studies have been inconsistent. Depressive symptoms occur in 40–50% of patients with AD and depression may be associated with an increased risk for AD and other dementias. A recent epidemiological study examining the association between depressive symptoms and incidence of dementia over a 17-years follow-up period showed an increase of AD and dementia in participants who were depressed at baseline (*Saczynski et al., 2010*). One episode of depression conferred an 87–92% increase in dementia risk, while having more episodes nearly doubled the risk. It has then been suggested that preventing the recurrence of depression in older adults may prevent or delay the onset of dementia (*Dotson et al., 2010*).

TRAUMATIC BRAIN INJURIES

Head injury and moderate to severe head trauma have been associated with an increased risk of develop AD as well as other forms of dementia later in life. Moderate head injuries are associated with twice the risk of developing AD and severe head injuries are associated with 4.5 times the risk (*Lye and Shores, 2000*). It has been proposed that traumatic brain injury leads to accumulation of APP with its proteolytic enzymes at sites of axonal injury, increased Aβ production and deposition into extracellular plaques (*Chen et al., 2004*). Some studies also suggest that ApoE4 carriers who experienced moderate or severe head injury have a higher risk to develop AD (*Katzman et al., 1996*).

EPIGENETIC MODIFICATIONS

Epigenetic modifications, defined as changes in gene expression that do not alter the nucleotide sequence of DNA, are the results from gene-environment interactions and are involved in the regulation of chromatin structure (*Goldberg et al., 2007*). A key feature that distinguishes epigenetic modifications from genetic changes is their reversible nature. Epigenetic alterations such as DNA methylation and histone modifications have been widely implicated in several age-related diseases, especially in cancer progression. Since the majority of late-onset AD cases is sporadic, occurs in patients without a family history of the disease and is characterized by differential susceptibility, epigenetic and environmental factors may play a role in the etiology of the disease. Recently, epigenetic phenomena have been recognized as a major contributor to the aging phenotype (*Fraga et al., 2005*) and epigenetic modifications seem to be involved in the disruption of synaptic signaling and neuronal survival. The heterogeneity noticed in clinical phenotypes of AD patients with PSEN1 mutations suggests that other factors, both genetic and epigenetic, must contribute to disease phenotype (*Larner and Doran, 2006*). AD brain cells also present epigenetic changes on gene expression associated with an increases susceptibility to oxidative stress. Studies on *post-mortem* human brain and peripheral leukocytes, as well as transgenic animal models, showed that aging and AD present epigenetic alterations, including abnormal DNA methylation and histone modifications (*Chouliaras et al., 2010; Arosio et al., 2012*). The hypothesis that epigenetic mechanisms can modulate AD risk is confirmed also by results obtained in twin studies. Interestingly, a recent study showed that pharmacological inhibition of DNA methylation in the hippocampus impairs memory consolidation in mice (*Day and Sweatt, 2011*). Studies reported that the deregulation of histone acetylation is related to learning and memory impairment in aged mice models, suggesting that epigenetic regulation is important in both normal aging and neurodegenerative processes (*Fischer et al., 2007; Peleg et al., 2010*). The role of epigenetic in aging process is a promising field of research, and since epigenetic alterations are more reversible than genetic alterations this area will be critical in future long-term studies.

CARDIOVASCULAR DISEASES

Growing evidences suggest that the health of the brain depends closely from the overall health of the heart and blood vessels. In fact, the brain has one of the richest networks of blood vessels and is necessary ensuring the maximum supply of oxygen and nutrient for its correct functionality. Some data indicate that cardiovascular disease risk factors such as physical inactivity, high cholesterol levels, diabetes, smoking and obesity, especially if present in the midlife, are associated with a higher risk to develop AD and other dementias (*Kivipelto et al., 2005*). Unlike genetic risk factors, several of these cardiovascular disease factors are reversible. Cerebrovascular changes such as infarcts, stroke and vasculopathy increase the risk of dementia (*Pendlebury and Rothwell, 2009*). Stroke may lead to cognitive impairment directly damaging brain regions implicated in memory functions and inducing inflammatory processes. Experimental animal models of cerebral ischemia demonstrated the presence of APP and tau in the area of ischemic damage and high level of amyloid results in progressive increases in infarct size, neuroinflammation, and cognitive deficits (*Whitehead et al., 2007*). Other studies also indicated that soluble APP and Aβ42 accumulates in patients with multiinfarct dementia (*Jendroska et al., 1997*). Brain ischemia and the following oxidative stress induce the expression and activity of both β- and γ-secretases, promoting production and aggregation of Aβ peptide which is toxic for ischemic neuronal cells (*Pluta et al., 2013*).

Hypertension, especially in midlife, may increase the risk of AD and accelerate the cognitive decline in patients at risk for dementia (*Goldstein et al., 2013*). The decrease of the vascular integrity of the blood-brain barrier (BBB) in patients with hypertension, determine the protein extravasation into brain tissue leading to cell damage, reduction in synaptic and neuronal functions, apoptosis and an increase of Aβ accumulation.

TYPE 2 DIABETES

Observational studies showed that type 2 diabetes nearly double the risk of AD (*Luchsinger et al., 2001*). In cases of hyperinsulinemia, insulin can cross the blood brain barrier and compete with Aβ for the insulin degrading enzyme (IDE), thereby reducing Aβ clearance from the brain and increasing its deposition (*Craft, 2007*). Moreover, a study showed a reduction in IDE gene expression and protein levels in the hippocampus of apoE4 AD patients, suggesting that IDE plays a critical role in the degradation of Aβ in the human brain (*Cook et al., 2003*). Diabetes and impairment of glucose tolerance lead also to the formation of advanced glycosylation end products (AGEs). The glycosylation of Aβ enhances its propensity to aggregate leading to amyloid plaques formation and neuronal damage (*Yan et al., 1996*). Insulin is also produced in the brain and alternatively may have a beneficial effect in amyloid clearance. Elevated insulin blood levels may inhibit brain insulin production, resulting in lower rate of amyloid clearance. Antidiabetic drugs such as glitazones, which decrease insulin resistance and peripheral insulin levels, may also be beneficial in AD.

PLASMA LIPID LEVELS

Epidemiologic studies examining the association between cholesterol and AD, such as the therapeutic effectiveness of statins for AD and mild cognitive impairment, have reported conflicting results (*Shepardson et al., 2011*). The disruption of cholesterol homeostasis in neuronal membranes caused by oligomeric Aβ may induce AD pathological alterations including enhanced phosphorylation of tau, impairment of synaptogenesis and synaptic plasticity, and neurodegeneration (*Michikawa, 2003*). Many experimental studies suggest that hypercholesterolemia accelerates the production of Aβ by increasing the amyloidogenic processing of APP by β- and γ-secretases. The mechanism whereby serum hypercholesterolemia leads to an increased neuronal content of cholesterol is unknown, but may be mediated by some cholesterol derivatives implied in its excretion pathways, known

as oxysterols (*Björkem et al., 2006*). One of these products is 27-hydroxycholesterol which is, in contrast to cholesterol, able to cross into the brain and considered be the link between circulating cholesterol and dementia (*Ghribi, 2008*). Nevertheless, dyslipidemia increases the risk of vascular disease, which in turn is associated with increased risk of AD, and in people suffering of cardiovascular and cerebrovascular disease, statins are the first-line treatments for reducing cholesterol levels. Statins may also be beneficial in preventing dementia however there is not clear effect on the treatment or prevention of this disease. The potential mechanisms, by which statins can act, may be lowering brain cholesterol levels leading to reduced neurofibrillary tangles and inflammation (*Wong et al., 2012*).

CIGARETTE SMOKING

The scientific literature has been reported conflicting results regarding the association between smoking and the increased risk of AD (*Cataldo et al., 2010*). Smoking is a strong risk factor for cerebrovascular diseases but the mechanism by which cigarette smoking can impair cognitive function and predispose to dementia is unknown. However, an *in vivo* study showed that smoking induces the oxidative stress, affect synaptic transmission, impair the stability of the cytoskeleton and increases the amyloidogenic processing of APP (*Ho et al., 2012*). All these pathological alterations could induce neurodegeneration and might predispose the brain to AD and dementia.

PSYCHOLOGICAL STRESS

Evidences suggest that chronic psychological stress can alter brain morphology exerting a detrimental effect on its functions such as memory, and might increase the risk of AD (*Aleisa et al., 2006*). The hippocampal region of the brain is involved in the response to stress (*Sapolsky et al., 2000*). The corticosterone hypersecretion caused by stress down-regulates the corticosteroid receptors in the hippocampus, reduces the feed-back inhibition

of the adrenocortical axis that leads to further hormones hypersecretion, finally causing permanent loss of hippocampal neurons (*McEwen, 2002*). In addition, associations between high concentrations of cortisol, impaired cognitive function, and hippocampal atrophy have been found in several studies of people with dementia, major depression and post-traumatic stress disorder (*Hull, 2002*).

PHYSICAL AND INTELLECTUAL ACTIVITIES

Epidemiological and experimental data suggest that physical exercise may promote brain health. However, conflicting results have emerged and some studies indicated that physical activity has a beneficial effect while others showed no association between exercise and healthy brain. Physical activity could increase cerebral blood flow, oxygen levels and glucose utilization (*Fratiglioni et al., 2004*). In animal models exercise promotes structural brain changes, such as an increase in capillary density, increased brain-derived neurotrophin factor (BDFN) gene expression and new cells formation in the hippocampus. Despite these results in animals, the fitness interventions in humans have produced less reliable effects on cognitive performance (*Colcombe and Kramer, 2003*). Reports indicate also that elderly people with higher levels of education had a lower incidence of dementia. Cognitive activity was suggested to decrease the risk of cognitive decline and several studies found that people engaged in cognitively stimulating activities, active lifestyle and rich social network were less likely to develop dementia (*Acevedo and Loewenstein, 2007*). Mental stimulation seems to selectively increase synaptogenesis, whereas physical exercise may enhance other components of the brain, such as vasculature. Although an active and socially integrated lifestyle in late life protects against dementia and AD, further researches are necessary to better define the mechanisms of these association.

1.3.4 DIET, INSULIN AND ALZHEIMER'S DISEASE

A growing body of evidence support that unhealthy diet can be considered a reversible risk factor for AD, although preclinical and clinical data are divergent. The first epidemiologic study providing that diet is linked to cognitive impairment and dementia, found a positive association between total calories and fat intake and the incidence of the disease (*Grant, 1997*). This correlation was supported by another study, where the intake of cholesterol and saturated fats in the middle-age population increased the risk of impaired cognitive functions (*Kalmijn et al, 1997*). The hypothesized mechanism of excess dietary fat late in life on neuronal damage was a combination of oxidative stress and inflammation. AD seems to be linked to excessive dietary intake of refined carbohydrates and high-saturated fats animal products, and low intake of fruits and vegetables containing fibers, vitamins, polyphenols and other antioxidant substances. These important studies pointed toward a strong environmental component to AD and suggested that dietary improvement might help to prevent the disease. However, follow-up studies have failed to confirm the link between healthy dietary modification and decreased risk of dementia, and several studies exploring preventive strategies with specific vitamin supplementation show no appreciable results.

Fatty acids can be categorized into saturated (SFA) and unsaturated (UFA). Elevated SFA levels could have negative effects on age-related cognitive decline, and epidemiological evidences suggest a possible association between monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA; particularly, n-3 PUFA) consumption and a reduced risk of cognitive impairment and dementia. In a recent longitudinal prospective study it has been found that abundant dietary SFA intake later in life impairs cognitive performance concerning specific learning and memory tasks (*Eskelinen et al., 2008*). Dietary fat intake at midlife is associated with the risk of dementia especially among the apoE4 carriers, which may be more susceptible to environmental factors (*Kivipelto et al., 2008*).

Evidences suggest that also high-carbohydrate diet (HC) can increase the risk of AD. This view is supported by the role of insulin/insulin-like-growth factor (IGF) signaling in aging and the similar aspects characterizing AD and type II diabetes. In general, insulin signaling

and metabolism are reduced in the aging of non-pathologic brains. Moreover, the higher serum glucose levels in normal aging may directly damage hippocampal structures, up-regulate the tau kinase GSK-3β and reduce levels of insulin-degrading enzyme in AD brains (*Wu et al., 2008*). Patients with advanced AD showed high insulin levels and low rates of glucose disposal. Furthermore, brain levels of insulin receptors, glucose transport proteins and other insulin pathway components are reduced in some AD cases (*Messier and Teuntenberg, 2005*). The consequent resistance to insulin signaling renders neurons energy deficient and more vulnerable to oxidative insults, impairing synaptic plasticity. Endogenous insulin signaling is important for maintaining relatively low phosphorylation levels of Tau and the down-regulation of insulin signaling may be involved in Tau hyperphosphorylation and aggregation. HC diet leads to increased levels of insulin and triglyceride rich lipoproteins, probably related with the reduction of lipid metabolism by lipoprotein lipases (LPL) (*Campos et al., 1995*). ApoE4 genotype is frequently associated with increased insulin sensitivity and elevated plasma lipid levels through the inhibition of LPL activity. HC diet and apoE4 can synergistically alter lipids metabolism and homeostasis within the CNS, compromising the integrity of cellular membranes and decreasing the function of membrane proteins such as glucose transporters and APP. The results of all these processes lead to decreased glucose utilization, altered APP processing, consequent Aβ deposition and risk to develop cognitive decline.

1.4 PEPTIDYL-PROLYL CIS/TRANS ISOMERASE

Protein phosphorylation is a central mechanism for the regulation of key cellular functions and its deregulation seems contribute to age-related pathological conditions such as AD. Recent studies suggest that dysfunction of the ubiquitous protein peptidyl-prolyl *cis/trans* isomerase (PIN1) may play a role in this process, supporting a direct involvement of PIN1 in neurodegenerative diseases. In particular, PIN1 interacts with phosphorylated serine or threonine preceding proline motifs (pSer/Thr-Pro), promoting the *cis/trans* isomerization of the peptide bond and increasing the accessibility for the dephosphorylation by

phosphatases. This conformational change modulates catalytic activity, phosphorylation status, stability and localization of several proteins. PIN1 is considered a mitotic regulator in the signaling of processes including cell cycle, transcription and neuronal survival (*Lu et al. 1999*). An interesting feature of degenerative neurons is an increased mitotic phosphorylation of certain proteins on Ser/Thr-Pro motifs associated with the activation of kynases such as Cdk5 and GSK3β. PIN1 is also modified by oxidation, which causes its inactivation in early stages of AD, suggesting that PIN1 has an important role in the response to oxidative stress (*Sultana et al., 2006*).

PIN1 accumulates in neurofibrillary tangles in AD brains and the consequent depletion of soluble PIN1 may contribute to neuronal death. It has been shown that PIN1 specifically interacts with phosphorylated tau and promotes tau dephosphorylation through its cis/trans isomerization (*Liou et al., 2003*). PIN1 is a modulator of tau metabolism and may contribute to the pathological processes of taupathies, including AD. Recent studies show that, although PIN1 expression remains constant, there is a deregulated post-translational modification of this protein in AD brains. These results suggest that PIN1 post-translational modifications may also represent interesting biomarkers to follow the severity of AD and tauopathies (*Ando et al., 2013*). In AD brain, depletion of PIN1 or its oxidative modification and inactivation may lead to NFTs formation and Aβ deposition. Knockout *PIN1* mice have age-dependent neurodegeneration with increased levels of phosphorylated tau (p-tau) and insoluble Aβ, suggesting that PIN1 has a protective role in neurodegeneration (*Pastorino et al.2006*). It could be speculated that PIN1 acts as an early factor in the development of Aβ pathology, because the absence of the *PIN1* gene causes increased amyloidogenic processing of APP. Another study showed that Aβ insult induces an up-regulation of PIN1 protein associated to decreased p-tau (*Bulbarelli et al. 2009*). Thus, in physiological conditions, PIN1 might protect from Aβ toxicity promoting the trans conformation of APP and increasing its non-amyloidogenic processing. However, the role of PIN1 in cellular pathways implicated in the protection or promotion of neurodegenerative disorders it is not completely elucidated. Studies on *post-mortem* human brains showed a down-regulation of PIN1 protein levels in advanced AD, with an inverse correlation between PIN1 and tau accumulation in the hippocampus. Nevertheless, PIN1

function may vary on the course of the disease and it has been reported that frontal cortex of AD brains has increased PIN1 gene expression associated to high p-tau levels (*Wang et al., 2007 b*). A recent study also showed a significant increase of PIN1 gene expression associated to a decreased epigenetic methylation of PIN1 gene promoter in peripheral blood mononuclear cells (PBMCs) of late onset AD patients, especially in patients carrying at least one copy of the ε4 allele (*Arosio et al., 2012*). It is possible suppose that the decrease of PIN1 may play a role in the initial accumulation of p-tau and Aβ in early stages of AD pathogenesis, while the increasing p-tau in later stages may induce a protective compensatory up-regulation of PIN1. Further studies are necessary to elucidate PIN1 functions on the molecular events of AD progression.

1.5 SIRTUIN 1

Sirtuins are a family of highly conserved proteins with deacetylase activity and involved in mechanisms known to promote healthy ageing and longevity. These enzymes belong to the class III of NAD-dependent histone deacetylases (HDAC) that remove acetyl groups from lysines both on histones and nonhistone targets. In humans, sirtuins are present as seven isoforms (SIRT1-SIRT7) which differ in catalytic activity, cell localization and tissue expression. The most investigated member of the sirtuins family is SIRT1, which plays a role in several physiological and pathological conditions. In particular, SIRT1 is involved in the regulation of numerous neuroprotective functions, including antioxidant and anti-inflammatory response, anti-apoptotic signaling, regulation of insulin and glucose homeostasis, gene transcription and mitochondrial metabolism. SIRT1 is a nuclear protein predominantly expressed in neurons, with high levels in the cortex and hippocampus, and low levels in white matter (*Ramadori et al., 2008*).

Increasing evidence suggest that SIRT1 function is necessary for the maintenance of synaptic plasticity, learning and memory, speculating a role of this protein in AD. Several studies on *in vitro* and *in vivo* models of AD prove a protective role of SIRT1 against neurodegeneration through the reduction of Aβ accumulation. SIRT1 was initially found to

be protective against AD in calorie restriction studies, in which calorie restriction reduced Aβ generation and senile plaques formation in transgenic AD mice (*Patel et al., 2005*). The over-expression of SIRT1 in the hippocampus of AD transgenic mice model protects against neuronal degeneration and cognitive impairments (*Kim et al., 2007*). Interestingly, SIRT1 can attenuate AD onset and Aβ deposition also through the direct activation of the transcription gene ADAM10 encoding for the α-secretase, enhancing the non-amyloidogenic processing of APP (*Donmez et al., 2010*).

SIRT1 is also involved in the reduction of the tau-related AD phenotype and its inhibition leads to increased levels of phosphorylated tau, suggesting an inverse correlation between SIRT1 activity and tau accumulation. Furthermore, SIRT1 mRNA and protein level were found decreased in the parietal cortex of AD patients, supporting that SIRT1 diminution may be an early event in the disease onset (*Julien et al., 2009*). SIRT1 displays protective effects against AD also by inhibiting mitochondrial dysfunction and by preventing inflammation. In fact, SIRT1 suppress the activity of the nuclear factor kβ in microglia, reducing neuronal damages resulting from the release of inflammatory cytokines induced by Aβ peptides exposure (*Chen et al., 2005*). Although SIRT1 activation exerts a protective role against neurodegeneration, it has also been show that its inhibition may have protective effects on neurons (*Li et al., 2008*).

SIRT1 deacetylase activity makes it a potential target for AD therapy. In fact, increasing evidences support that the abnormal histone acetylation is involved in the pathology of AD. HDAC inhibitors have been reported to improve the memory and cognition in mouse model of AD. The potential mechanism is the inhibition of tau hyperphosphorylation induced by Aβ deposition, or the regulation of the expression of important genes in the learning and memory processes (*Xu et al., 2011*). Because of sirtuins are proteins regulating several different pathways in the cells, further studies are necessary to elucidate the specific functions of SIRT1 on molecular events implicated in AD progression.

1.6 PRESENILIN 1

Presenilins (PSEN 1 and 2) are integral membrane proteins playing a crucial role in the AD neurodegenerative process. These enzymes provide the active catalytic components of the γ-secretase complex, responsible for the APP cleavage into Aβs of different lengths (*De Strooper et al, 1998*). PSEN are ubiquitously expressed in neurons and peripheral tissues, mainly localized in the endoplasmic reticulum and Golgi apparatus. The majority of mutations associated to familial AD cases have been identified in *PSEN1*, whereas only a smaller number of mutations concern *PSEN2* and *APP* genes (*Sorbi et al., 2001*). The altered APP cleavage related to PSEN mutations induces a selective enhancement of Aβ42 peptides rate, often together with a descrease of Aβ40 generation, suggesting a toxic gain of function mechanism in accordance with the amyloid-cascade hypothesis. In addition, *PSEN* mutations cause a partial loss of neuronal functions that could contribute to the neurodegenerative processes. It seems that the increase of Aβ42 and the loss of PSEN function may independently or in concert contribute to the pathogenesis of the disease. These observations might explain why patients with PSEN mutations display an earlier onset and a faster progression of AD than those with APP mutations. However, the complete loss of PSEN function in the brain of transgenic mice results in a neurodegeneration without Aβ deposition, leading to the theory that Aβ may be not sufficient for AD development (*Saura et al, 2004*).

Besides its involvement in Aβ formation, PSEN regulates the cleavage of other proteins, modulating different signaling pathways. More importantly, PSEN is implicated in the maintenance of synaptic functions, in memory formation and synaptic plasticity. The loss of PSEN activity results in hippocampal-dependent spatial and memory impairments, with inflammation and progressive neuronal degeneration. It has also been reported that PSEN1 protein levels are reduced in the association neocortex and hippocampus of AD brains (*Davidsson et al., 2001*). The functional loss or mutations of PSEN1 are associated to increased tau phosphorylation, likely through the activation of CDK5 and GSK3β kinases, with a parallel impairment of axonal transport (*Pigino et al., 2003*). It is plausible that a

large number of distinct PSEN mutations is more consistent with a partial loss of function than a toxic gain of function pathogenic mechanism of this enzyme.

It has been suggested that a potential therapeutic strategy for the treatment of AD is the counteraction of the Aβ production by modulating or inhibiting PSEN-mediated γ-secretase activity (*Ballard et al., 2011*). However, disruption or loss of γ-secretase function is also involved in the impairment of many essential physiological processes. Elucidation of the roles played by presenilins in the brain is necessary for the development of effective therapeutic strategies in neurodegenerative disorders.

1.7 BRAIN-DERIVED NEUROTROPHIC FACTOR

Neuropathological disorders such as AD are characterized by a decreased neuronal plasticity defined as "the dynamic capacity of neurons or glial cells to improve or depress the synaptic efficacy through biochemical or morphological changes". A family of protein factors extremely important in the structural and functional plasticity of the brain is represented by neurotrophins. A current hypothesis sustains that cognitive impairment may be associated with altered trophic support by neurotrophins to neuronal activity and survival (*Arancio and Chao, 2007*). In fact, neurotrophins play an important role for development, differentiation and protection of neurons against several toxic insults. The most widely distributed neurotrophin in the CNS is the brain-derived neurotrophic factor (BDNF), highly expressed in brain areas with high degree of plasticity such as hippocampus and cortex (*Tapia-Arancibia et al. 2008*). BDNF regulates synaptic transmission and neurotransmitters release, but in turn neuronal activity regulates the expression of BDNF. This neurotrophine is involved in the regulation of axonal and dendritic growth, having a critical role also in the memory formation and long-term potentiation. Several studies suggest that BDFN is required for the hippocampus-mediated learning, probably through the induction of structural changes in the brain. In addition, BDNF is implicated in the control of gene transcription by the interaction with the p75 receptor, and in the regulation of intracellular signaling by the activation of the TrkB

receptor (*Reichardt, 2006*). The global effects on neuronal differentiation, plasticity and survival implicate that BDNF may be considered a key molecule in the pathology of neurodegenerative disorders such as AD.

BDNF is synthesized in the endoplasmic reticulum as a precursor protein proBDNF which is proteolitically cleaved to generate either the truncated form, or the mature form mBDNF that is naturally found as a dimer. Recent studies revealed that mBDNF and proBDNF may elicit opposite biological effects. Specifically, the interaction between mBDNF and TrkB receptor promotes cell survival, whereas binding of proBDNF to the p75 receptor triggers apoptotic processes (*Greenberg et al., 2009*). In addition, mBDNF and proBDNF have different effects on morphological neuroplasticity. Indeed, in the hippocampus mBDNF supports dendritic spines formation whereas proBDNF induces spine pruning. With respect to AD, BDNF has been shown to promote survival and differentiation of basal forebrain cholinergic neurons stimulating the release of acetylcholine, a neurotransmitter defective in AD patients. These preclinical observations suggest that deficits of BDNF synthesis might participate in the impairment of the cellular homeostasis that leads to AD. Sublethal doses of Aβ42 down-regulate BDNF expression in cultured cortical neurons and impair BDNF intra-cellular trafficking (*Poon et al., 2011*). In contrast, BDNF is up-regulated in astrocytes exposed to Aβ42 suggesting that Aβ has a distinct effect on the expression and production of BDNF depending on the CNS cellular lineage. The up-regulation of BDNF mRNA expression and protein is also found in microglia and astrocytes of transgenic mice model of AD (*Burbach et al., 2004*). These findings corroborate the hypothesis that an increase in BDNF levels might be a compensatory mechanism to the amyloid-induced toxicity in the early stages of AD.

Several clinical evidences have shown that the expression of BDNF and its receptor trkB are decreased in *post-mortem* brain regions such as hippocampus, temporal and frontal cortices of AD patients. Interestingly, neurons containing neurofibrillary tangles do not show BDNF-immunoreactive material, whereas neurons without tangles display intense BDNF staining (*Murer et al., 1999*). The precursor form of BDNF is also decreased in the hippocampus and parietal cortex even in pre-clinical stages of AD.

Despite the controversial findings of genetic studies regarding the association between BDNF polymorphisms and the risk of AD, large evidence suggests that a reduced neurotrophic support may play a role in cognitive decline and AD. Decreased serum concentration of BDNF has been consistently described in AD patients as compared to healthy controls (*Forlenza et al., 2010*). In contrast, other studies have reported increased serum BDNF levels in patients with early AD (*Angelucci et al., 2010*), suggesting that there might have a compensatory increase in BDNF levels. Thus, additional studies are necessary to clarify the dynamics of BDNF changes in early stages of AD.

1.8 OXIDATIVE STRESS AND ALZHEIMER'S DISEASE

In earliest stages of AD pathogenesis a major role is probably played by the interaction of impaired energy metabolism and oxidative stress. Reactive oxygen species (ROS) are abundantly produced under physiological conditions by cells, playing helpful roles in signal transduction or as defense against infectious agents. Although ROS are chemically unstable and highly reactive, their levels are kept low by efficient antioxidant systems. Oxidative stress is caused by an imbalance in the pro-oxidant and antioxidant systems, when ROS are produced in excess or there is a reduction of the endogenous antioxidant defenses or inability to repair oxidative injuries. ROS damage several biological substrates such as proteins, DNA, RNA, or polyunsaturated fatty acids of cellular membranes. All of these markers of oxidative stress have been described in AD brain, suggesting the contribution of oxidative homeostasis imbalance to neuronal death and dysfunctions (*Nunomura et al., 2006*). ROS are also able to induce protein misfolding, Aβ deposition, tau phosphorylation and impaired mitochondrial functions.

Several studies showed that oxidative metabolic reactions and their by-products can play a role in the pathogenesis of neurodegenerative diseases. An explanation of the brain susceptibility to oxidative stress is due to its high oxygen metabolism rate, which generates higher levels of oxygen-reactive products. In addition, the brain has more limited antioxidant enzymatic and non-enzymatic systems than other organs, and a large amount of

iron, excitatory neurotransmitters and unsaturated lipids, which represent hot spots for oxidation. Several studies are consistent with the hypothesis that oxidative damage could be a causative early phenomenon on neurodegenerative pathogenesis and not a secondary consequence. In fact, in early stages of the disease Aβ peptides induce ROS generation in the mitochondria, deregulation of calcium homeostasis with energy failure subsequent oxidative stress prior to the development of plaques (*Reddy, 2006*). (**Figure 6**)

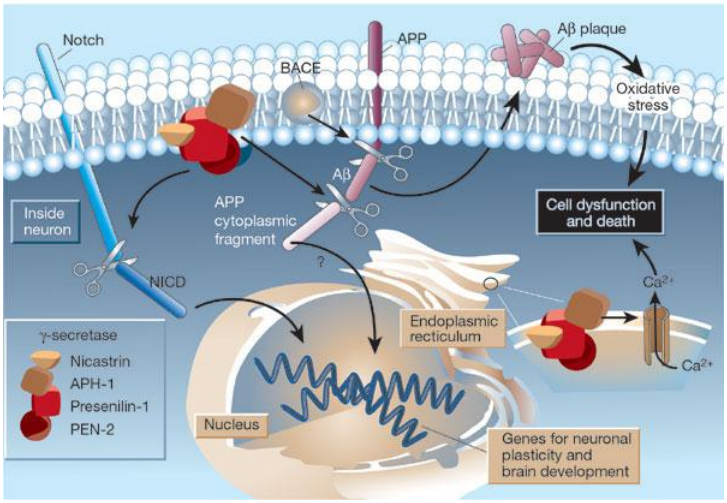


Figure 6. Aβ and oxidative stress.

Several studies revealed that polymorphisms in antioxidant genes might be a risk factor for AD development, and a small variation in antioxidant systems is associated with relevant modifications of the disease risk (*Piacentini et al., 2012*). Nevertheless, the relationship between antioxidant status and the susceptibility to neurodegenerative disorders have been still poorly investigated. Data concerning the imbalance between oxidative stress markers and antioxidant scavengers are conflicting. Recent studies showed that low total antioxidant status is associated with increased risk to develop atrophy in medial temporal lobe structures, such as hippocampus and entorhinal cortex. The risk of temporal lobe atrophy is increased by the presence of ε4 allele. In fact, apoE4 genotype plays an important role in the antioxidant status and its interaction with oxidative stress is related to

impaired lipid homeostasis contributing to neurodegeneration (*Zito et al., 2013*). Numerous studies demonstrated that AD patients have a global oxidative imbalance status with increased biological markers of oxidative stress, even if the antioxidant enzymes levels were unchanged (*Gironi et al., 2011*). However, the antioxidant capacity is significantly elevated in AD *post-mortem* brains and directly related to disease severity. Due to the high redundancy of the antioxidant system and the dual role of some antioxidant scavengers, the up-regulation of antioxidant defense might be a counteracting mechanism against the increased oxidative stress. In fact, in contrast to the general accepted role of the pathologic hallmarks, also the aggregation of Aβ and tau seems to be a compensatory and protective response to oxidative insults. Although the exact mechanism is unknown, Aβ plaques and NTFs oppose to oxidative stress probably chelating redox-active metals such as iron and copper.

1.9 ENDOGENOUS ANTIOXIDANT SYSTEMS

1.9.1 SUPEROXIDE DISMUTASE, GLUTATIONE PEROXIDASE, CATALASE AND GLUTATIONE

Cellular ROS are controlled by enzymatic and non-enzymatic antioxidants. As major antioxidant enzyme, superoxide dismutase (SOD) plays a crucial role in scavenging superoxide anion radicals primarily produced in the mitochondria as byproducts of oxygen metabolism and also deriving from extracellular oxidative insults. SOD exists in three different isoforms in the brain, with similar functions but different protein structures, metal cofactor requirement and cellular compartmentalization. The copper-zinc superoxide dismutase (Cu/Zn SOD or SOD1) is localized in the cytosol, lysosomes and mitochondrial intramembrane space. The manganese superoxide dismutase (Mn SOD or SOD2) is localized in the mitochondrial matrix, and the SOD3 is localized in the extracellular space. The function of SOD is to convert superoxide into oxygen and hydrogen peroxide. Other important antioxidant enzymes are glutathione peroxidase and catalase. Glutathione

peroxidase catalyzes the reduction of hydrogen peroxide or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH). In mammalian tissues, there are four major selenium-dependent glutathione peroxidases (GPX) and among these isoforms GPX1 is localized in glial cells, in which its activity is tenfold higher than in neurons. Catalase is a ferriheme-containing enzyme that converts the hydrogen peroxide into water. It is localized in peroxisomes and may also be found in cytoplasm and mitochondria. It has a minor role in the scavenging of hydrogen peroxide at low levels but becomes more important at higher levels of hydrogen peroxide production (*Gandhi and Abramov, 2012*). The main non-enzymatic antioxidant in CNS is glutathione (GSH), a small tripeptide composed by glutamate, cysteine and glycine characterized by a reactive thiol group and present in millimolar concentration in the brain. Free glutathione is mainly present in its reduced form GSH, but under oxidative conditions it can be converted into the oxidized form GSSG. Reduced GSH can act directly with free radicals, notably superoxide radicals, hydroxyl radicals, nitric oxide, and carbon radicals for their removal. GSH peroxidase and GSH reductase can act enzymatically to remove the hydrogen peroxide and maintain GSH in a reduced state (*Dringen and Hirrlinger, 2003*). GSH is essential to preserve the cellular reducing environment to provide the correct activity of several enzymes. An important non-enzymatic antioxidant molecule in the central nervous system is the lipid soluble vitamin E. Although its function is not fully understood, it seems to neutralize the effect of peroxide and prevent lipid peroxidation in membranes.

1.9.2 THIOREDOXIN AND GLUTAREDOXIN

Thioredoxin (TRX) is a multifunctional and ubiquitous small protein (12 kDa) containing an active thiol (reduced)/disulphide (oxidized) site with oxido-reductase activity. The major TRX isoforms are cytosolic TRX1 and mitochondrial TRX2. Thioredoxins have a highly conservative amino acid sequence in the active centre (-Cys-Gly-Pro-Cys-) containing two cysteine residues that are oxidized into corresponding disulphides. The disulphides formed in the active centres of TRX1 and TRX2 are reduced by the NADPH-

dependent enzyme thioredoxin reductase (TrxR), which is present as two major isoforms cytosolic (TrxR1) and mitochondrial (TrxR2). The TRX system plays a key role in cell function by limiting oxidative stress directly via antioxidant effects and indirectly by protein–protein interactions. Cellular redox regulation of many cellular processes is provided by the cooperation between the TRX and glutathione system (*Cho et al., 2003*). TRX is predominantly a cytosolic protein that can translocate to the nucleus in response to stress conditions. Into the cells TRX regulates the activity of several redox-sensitive transcription factors. TRX is able to inhibit apoptosis signaling not only by scavenging intracellular ROS but also by inhibiting the activity of apoptosis signal-regulating kinase 1 (ASK1) in a redox-sensitive manner. Reduced TRX1 forms a complex with the N-terminal domain of ASK1, suppressing its activity and stimulating its degradation (*Liu and Min, 2002*). Studies suggest that TRX1 is involved in the nerve growth factor (NGF) signaling pathway. NGF is a neurotrophic factor which has profound effects on CNS promoting of neuronal survival and differentiation. NGF activates TRX1 expression and also induces nuclear translocation of TRX1. Thus, thioredoxin appears to be a neurotrophic co-factor that intensifies the effect of NGF on neuronal differentiation and regeneration (*Masutani et al., 2004*).

Glutaredoxin (GRX) is a 12 kDa dithiol protein structurally similar to TRX, with a redox-active site characterized by the amino acid sequence -Cys-Pro-Tyr-Cys-. Mammalian cells contain three isoforms of GRX: the classical dithiol cytosolic GRX1, mitochondrial and nuclear GRX2, and a monothiol GRX5 localized in the mitochondria. In presence of an oxidative environmental, the disulphide site of GRX is reduced by two molecules of glutathione which in turn is reduced by the NADPH-dependent glutathione reductase enzyme. GRX possesses the same principal functions of TRX in the maintenance of the environmental redox homeostasis and participates in cellular processes such as cellular differentiation, regulation of transcription factors and apoptosis. GRX, such as TRX, acts as negative regulator of ASK1 by binding the C-terminal domain of the kinase and leading to an inactive complex (*Lillig and Holmgren, 2007*).

1.9.3 ROLE OF THIOREDOXIN AND GLUTAREDOXIN IN ALZHEIMER'S DISEASE

The role of TRX1 in AD seems to be important since brain tissues of AD patients show low TRX1 levels, increased TrxR activity and a marked accumulation of Aβ peptide (*Lovell et al., 2000*). These data suggest that loss of TRX1 may contribute to the increased neuronal oxidative stress and cell death observed in AD. Another study reported increased GRX1 and decreased TRX1 neuronal levels in the hippocampus and frontal cortex of AD brains. The loss of TRX1 function was also found in transgenic mice overexpressing Aβ. Furthermore, in human SH-SY5Y neuroblastoma cells the exposure to Aβ peptides caused a strong and early oxidation of both intracellular TRX1 and GRX1, whereas overexpression of these proteins completely protected cells from the Aβ toxicity (*Akterin et al., 2006*). Thus, Aβ toxicity might be mediated by oxidation of TRX1 and GRX1 and subsequent induction of apoptosis process through the activation of ASK1. These data suggest that the deregulation of TRX1 and GRX1 antioxidant proteins could be an important event in the pathogenesis of AD. Furthermore, the gradual loss of antioxidant functions during aging can contribute to oxidative stress and neuronal impairment of hippocampus and frontal cortex (*Venkateshappa et al., 2012*). TRX plays also a role in immune responses acting as chemokine and cytokine, and might be an important link between oxidative stress and inflammation. All these findings imply that acquired or genetic dysfunction of TRX and/or GRX could predispose neurons for degeneration and promote AD pathogenesis.

2. AIM OF THE STUDY

AD is a multifactorial disease characterized by the interaction between genetic and environmental risk factors. Several evidences suggest that lifestyle factors such as dietary habits may increase cognitive impairment and the risk to develop AD, especially in apoE4 carries (*Kivipelto et al., 2008*). However, the mechanisms by which apoE4 interacts with the environmental risk factors leading to an increased susceptibility to the neurodegeneration are still unknown.

The human apoE3 and apoE4 targeted replacement (h-apoE TR) mice have been used as a model to study the role of apoE genotype in neurodegenerative processes related to AD. ApoE4 is associated with age-dependent memory impairments and loss of synaptic integrity in h-apoE TR mice (*Bour et al., 2008; Klein et al., 2010*). A recent study demonstrated that apoE4 mice fed with HCD show cognitive deficits starting at 6 months of age, suggesting a synergic interaction between HCD and apoE4 in the impairment of spatial and learning memory. In this animal model, the hippocampus of apoE4 HCD mice present decreased levels of proteins involved in the regulation of memory processes, such as Arc, β-catenin and BDNF, and increased levels of the kinase GSK3β although not associated to changes in tau phosphorylation rate. In contrast, apoE3 mice are resistant to the deleterious effects of HCD on both behavior and memory-related proteins (*Maioli et al., 2012*).

Several studies in asymptomatic elderly individuals demonstrated a significant correlation between apoE4 genotype, increased Aβ deposition and neurodegeneration. It has been suggested that the decrease of brain volume in the earliest stages of AD, especially in the entorhinal cortex, is induced by Aβ deposition only in individuals with phosphorylated tau (p-tau). A recent study hypothesized that, during preclinical AD, apoE4 genotype influences Aβ production but not tau phosphorylation and that Aβ-associated neurodegeneration occurs only in presence of p-tau (*Desikan et al., 2012*). From this point of view, apoE4 genotype can be a critical link between Aβ accumulation and tau phosphorylation in AD brain neurodegeneration.

Increasing evidences suggest that the oxidative stress may play a role in the earliest phases of AD pathogenesis probably affecting the protective functions of cellular antioxidant systems. The Aβ deposition is implicated in oxidative stress and neuronal death, although the mechanism underlying its toxicity is not completely understood. The endogenous antioxidant proteins TRX1 and GRX1 seem to play an important role in the protection of neurons against the alteration of redox homeostasis and neuronal apoptosis. Although the role of these proteins in AD is largely unknown, some researches suggest their involvement in cellular protection against AD-associated oxidative stress. An important study demonstrated that the neurons of hippocampus and frontal cortex of AD patients present a significant decrease of TRX1 and increase of GRX1 protein levels (*Akterin et al., 2006*). The same study also showed that increasing levels of TRX1 and GRX1 by transient SH-SY5Y cells transfection protects against Aβ42 toxicity. The Aβ induces an early and strong oxidation of theses antioxidant proteins and activates apoptosis pathways, confirming the involvement of these antioxidant proteins in cellular protection. These data suggest that deregulation of TRX1/GRX1 antioxidant systems could play an important role in the pathogenesis of AD.

PIN1 plays a central role in the regulation of cellular processes related to aging. In early stages of AD PIN1 is oxidized, suggesting its function in the response to oxidative stress (*Sultana et al., 2006*). PIN1 is also involved in the induction of tau phosphorylation and, in physiological conditions, might protect against Aβ toxicity promoting the non amyloidogenic process of APP. Recent studies reported a down-regulation of PIN1 protein levels in *post-mortem* AD brains, although PIN1 function may vary on the course of the disease. In fact, it has been reported that frontal cortex of AD brains has increased PIN1 gene expression associated to high p-tau levels (*Wang et al., 2007 b*). A recent study also showed that PBMCs of late onset AD patients display an increased PIN1 gene expression levels (*Arosio et al., 2012*).

SIRT1 is a deacetylase enzyme that presents neuroprotective, antioxidant and transcriptional functions, plays an important role also in the synaptic plasticity, learning and memory. SIRT1 seems to be involved in the reduction of Aβ deposition and tau phosphorylation. It has been reported that SIRT1 gene expression is decreased in the

parietal cortex of AD patients (*Julien et al., 2009*). These data support the involvement of SIRT1 in the normal brain physiology and in the pathogenesis of neurological disorders.

PSEN1 plays a crucial role in the amyloidogenic processing of APP mediated by the enzyme complex γ-secretase. It is a genetic risk factor strongly associated to familial cases of AD. PSEN1 also modulates different proteins involved in cellular signaling pathways and it is implicated in synaptic function and plasticity. It has been demonstrated a reduced PSEN1 protein level in the hippocampus of AD patients (*Davidsson et al., 2001*). The impairment and loss of PSEN1 functions seem also to be involved in tau phosphorylation (*Pigino et al., 2003*).

Deficits of BNDF neurotrophin synthesis might participate in the impairment of cellular survival and differentiation leading to AD pathogenesis. In fact, clinical evidences showed a decrease of BDNF levels in the hippocampus, and frontal and temporal cortices of AD patients (*Murer et al., 1999*). The precursor pro-BDFN is decreased in the hippocampus and parietal cortex even in pre-clinical AD stages. However, some studies reported an increased BDFN level in serum of early AD patients and an up-regulation of BDNF gene expression and protein levels in transgenic mice model of AD (*Burbach et al., 2004*). These results suggest that further studies are necessary to clarify the changes of BNDF in AD process.

2.1 SPECIFIC AIMS

Based on these considerations on the complex mechanisms underlying AD pathogenesis, the aim of this research project was to evaluate the expression modifications of different proteins implicated in neurodegenerative processes, using *in vitro* and *in vivo* models of AD.

1) **Study of the effects of amyloid beta peptides and apoE genotype on the PIN1, SIRT1, PSEN1 and BDNF gene expression**

The present study investigated the modulation of these proteins involved in neurodegenerative processes evaluating the gene expression levels by Real Time-PCR assay, using as *in vitro* model the SH-SY5Y human neuroblastoma cell line exposed to Aβ peptides for 5, 24 and 48 hours.

It has been investigated the effect of the apoE genotype on the modulation of PIN1, SIRT1, PSEN1 and pro-BDNF in brain areas involved in AD pathology, using 18 months old apoE3 and apoE4 transgenic mice model fed with normal diet. The levels of gene expression were measured in the hippocampus, and in the frontal, entorhinal and parietal cortices of apoE mice using the apoE3 as control group.

2) **Study of the effects of the lipoproteins apoE3 and apoE4 on the antioxidant proteins TRX1 and GRX1**

The first aim of this study was to investigate the effect of human recombinant proteins apoE3 and apoE4 on the levels of TRX1 and GRX1 in an *in vitro* model. The protein variations were assessed by immunoblotting, using BE(2)-M17 human neuroblastoma cells exposed to the lipoproteins for 5, 24 and 48 hours.

The second aim was to study the interaction of apoE genotype on the regulation of TRX1 and GRX1 antioxidant system. In particular, the interaction of the genotype with different diets, normal diet (ND) and high carbohydrate diet (HCD), was studied. The protein levels in the hippocampus of 6 months old apoE3 and apoE4 ND and HCD mice model were measured, using apoE3 ND as control group.

3. MATERIALS AND METHODS

3.1 CELL CULTURES

Human neuroblastoma cell lines SH-SY5Y and BE(2)-M17 were used.

SH-SY5Y cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Lonza, Italy), supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

BE(2)-M17 cells were cultured in Opti-MEM medium (Gibco, Sweden) supplemented with 10% (v/v) of FBS.

Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. In all experiments, cells were seeded and allowed to reach 80% confluence before starting the treatment.

3.2 CELL TREATMENTS

Cells were exposed to different schedule of amyloid beta (25-35) peptides and apolipoprotein E3 and E4 as follows.

- AMYLOID BETA (25-35) PEPTIDE. Amino acid sequence: Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met. Aβ 25-35 peptides (Sigma-Aldrich, Italy), were dissolved in sterile distilled water to obtain a stock solution at concentration of 1 mM. The resulting unaggregated peptides were incubated at 37 °C for 72 h, gently mixing once in a while to favour the aggregation. The stock solution was stored at -80 °C until use and diluted into the cell medium supplemented with 2,5% FBS at final concentration of 25 μM.

- APOLIPOPROTEIN E3 AND E4: Human recombinant apoE3/4 isoforms (Reliatech, Sweden) were dissolved in a sterile water solution of sodium phosphate pH 7.8 or sterile distilled water, respectively, to obtain a stock solution of 10 μM. The solution was stored to -20°C until use and diluted in serum free Opti-MEM medium at final concentration of 100 nM.

3.3 MTT CELL VIABILITY ASSAY

Cell viability was measured using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (*Mosmann, 1983*). Briefly, cells were plated 24 h before the treatment at 37°C on 24-well plates at a density of 3 x 10^4 cells/well and grown to subconfluence as previously described. After removal of the growth medium, the cells were rinsed with PBS and treated with 25μM Aβ 25-35 in DMEM containing 2% FBS, for 5, 24 and 48 h. After the required time of exposure, the medium was removed and replaced with fresh DMEM without phenol red and serum, containing MTT solution (0.5 mg/mL in PBS). The cells were then incubated in the dark at 37°C and 5% CO_2 for 3 h. After removal of the supernatant, a DMSO–EtOH (4:1) mixture was added to each well for the solubilization of formazan crystals. The optical densities were then read using a microplate spectrophotometer (GENIos Tecan) at 590 nm. Data were expressed as a percentage of the OD value of treated cells compared to untreated ones.

3.4 REVERSE TRANSCRIPTION AND REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (qRT-PCR)

Total RNA was extracted according to the method described by Chomczynski and Sacchi (*1987*). Briefly, single tissue samples were homogenized in 10-20 volumes of TRI Reagent solution containing phenol and guanidine thiocyanate (Ambion Inc. Italy). After incubating for 5 min at room temperature, a volume of 100 μl of chloroform was added to the

homogenate, mixed well, incubated at room temperature for 15 min, and then centrifuged at 12,000 x g for 15 minute at 4°C. The aqueous phase was transferred to a fresh tube and mixed with 500 µl of isopropanol, incubated for 15 min at room temperature and the RNA pellet was isolated by centrifugation at 12,000 x g for 8 min at 4°C. The RNA was washed twice with 75% ethanol, centrifuged at 12,000 x g for 5 min, dried under vacuum and then dissolved in 25 µl of RNase-free water. Total RNA was digested with DNase RNasefree enzyme to eliminate genomic DNA content and then quantified by measurement of absorbance at 260 nm (1 OD/ml = 40 µg RNA/ml). The purity of the total RNA was provided by a ratio value OD260/OD280 > 2, and its integrity was assessed by electrophoresis with a 1% agarose gel. The RNA extraction protocol was the same for treated cells, previous removal of the culture medium and two washes with ice-cold PBS. A volume of 1 ml of TRI Reagent solution per 5-10 x 106 cells was used.

RNA samples were subjected to DNase treatment and converted to cDNA with the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) by using random hexamers (0.45 µg of total RNA in a final reaction volume of 20 µl). The cDNAs were subsequently diluted x 3 with Nuclease-free water. Relative abundance of each mRNA species was assessed by real-time RT-PCR employing 2 µl of the diluted cDNA samples in a final volume of 20 µl, using SYBR Green PCR or TaqMan Gene expression Master Mix on an StepOne Detection System (Applied Biosystems, Foster City, CA, USA). To provide precise quantification of initial target in each PCR reaction, the amplification plot was examined and the point of early logarithmic phase product accumulation was defined by assigning a fluorescence threshold above background, defined as the threshold cycle (Ct) number. Differences in threshold cycle number were used to quantify the relative amount of PCR target contained within each well. Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2^{-DDCt}) for statistical analysis. All data were normalized to the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. After PCR using SYBR Green dye, a dissociation curve (melting curve) was constructed in the range of 60 ºC to 95 ºC in order to evaluate the specificity of the amplification products. The primers used for PCR amplification are listed in the (**Table 1 and 2**). Each sample was run in

triplicate and data were analyzed using StepOne Software v. 2.2 (Applied Biosystems). Data are expressed as a normalized percentage of control group.

Table 1. Sequence of the designed primers used for PCR amplification of human PIN1, SIRT1, PSEN1, BDNF and GAPDH genes (Eurofins).

PRIMERS	**Forward (5' – 3')**	**Reverse (3' – 5')**
PIN1	GACGAGGAGAAGCTGCCGCC	CAGGCTCCCCCTGCCCGTTT
SIRT1	GCGATTGGGTACCGAGATAA	GTTCGAGGATCTGTGCCAAT
PSEN1	TTGCGGTCCTTAGACAGCTT	AGGACAACGGTGCAGGTAAC
BDNF	AGAAGGCAGCCCTAGGAAAC	GCATCGATGTCGAAAAACCT
GADPH	ATTCCACCCATGGCAAATTC	TGGGATTTCCATTGATGACAAG

Table 2. TaqMan probes for PCR amplification of mouse PIN1, SIRT1, PSEN1, TRX1 and GAPDH genes (Applied Biosystems) and sequence of the designed primers used for the amplification of mouse pro-BDNF and GAPDH genes (Eurofins).

	TAQMAN PROBES	
	PIN1	Mm00777269_mH
	SIRT1	Mm00490758_m1
	PSEN1	Mm00501184_m1
	TRX1	Mm00726847_s1
	GADPH	Mm99999915_g1
PRIMERS	**Forward (5' – 3')**	**Reverse (3' – 5')**
pro-BDNF	GCGGCAGATAAAAAGACTGC	CCTATGAATCGCCAGCCAAT
GADPH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

3.5 PROTEIN EXTRACTION AND IMMUNOBLOTTING

After treatments, cells were washed with ice-cold phosphate buffer saline (PBS), harvested and collected by centrifugation at 4 °C (2000 × g, 5 min). Cells were lysed on ice with a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA with freshly added protease inhibitor cocktail (1:500, Sigma–Aldrich) and phosphatase inhibitor cocktail I (1:100, Sigma–Aldrich). Lysates were stored frozen at −80 °C. Equivalent amounts of proteins were separated using 14% acrylamide gels, and transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany). Incubations with primary antibodies (**Table 3**) were performed overnight at 4°C. Membrane was incubated with secondary anti-rabbit or anti-mouse IgG (1:2000, Amersham Biosciences, England) for 1 h at 22°C. Immunoreactivity was detected by the ECL detection system (Amersham Biosciences, England). Some immunoblots were stripped using Restore™ Western Blot Stripping buffer (Pierce, USA) at room temperature for 15 min, and then re-blotted with other antibodies. The relative density of the immunoreactive bands was calculated from the optical density (OD) multiplied by the area of the selected band using ImageJ 1.383 software (NIH, MA).

Table 3. List of primary antibodies anti human proteins TRX1, GRX1 and β-ACTIN (internal control)

ANTIBODIES	
TRX1 (rabbit)	IMCO Corporation (Sweden) ATRX-08
GRX1 (goat)	IMCO Corporation (Sweden) AGRX-03
β-ACTIN (mouse)	Sigma Aldrich (MO, USA)

3.6 ANIMALS

In order to investigate the interaction of different genes and proteins involved in neurodegenerative disorders with the *apoE* genotype, we used as animal model the human *apoE* Targeted Replacement (TR) mice. These transgenic mice express human apoE3 and apoE4, under the control of the murine apoE regulatory sequences and on the C57BL/6J background. apoE3 and apoE4 TR mice were purchased by Taconic Farms (USA):

Homozygous B6.129P2-*Apoetm3(APOE*4)Mae* N8

Homozygous B6.129P2-*Apoetm2(APOE*3)Mae* N8

The model was created by targeting the murine apoE gene for replacement with the human apoE4 and apoE3 allele in E14TG2a ES cells and injecting the targeted cells into blastocysts. Resultant chimeras were backcrossed to C57BL/6 for seven generations (N7). The mice were backcrossed once more (N8) and embryo transfer derived. The colony was maintained through mating homozygotes. Research Breeding Agreement was delivered by Taconic Farms. The pups were suckled by mothers fed with different diets and after weaning, at 3 weeks of age, they were treated for 6 months with normal diet (ND) or high carbohydrate diet (HCD) containing 70% of carbohydrates (Mucedola s.r.l., Milano).

The mice were divided in groups of six, housed in individually ventilated cages (Tecniplast, Italia) with water and food *ad libitum* and controlled conditions of light (from 7.00 a.m. to 7.00 p.m.), temperature (22±2 °C) and humidity (65%). The experiments were performed using 6 months and 18 months old mice. The experimental protocol was approved by a local bioethics committee, while the procedures and animal comfort were controlled by the University Veterinary Service. All efforts were made to minimize animal suffering and the number of animals used was kept to a minimum by the experimental design. All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and National (Ministry of Health) laws and policies (authorization no. 139/2012-B). Animal experiments were carried out in the Department of Pharmacology of the University of Bologna with the approval of the local Ethical committee (Veterinary Service of the University of Bologna).

3.7 TISSUES SAMPLING

Mice were killed by cervical dislocation and their brains were quickly removed. The brain areas (hippocampus, entorhinal, parietal and frontal cortices) were rapidly dissected out, frozed in dry ice and stored at -80°C until use for RNA and proteins extraction.

3.8 IMMUNOBLOTTING

The dissected brains were homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA) with protease and phosphatase inhibitor cocktail (Sigma-Aldrich, MO) at dilution 1:500 added freshly, and incubated 30–60 minutes on ice before centrifugation (13600 g for 10 min) at 4°C. Protein levels were quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were mixed with equal volume of tricine gel sample buffer (0.16 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue, 0.1 M DTT added fresh) and then electrophoresis was performed. Equal amounts of protein (40 μg) were separated using 12% acrylamide gel under reducing conditions and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany). After transfer, the membrane was blocked in 5% nonfat milk buffer in Tris-Buffered saline (TBS) for 1h at room temperature and then incubated overnight at 4°C with the primary antibody at proper dilutions (**Table 3**). After being probed with the primary antibody and washed with TBS-Tween buffer (TBS and 0.01% Tween 20), membranes were incubated with peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat immunoglobulin G (IgG) at 1:2000 dilution (Amersham Biosciences, Little Chalfont, UK) in blocking buffer for 2 hr at 22°C. Immunoreactivity was detected by the ECL detection system (Amersham Biosciences, Little Chalfont, UK). Some immunoblots were stripped using RestoreTM Western Blot Stripping buffer (Pierce, Rockford, IL, USA) at room temperature for 15 minutes, and then re-blotted with other antibodies. The relative density of the immunoreactive bands was calculated from the optical density (OD) multiplied by the area of the selected band using ImageJ 1.383 software (NIH, MA). The

variations of proteins levels were then evaluated with respect to β-actin as internal control. The results were expressed as the ratio of protein levels compared to the controls, as 100%.

3.9 DATA ANALYSIS

Results were expressed as mean ± S.E.M (standard error of the mean). Data of experimental groups were processed by using Student's t-test or by analysis of variance (one-way and two-way ANOVA) followed by Dunnett *post hoc* test. Differences were considered statistically significant at *$p < 0.05$. Statistical data analyses were performed using the Program GraphPad version 4.03 (GraphPad Software, San Diego, CA, USA).

4. RESULTS

4.1 VIABILITY OF SH-SY5Y CELLS EXPOSED TO Aβ

The exposure of SH-SH5Y neuroblastoma cells to 25 μM Aβ peptides for 5, 24 and 48 hours showed a significant decrease of viability in a time-dependent manner: 88.05 ± 0.99 *versus* control 100 ± 0.70, $p<0.01$, at 5 hours; 81.27 ± 1.34 *versus* control 100 ± 1.06, $p<0.01$, at 24 hours; 79.80 ± 0.82 *versus* control 100 ± 0.83, $p<0.01$, at 48 hours (**Figure 7**).

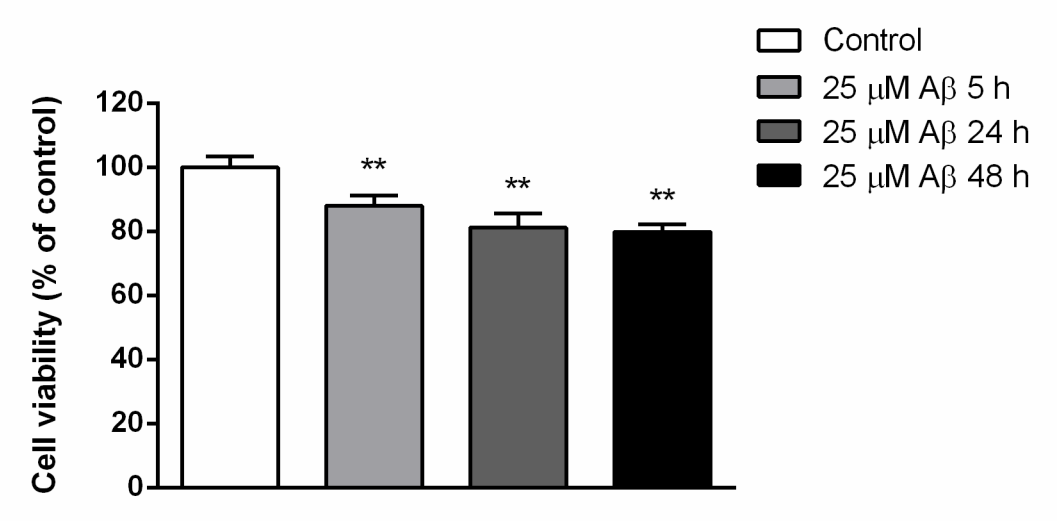


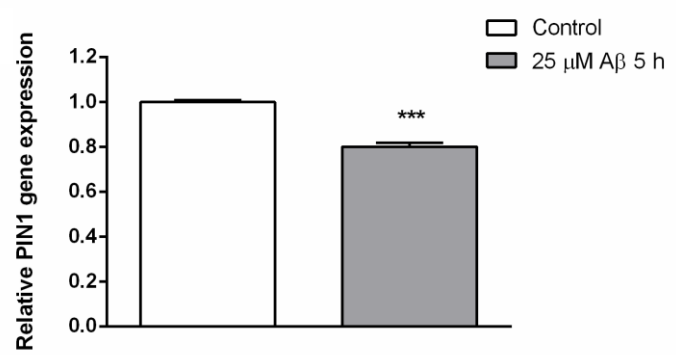
Figure 7. Viability of SH-SY5Y human neuroblastoma cells exposed to 25 μM Aβ (25-35) peptides for 5, 24 and 48 hours evaluated by the MTT assay. Data are expressed as percentage of absorbance values of treated cells compared to control untreated cells (100%) and reported as a mean ± S.E.M. of three independent experiments performed in triplicate. Data were analyzed by one-way Anova, followed by Dunnett *post hoc* test (**$p < 0.01$).

4.2 GENE EXPRESSION IN SH-SY5Y EXPOSED TO Aβ

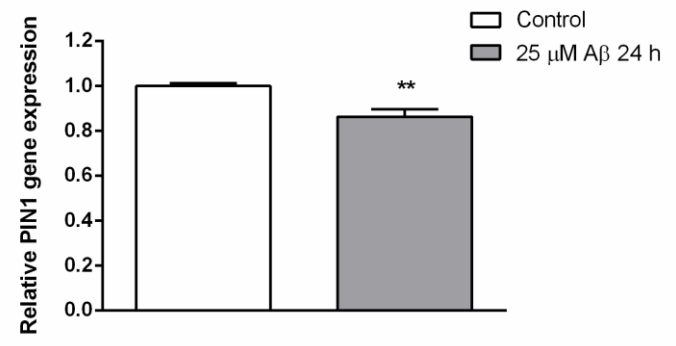
PIN1 gene expression

The level of PIN1 gene expression was significantly decreased in cells exposed to 25 μM Aβ for 5 hours (0.80 ± 0.04 *versus* control 1.00 ± 0.02, $p<0.001$) and for 24 hours (0.86 ± 0.08 *versus* control 1.00 ± 0.03, $p<0.01$) compared to unexposed control cells (**Figure 8a** and **b**, respectively). No changes of gene expression were observed after cell exposure to 25 μM Aβ for 48 hours (**Figure 8c**; **Table 4**).

a



b



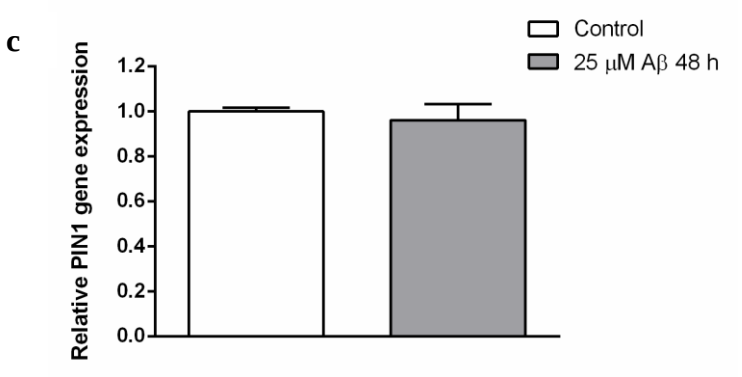


Figure 8. PIN1 relative gene expression was measured by real-time PCR in SH-SY5Y neuroblastoma cells exposed to 25 μM Aβ (25-35) peptides for 5 (**a**) 24 (**b**) and 48 (**c**) hours. Bars represent 2^{-DDCt} values calculated by Delta-Delta Ct (DDCt) method. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as mean ± S.E.M percent of control untreated cells for three independent experiments (***p <0.001; **p<0.01 *versus* controls; Student's t-test)

Table 4

	PIN1 gene expression level	
Time	**Control cells**	**Aβ exposed cells**
5 hours	1.00 ± 0.02	**0.80 ± 0.04*****
24 hours	1.00 ± 0.03	**0.86 ± 0.08****
48 hours	1.00 ± 0.04	0.96 ± 0.17

**p<0.01; ***p<0.001

SIRT1 gene expression

The level of SIRT1 gene expression was significantly decreased in cells exposed to 25 μM Aβ for 5 hours (0.78 ± 0.07 *versus* control 1.00 ± 0.04, p<0.001) and for 24 hours (0.74 ± 0.10 *versus* control 1.00 ± 0.08, p<0.001) compared to unexposed control cells (**Figure 9a** and **b**, respectively). No changes in the gene expression were observed following cell exposure to 25 μM Aβ for 48 hours (**Figure 9c**; **Table 5**).

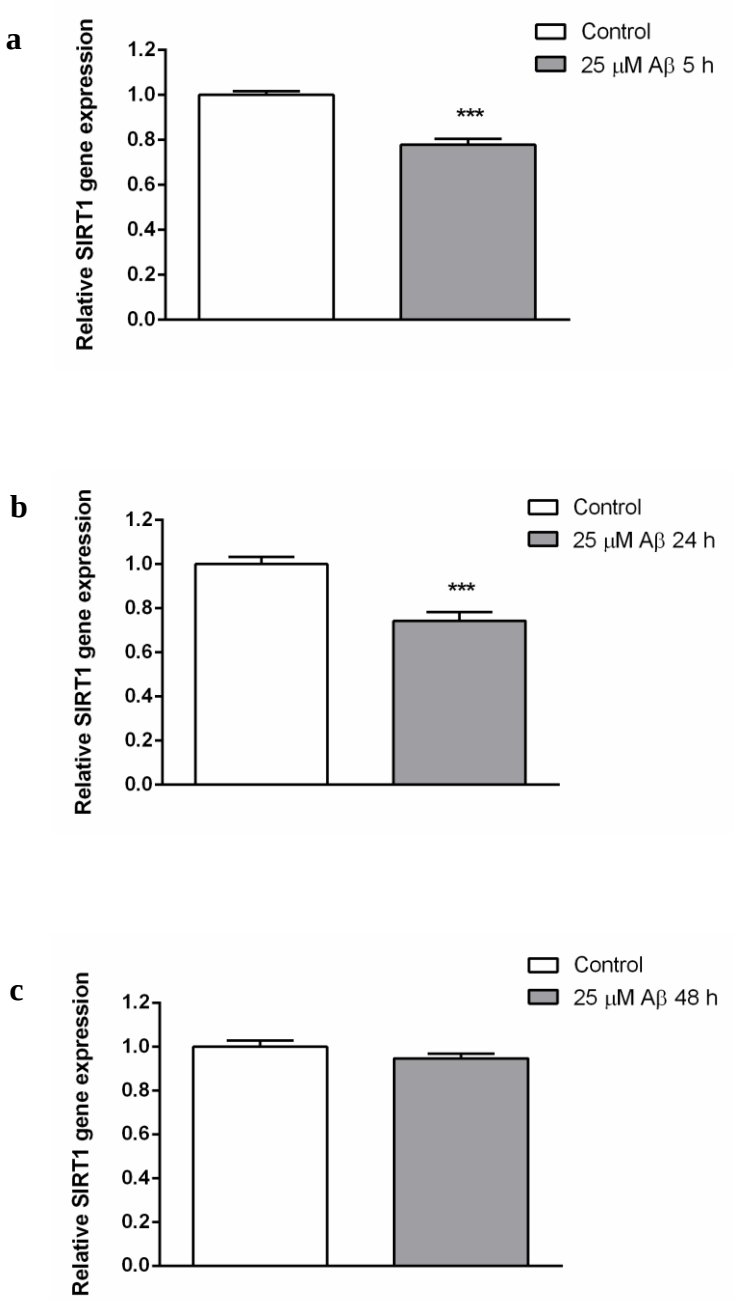


Figure 9. SIRT1 relative gene expression was measured by real-time PCR in SH-SY5Y neuroblastoma cells exposed to 25 μM Aβ (25-35) peptides for 5 (**a**) 24 (**b**) and 48 (**c**) hours. Bars represent 2^{-DDCt} values calculated by Delta-Delta Ct (DDCt) method. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as mean ± S.E.M percent of control untreated cells for three independent experiments (***p <0.001; *versus* controls; Student's t-test)

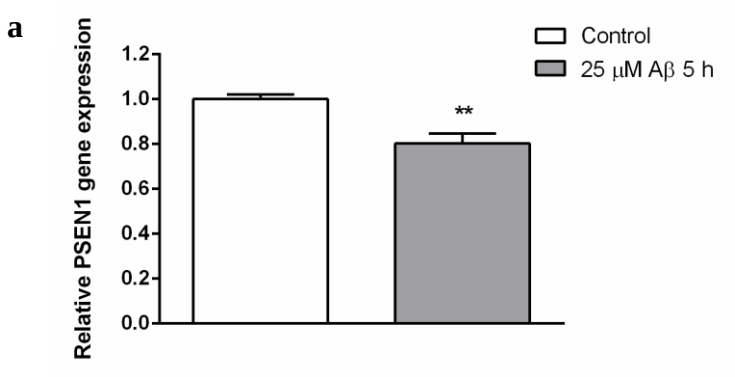
Table 5

	SIRT1 gene expression level	
Time	**Control cells**	**Aβ exposed cells**
5 hours	1.00 ± 0.04	**0.78 ± 0.07*** **
24 hours	1.00 ± 0.08	**0.74 ± 0.10*** **
48 hours	1.00 ± 0.07	0.95 ± 0.05

***p<0.001

PSEN1 gene expression

The PSEN1 gene expression was significantly decreased in cells exposed to 25 μM Aβ at all time-points of the experimental protocol compared to unexposed control cells: 5 hours (0.80 ± 0.10 *versus* control 1.00 ± 0.04, p<0.01); 24 hours (0.69 ± 0.06 *versus* control 1.00 ± 0.06, p<0.001); 48 hours (0.82 ± 0.03 *versus* control 1.00 ± 0.05, p<0.001) (**Figure 10a**, **b**, **c**, respectively; **Table 6**).



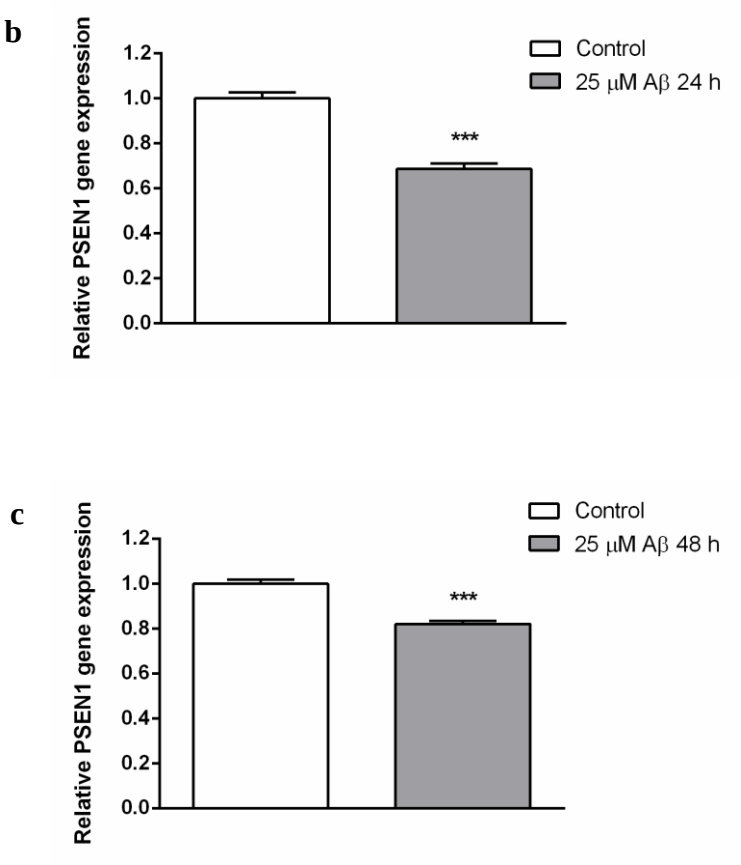


Figure 10. PSEN1 relative gene expression was measured by real-time PCR in SH-SY5Y neuroblastoma cells exposed to 25 μM Aβ (25-35) peptides for 5 (**a**) 24 (**b**) and 48 (**c**) hours. Bars represent 2^{-DDCt} values calculated by Delta-Delta Ct (DDCt) method. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as mean ± S.E.M percent of control untreated cells for three independent experiments (***p <0.001; **p<0.01 *versus* controls; Student's t-test)

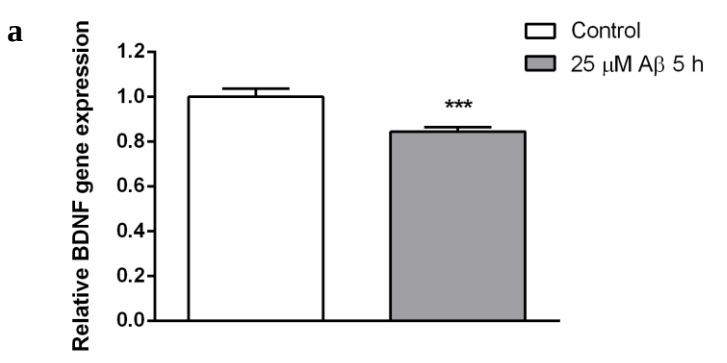
Table 6

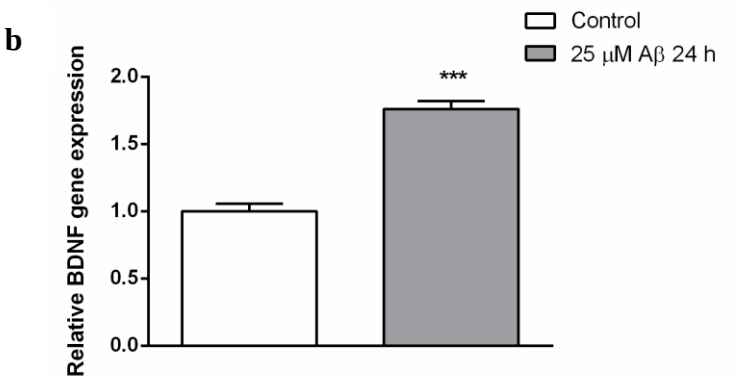
	PSEN1 gene expression level	
Time	**Control cells**	**Aβ exposed cells**
5 hours	1.00 ± 0.04	**0.80 ± 0.10****
24 hours	1.00 ± 0.06	**0.69 ± 0.06*****
48 hours	1.00 ± 0.05	**0.82 ± 0.03*****

**p<0.01; ***P<0.001

BDNF gene expression

The BDNF gene expression was significantly decreased in cells exposed to 25 μM Aβ for 5 hours compared to unexposed control cells (0.84 ± 0.05 *versus* control 1.00 ± 0.09, $p<0.01$) (**Figure 11a**). In contrast, the BDFN gene expression was significantly increased in cells exposed to Aβ for 24 hours (1.76 ± 0.15 *versus* control 1.00 ± 0.14, $p<0.001$) and 48 hours (1.72 ± 0.25 *versus* control 1.00 ± 0.13, $p<0.001$) (**Figure 11b** and **c**, respectively; **Table 7**).





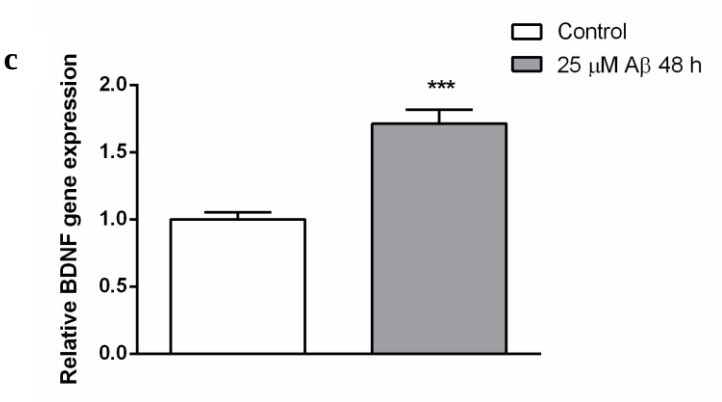


Figure 11. BDNF relative gene expression was measured by real-time PCR in SH-SY5Y neuroblastoma cells exposed to 25 μM Aβ (25-35) peptides for 5 (**a**) 24 (**b**) and 48 (**c**) hours. Bars represent 2^{-DDCt} values calculated by Delta-Delta Ct (DDCt) method. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as mean ± S.E.M percent of control untreated cells for three independent experiments (***p <0.001; *versus* controls; Student's t-test)

Table 7

	BDNF gene expression level	
Time	**Control cells**	**Aβ exposed cells**
5 hours	1.00 ± 0.09	**0.84 ± 0.05*****
24 hours	1.00 ± 0.14	**1.76 ± 0.15*****
48 hours	1.00 ± 0.13	**1.72 ± 0.25*****

****p<0.001

4.3 GENE EXPRESSION IN APOE3/E4 TRANSGENIC MICE

PIN1 gene expression

The PIN1 gene expression levels were significantly increased in the hippocampus of 18 months old apoE4 mice (1.23 ± 0.09 *versus* control apoE3 group = 1.00 ± 0.07, p<0.01) (**Figure 12a**). In contrast, apoE4 genotype is associated with a significant reduction of PIN1 gene expression in the entorhinal cortex (0.75 ± 0.05 *versus* control apoE3group = 1.00 ± 0.05, p<0.001) and in the parietal cortex (0.68 ± 0.10 *versus* control group apoE3=

1.00 ± 0.11, p<0.001) (**Figure 12c** and **d**, respectively). No significant changes of PIN1 gene expression were observed in the frontal cortex (**Figure 12b**; **Table 8**).

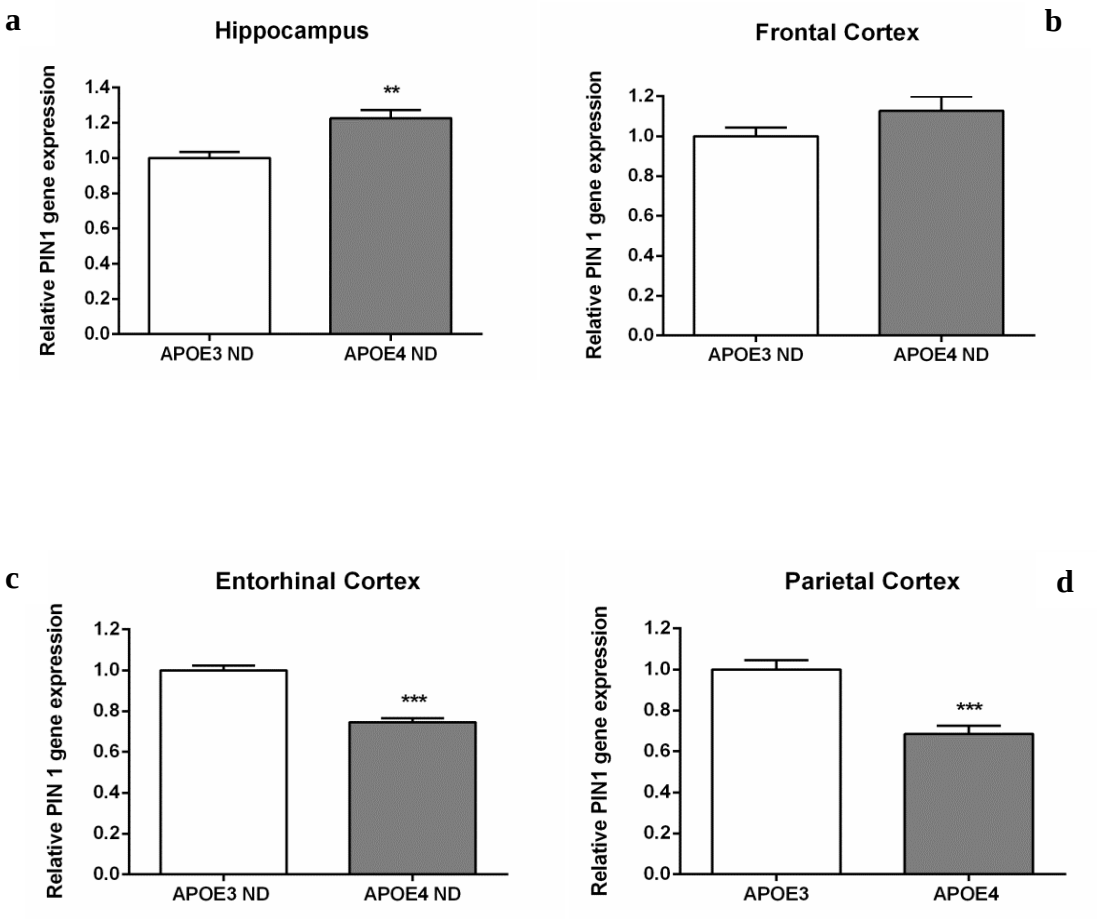


Figure 12. PIN1 gene expression was measured by real-time PCR in hippocampus (**a**), frontal cortex (**b**), entorhinal cortex (**c**) and parietal cortex (**d**) of 18 months old apoE 3 and apoE4 transgenic mice fed with normal diet (ND). Bars represent 2^{-DDCt} value calculated by Delta-Delta Ct (DDCt) method (n=6). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as means ± SEM relative to control apoE3mice (***p <0.001; **p<0.01. Student's t-test).

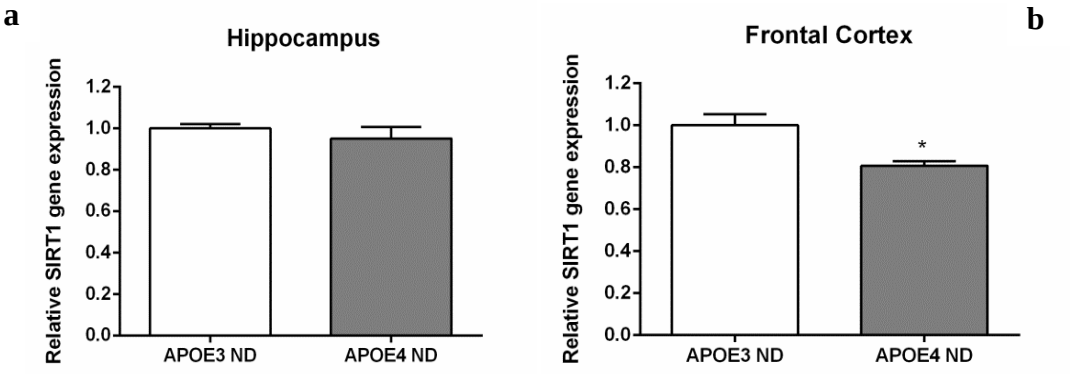
Table 8

	PIN1 gene expression level	
Brain areas	**APOE3 mice**	**APOE4 mice**
Hippocampus	1.00 ± 0.07	**1.23 ± 0.09****
Frontal cortex	1.00 ± 0.08	1.13 ± 0.14
Entorhinal cortex	1.00 ± 0.05	**0.75 ± 0.05*****
Parietal cortex	1.00 ± 0.11	**0.68 ± 0.10*****

**p<0.01; ***p<0.001

SIRT1 GENE EXPRESSION

The SIRT1 gene expression was significantly decreased in the frontal cortex of 18 months old apoE4 mice (0.81 ± 0.04 *versus* control apoE3 group = 1.00 ± 0.10, p<0.05) (**Figure 13b**). No changes were observed in the other examined brain areas (**Table 9**).



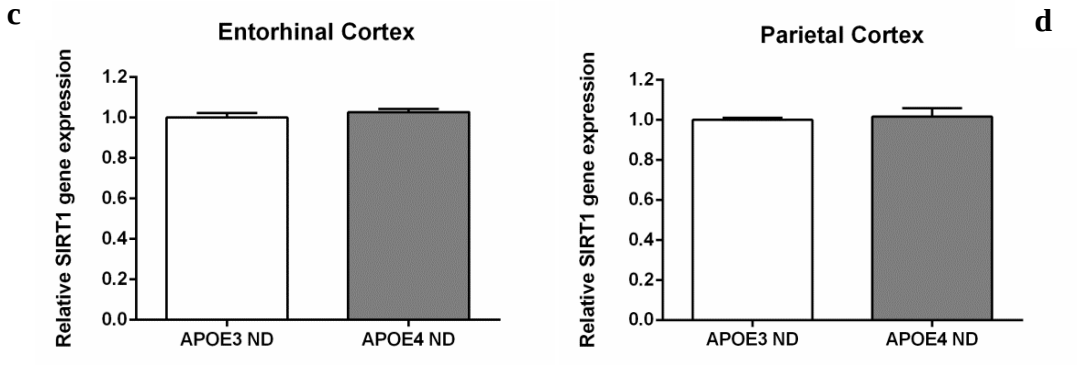


Figure 13. SIRT1 expression was measured by real-time PCR in hippocampus (**a**), frontal cortex (**b**), entorhinal cortex (**c**) and parietal cortex (**d**) of 18 months old apoE 3 and apoE4 transgenic mice fed with normal diet (ND). Bars represent 2^{-DDCt} value calculated by Delta-Delta Ct (DDCt) method (n=6). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as means ± SEM relative to control apoE3mice (*p<0.05. Student's t-test).

Table 9

	SIRT1 gene expression level	
Brain areas	**APOE3 mice**	**APOE4 mice**
Hippocampus	1.00 ± 0.04	0.95 ± 0.11
Frontal cortex	1.00 ± 0.10	**0.81 ± 0.04***
Entorhinal cortex	1.00 ± 0.06	1.03 ± 0.04
Parietal cortex	1.00 ± 0.03	1.02 ± 0.10

*p<0.05

PSEN1 gene expression

PSEN1 gene expression was significantly decreased in the brain areas of 18 months old apoE4 mice as follows: hippocampus (0.80 ± 0.05 *versus* control group apoE3= 1.00 ± 0.10, p<0.05) (**Figure 14a**); frontal cortex (0.79 ± 0.07 *versus* control group apoE3= 1.00 ± 0.12, p<0.05) (**Figure 14b**); entorhinal cortex (0.93 ± 0.02 *versus* control group apoE3=

1.00 ± 0.06, p<0.05) (**Figure 14c**). No gene expression changes were observed in the parietal cortex (**Figure 14d**; **Table 10**).

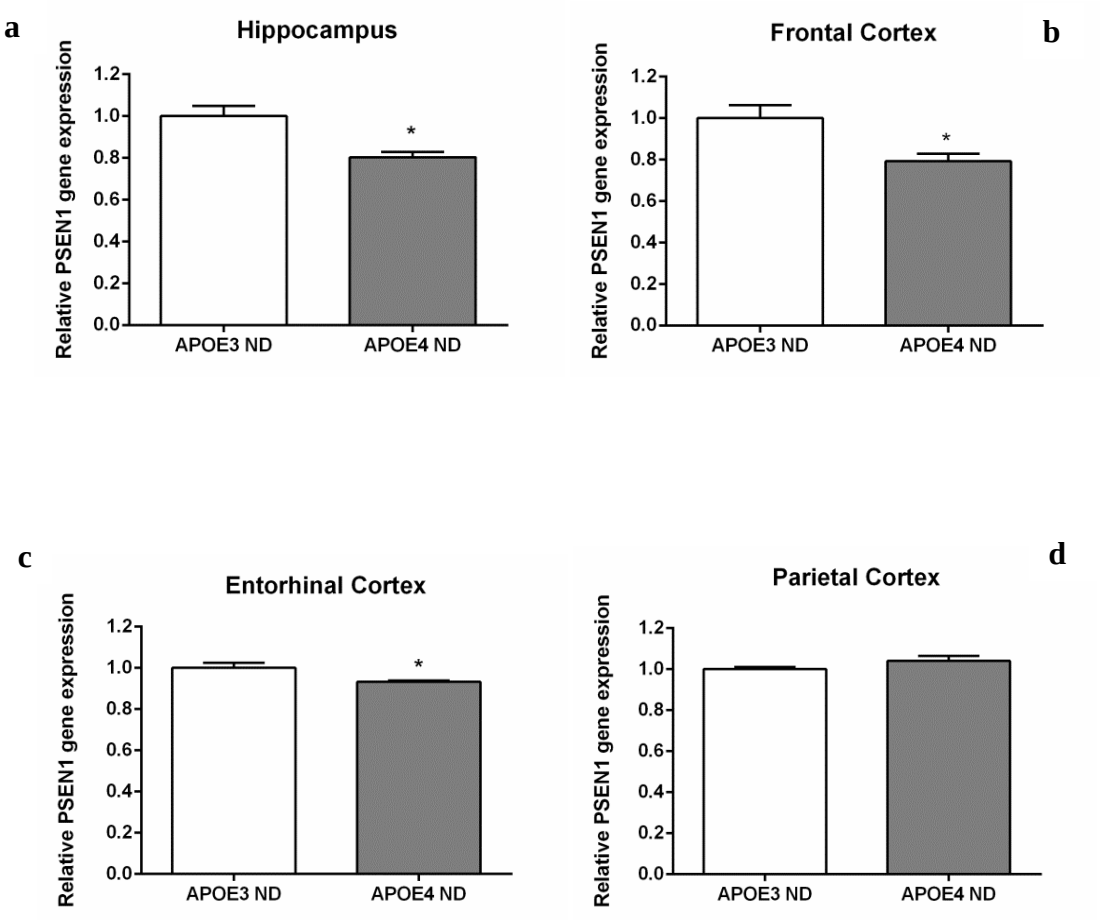


Figure 14. PSEN1 expression was measured by real-time PCR in hippocampus (**a**), frontal cortex (**b**), entorhinal cortex (**c**) and parietal cortex (**d**) of 18 months old apoE 3 and apoE4 transgenic mice fed with normal diet (ND). Bars represent 2^{-DDCt} value calculated by Delta-Delta Ct (DDCt) method (n=6). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as means ± SEM relative to control apoE3mice (**p <0.01; *p<0.05. Student's t-test).

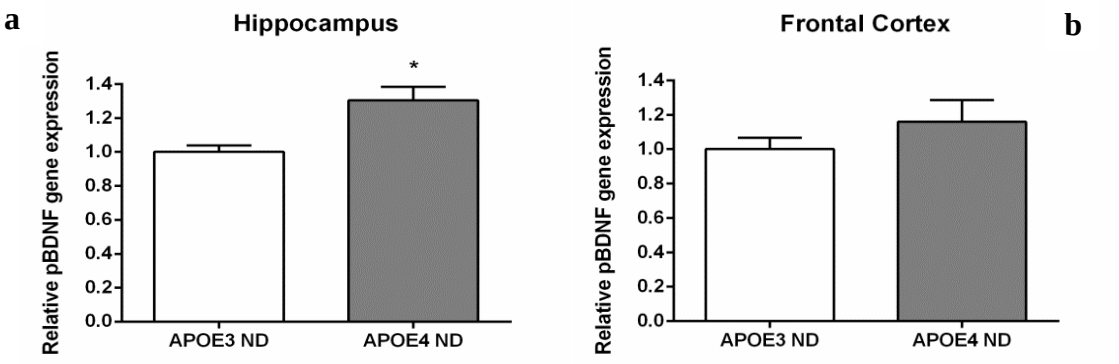
Table 10

	PSEN1 gene expression level	
Brain areas	**APOE3 mice**	**APOE4 mice**
Hippocampus	1.00 ± 0.10	**0.80 ± 0.05***
Frontal cortex	1.00 ± 0.12	**0.79 ± 0.07***
Entorhinal cortex	1.00 ± 0.06	**0.93 ± 0.02***
Parietal cortex	1.00 ± 0.03	1.02 ± 0.10

*p<0.05

pro-BDNF gene expression

The pro-BDNF gene expression was significantly increased in the hippocampus of 18 months old apoE4 mice (1.30 ± 0.16 *versus* control apoE3 group = 1.00 ± 0.08, p<0.05) (**Figure 15a**). In contrast, apoE4 genotype is associated with a significant reduction of pro-BDNF gene expression in the entorhinal cortex (0.85 ± 0.10 *versus* control group apoE3= 1.00 ± 0.04, p<0.01) (**Figure 15c**). No significant changes of pro-BDNF gene expression were observed in frontal and parietal cortices (**Table 11**).



c **d**

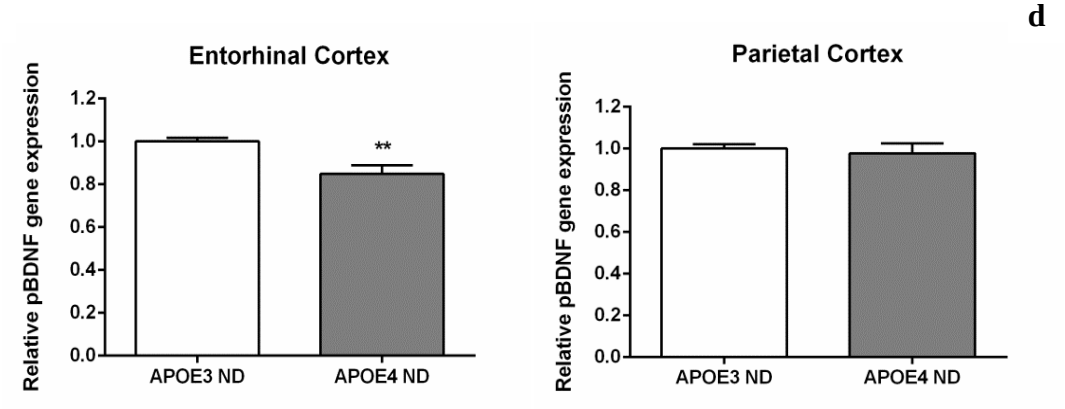


Figure 15. pro-BDNF expression was measured by real-time PCR in hippocampus (**a**), frontal cortex (**b**), entorhinal cortex (**c**) and parietal cortex (**d**) of 18 months old apoE 3 and apoE4 transgenic mice fed with normal diet (ND). Bars represent 2^{-DDCt} value calculated by Delta-Delta Ct (DDCt) method (n=6). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as means ± SEM relative to control apoE3mice (**p <0.01; *p<0.05. Student's t-test). t-test. (n=6)

Table 11

	p-BDNF gene expression level	
Brain areas	**APOE3 mice**	**APOE4 mice**
Hippocampus	1.00 ± 0.08	**1.30 ± 0.16***
Frontal cortex	1.00 ± 0.13	1.16 ± 0.25
Entorhinal cortex	1.00 ± 0.04	**0.85 ± 0.10****
Parietal cortex	1.00 ± 0.05	0.98 ± 0.12

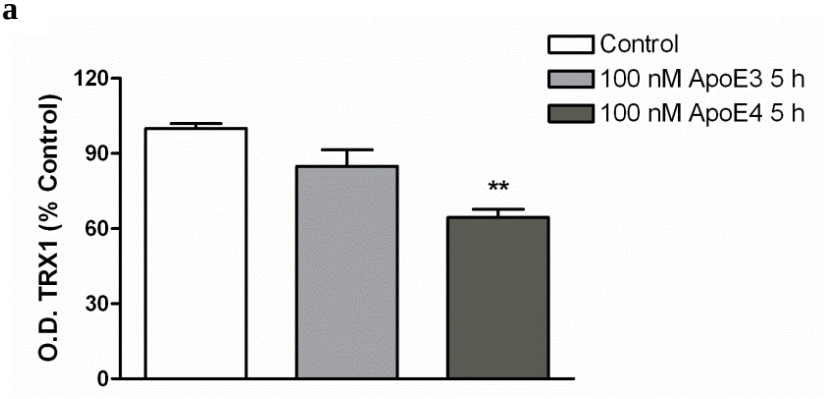
*p<0.05; **p<0.01

4.4 PROTEIN LEVELS IN BE(2)-M17 CELLS EXPOSED TO APOE3 AND APOE4

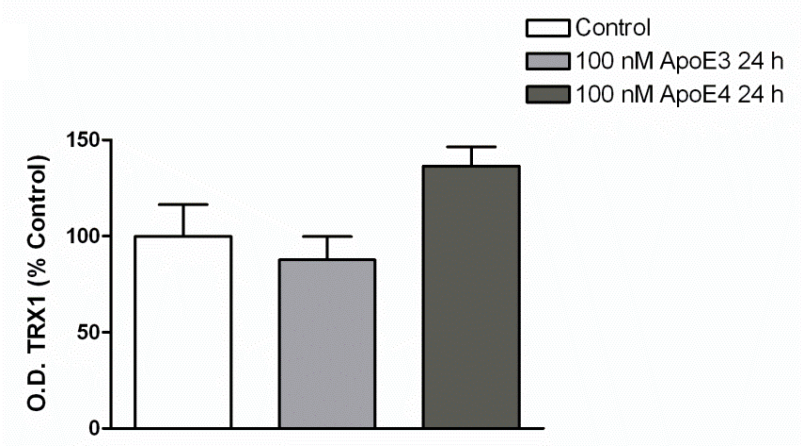
TRX1 levels

The western blot analysis showed a significant decrease of TRX1 protein levels in cells exposed to 100 nM apoE4 human recombinant protein for 5 hours compared to unexposed control (64.5 ± 3.19; OD% of control 100 ± 1.95, $p<0.01$; **Figure 16a**); cell exposure to 100 nM apoE3 induced no changes of TRX1 protein levels. The levels of the protein were significantly increased in cells exposed to apoE4 for 48 hours (172.5 ± 10.8; OD% of control 100 ± 6.79, $p<0.01$) (**Figure 16c**). No changes on TRX1 protein levels were observed in cells exposed to 100 nM apoE4 for 24 hours (**Figure 16b**; **Table 12**).

a



b



c

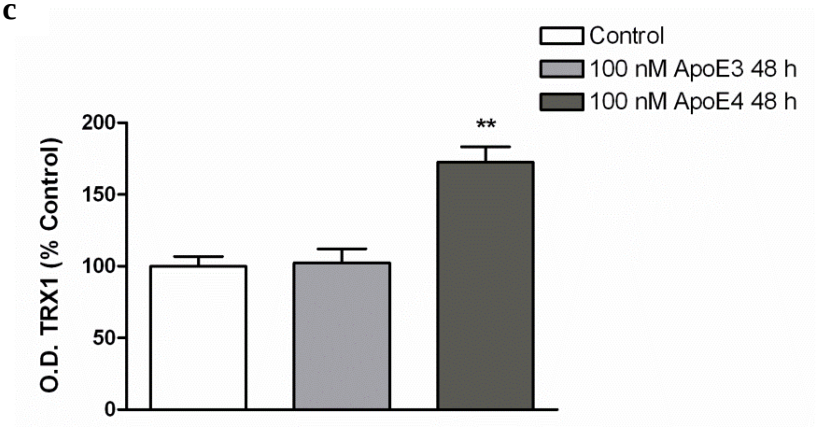


Figure 16. Immunoblotting analysis of TRX1 protein levels (12 kDa) in BE(2)-M17 human neuroblastoma cells exposed to 100 nM apoE3 and apoE4 for 5 (**a**), 24 (**b**) and 48 (**c**) hours. Bars represent the mean ± S.E.M of optical density (O.D.) normalized to the internal loading control β-actin (42 kDa) and expressed as percentage of control untreated cells. **p < 0.01 (one one-way Anova, followed by Dunnett *post hoc* test; n=6).

Table 12

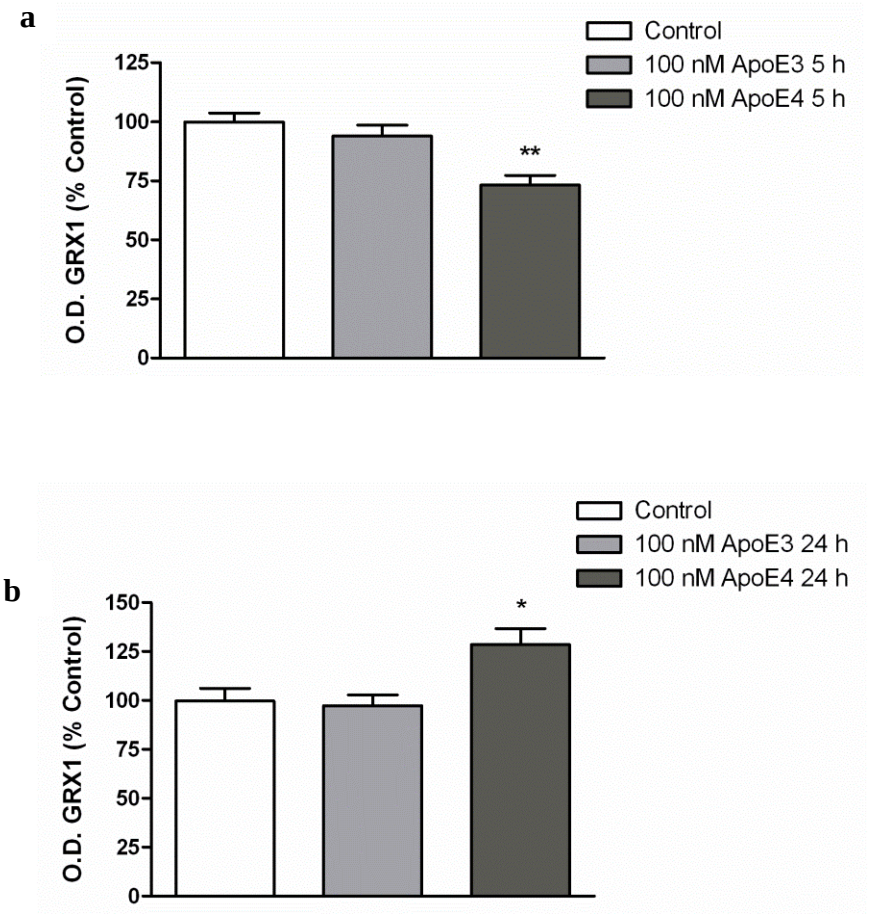
	TRX1 protein levels		
Time	**Control cells**	**ApoE3 exposed cells**	**ApoE4 exposed cells**
5 hours	100 ± 1.95	84.9 ± 6.55	**64.5 ± 3.19****
24 hours	100 ± 16.5	87.7 ± 12.2	136.4 ± 10.0
48 hours	100 ± 6.79	102.3 ± 9.86	**172.5 ± 10.8****

**p<0.01

GRX1 levels

The western blot analysis showed a significant decrease of GRX1 protein levels in cells exposed to 100 nM apoE4 human recombinant protein for 5 hours compared to unexposed control cells (73.3 ± 4.07; OD% of control 100 ± 3.75, p<0.01) (**Figure 17a**). The protein levels were significantly increased in cells exposed to apoE4 for 24 hours (128.6 ± 8.05; OD% of control 100 ± 6.61, p<0.05) and 48 hours (175.9 ± 4.96; OD% of control 100 ± 8.54, p<0.01) (**Figure 17b** and **c**, respectively). No changes on GRX1 protein levels were

observed in cells exposed to 100 nM apoE3 human recombinant protein at 5 and 24 hours. However, the levels of GRX1 were increased after the apoE3 exposure for 48 hours (149.3 ± 18.5; OD% of control 100 ± 8.54, $p<0.05$) (**Figure 17c**; **Table 13**)



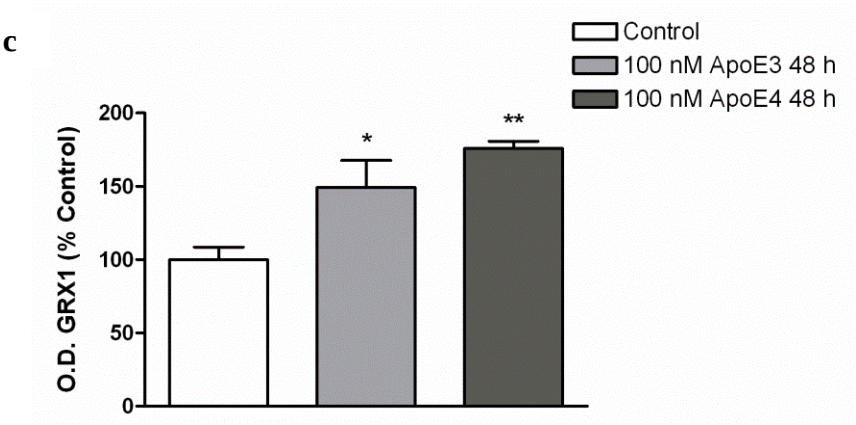


Figure 17. Immunoblotting analysis of GRX1 protein levels (12 kDa) in BE(2)-M17 human neuroblastoma cells exposed to 100 nM apoE3 and apoE4 for 5 (**a**), 24 (**b**) and 48 (**c**) hours. Bars represent the mean ± S.E.M of optical density (O.D.) normalized to the internal loading control β-actin (42 kDa) and expressed as percentage of control untreated cells. *p<0.05; **p < 0.01 (one one-way Anova, followed by Dunnett *post hoc* test; n=6).

Table 13

	GRX1 protein levels		
Time	**Control cells**	**ApoE3 exposed cells**	**ApoE4 exposed cells**
5 hours	100 ± 3.75	94.0 ± 4.67	**73.3 ± 4.07****
24 hours	100 ± 6.61	97.3 ± 5.54	**128.6 ± 8.05***
48 hours	100 ± 8.54	**149.3 ± 18.5***	**175.9 ± 4.96****

*p<0.05; **P<0.01

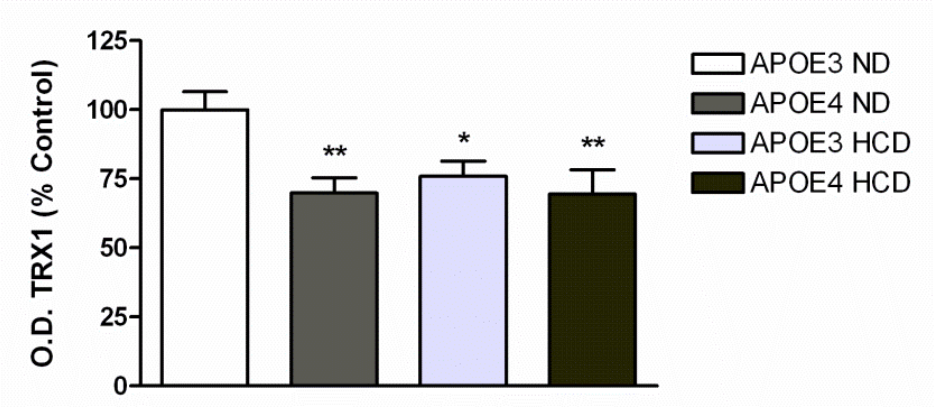
4.5 PROTEIN LEVELS IN THE APOE3/E4 TRANSGENIC MICE

TRX1 and GRX1 levels

The western blot analysis showed a significant decrease of TRX1 protein levels in the hippocampus of 6 months old apoE4 mice ND and HCD compared to the control group apoE3 ND, with a main effect of the genotype (apoE4 ND= 69.9 ± 5.48; apoE4 HCD= 69.5 ± 8.87; OD% of control 100 ± 6.50, p<0.01) (2-Way Anova: p<0.05). The levels of the protein were significantly decreased also in apoE3 HCD, although in a smaller extent

compared to apoE4 mice experimental groups (75.9 ± 5.57 OD% of control, $p<0.05$) (**Figure 18a**). The western blot analysis of GRX1 protein levels showed no significantly differences between experimental groups (**Figure 18 b**). (**Table 14**)

a



b

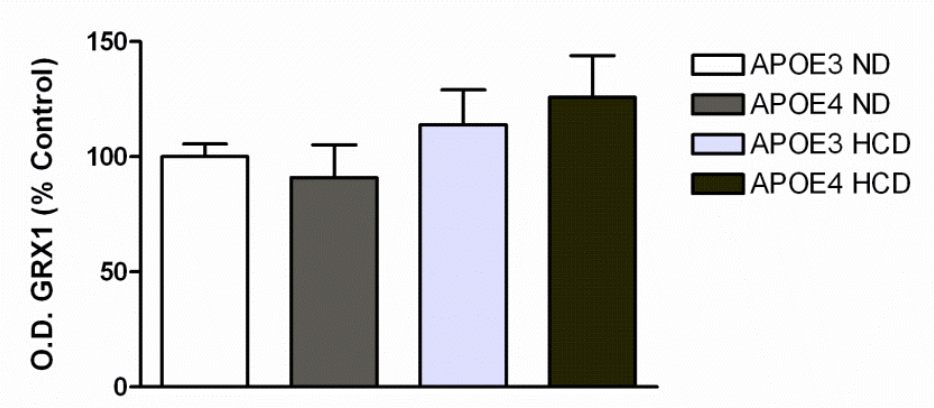


Figure 18. Hippocampal samples from 6 months old apoE3 and apoE4 mice fed with normal diet (ND) or high carbohydrate diet (HCD) were analyzed by Western immunoblotting for TRX1 (**a**) and GRX1 (**b**) protein levels. TRX1 levels (12 kDa) are decreased in apoE4 ND and HCD mice, and in apoE3 HCD compared to control group apoE3 ND mice (*$p<0.05$; **$p< 0.01$, main effect of the genotype). No significant differences were found for GRX1 protein levels (12 kDa). Results represent the mean ± S.E.M of optical density (O.D.) normalized to the internal loading control β-actin (42 kDa) and expressed as percentage of control. 2-way Anova= *$p<0.05$, main effect of the genotype; one-way Anova, Dunnett post-test, *$p<0.05$; ** $p<0.01$ (n=8)

Table 14

	APOE3 ND	APOE4 ND	APOE3 HCD	APOE4 HCD
TRX1 protein levels	100 ± 6.5	**69.9 ± 5.48****	**75.9 ± 5.57***	**69.4 ± 8.87****
GRX1 protein levels	100 ± 5.5	90.9 ± 14.3	114 ± 15.1	125.8 ± 18.1

*p<0.05; **p<0.01

4.6 TRX1 GENE EXPRESSION IN THE APOE3/E4 TRANSGENIC MICE

The TRX1 gene expression was increased in the hippocampus of apoE4 ND and apoE4 HCD mice compared to the control group apoE3 ND (apoE4 ND= 2.10 ± 0.55; apoE4 HCD= 1.97 ± 0.51 *versus* the control apoE3 group ND= 1.00 ± 0.23, p<0.05), with a main effect of the genotype (2-Way Anova) (**Figure 19**). No changes were observed in apoE3 HCD mice (1.46 ± 0.16 *versus* the control apoE3 group ND).

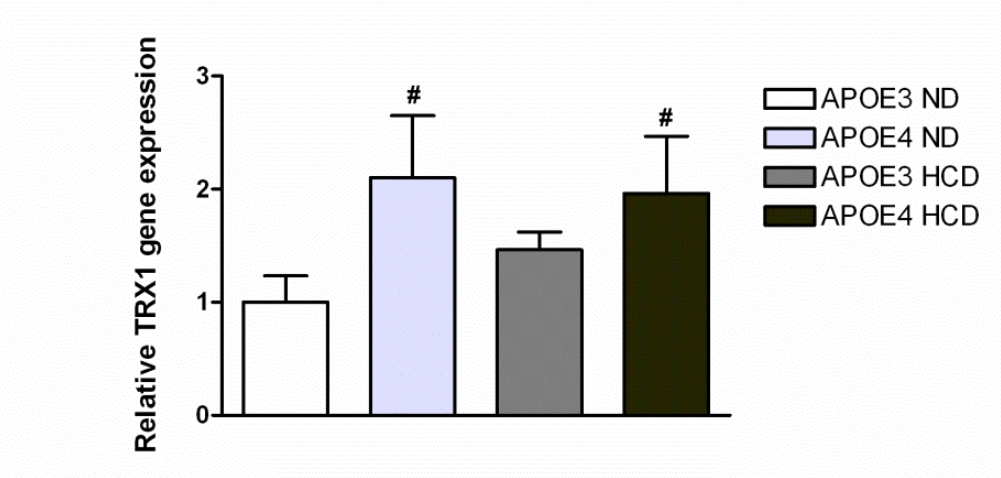


Figure 19. TRX1 relative gene expression was determined by real-time PCR in hippocampus of 6 months old apoE 3 and apoE4 transgenic mice fed with normal diet (ND) or high carbohydrate diet (HCD). Bars represent 2-DDCt value calculated by Delta-Delta Ct (DDCt) method (n=6). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); data are expressed as means ± SEM relative to control apoE3mice (2-way Anova= #p<0.05, main effect of the genotype)

5. DISCUSSION

5.1. METHODOLOGICAL CONSIDERATIONS

5.1.1 Aβ 25-35 PEPTIDES

Several evidences suggest that the excessive production of Aβ peptides and its aggregation into toxic fibrillar deposits play a central role in the etiology of AD. Potential toxic mechanisms of Aβ include altered activity of neurotransmitter receptors and signaling molecules with impairment of axonal transport, disrupted intracellular calcium homeostasis and mitochondrial functions (*Huang and Mucke, 2012*). In support of this hypothesis, several *in vitro* and *in vivo* investigations reported neurotoxic effects of Aβ fragments in neurons of brain regions involved in AD. Nevertheless, the neurodegenerative processes induced by Aβ in these studies have been observed only in specific experimental conditions so that the data are somewhat inconsistent.

Human Aβ peptides are present in different assembly states, including monomers, oligomers, as well as mature fibrils, that may be deposited in brain tissues. Several studies focusing on functional Aβ effects used synthetic Aβ peptides. However, it is still unknown the Aβ concentration that should be considered normal or physiological and which abnormally high or pathological. It has been proposed that the aggregation state also influences the toxic properties of Aβ, but which form of Aβ is the most toxic and how its accumulation causes neuronal dysfunction in the brain are unresolved topics (*Holscher et al., 2007*). Data obtained in different experimental models suggest that soluble aggregates of Aβ cause more damages to synaptic and cognitive functions than insoluble fibrils (*Roberson and Mucke, 2006*). However, it is impossible to measure Aβ oligomers levels in the brains of living people, and the kinetic of Aβ peptides assembly in the brain certainly differs from that observed *in vitro*. In the earliest stages of amyloid plaques deposition it is likely that Aβ assembly as small oligomeric species more toxic than large fibrillar products. It has been suggested that Aβ 25-35 peptide, largely used in the cellular neurotoxicity studies, represents the biologically active fragment of Aβ. This peptide differs from the other Aβ peptides used in experimental studies since it displays rapid aggregation properties forming stable fibrils with large β-structure and it is neurotoxic immediately

upon dissolution (*Millucci et al., 2009*). The Aβ 25-35 is also the shortest peptide that retains the toxicity of the full-length Aβ 1-42 (*D'Ursi et al., 2004*). Moreover, immunohistochemical analyses demonstrated the presence of Aβ 25–35 peptides in the senile plaques and in the hippocampal neurons of AD brains, but not in age-matched controls. These results strengthen the hypothesis that soluble Aβ40 is released from plaques and converted by proteolysis to the toxic Aβ 25–35 (*Kubo et al., 2002*). On the basis of these experimental considerations, in the present study we used Aβ 25–35 aggregated peptides to induce AD-like neurotoxic insults in SH-SY5Y neuroblastoma cells as *in vitro* model, and then investigate the modulation of PIN1, SIRT1, PSEN1 and BNDF gene expression induced by the Aβ exposure. In particular, Aβ 25–35 peptides were "aged" and aggregated in water through the incubation at 37 °C for 72 h. The consequent aggregation produces a conformational change of peptides that form β-structures with increased neurotoxicity as demonstrated by *in vitro* and *in vivo* studies (*Croce et al., 2011; Maurice et al., 1996*). The *in vitro* effects of neurotoxic 25 μM Aβ aggregates result in a reduced time-dependent cell viability, starting to 5 hours after the exposure; the maximum level of toxicity corresponds to a mortality rate around 20%. This significant but not so elevated neurotoxicity is useful to investigate the effect of the aggregated Aβ 25-35 peptides on the early modulation of different genes related to the neurodegenerative processes which can lead to AD.

5.1.2 APOE3 AND APOE4 TRANSGENIC MICE

Human apoE targeted replacement (h-apoE TR) mice is a validated animal model produced using a gene targeting strategy which allows the expression of apoE3 or apoE4 mRNA with a similar levels and tissue distribution (*Sullivan et al., 1997*). This transgenic line is largely used to study the role of apoE in neurodegenerative processes related to the onset of AD. In particular, the spatial memory performances are sensitive to the apoE isoform, with apoE4 mice showing age-dependent memory impairments in spatial and avoidance memory tasks (*Bour et al., 2008*). It has been also demonstrated that the synaptic and cholinergic deficits

induced by apoE4 preceded the amyloid plaques formation (*Buttini et al., 2002*). Based on these data, it has been hypothesized that apoE4 displays neurotoxic properties independently by Aβ and plaques deposition. Both *in vitro* and *in vivo* studies suggest that several mechanisms apoE4-mediated could contribute to the pathogenesis of AD. These include the modulation of the Aβ deposition and Aβ clearance, the alteration of the antioxidant system, the impairment of the neuronal signaling pathways, the disruption of cytoskeletal structure with increased phosphorylation of tau, and the impairment of glucose metabolism and mitochondrial function (*Huang, 2011*). Even if the association of the apoE4 genotype with increased risk to develop AD has been largely established, the exact pathogenic mechanism is still poorly understood. In the brain, apoE has been implicated in lipid metabolism, neuronal repair and remodeling processes. In general, apoE3 is neurotrophic or neuroprotective, whereas apoE4 and its fragments are toxic. The neurotoxicity of apoE4 fragments increases the tau phosphorylation, alter the cellular signaling pathways and/or lipid metabolism leading to impaired neuronal functions especially in the hippocampus (*Li et al., 2009*)

The apoE4 genotype is associated to loss of synaptic integrity, spine density and dendritic arborization in mice, and represents the major genetic risk factor for sporadic AD in humans. In particular, a recent study showed that apoE4 mice fed with HCD (high carbohydrate diet) have cognitive deficits starting at 6 months of age, with decreased levels of proteins that regulate memory processes such as Arc, β-catenin and BDNF, in the hippocampus, one of the first brain regions that degenerates in earliest stages of AD pathogenesis (*Maioli et al., 2012*).

Based on these experimental considerations, in the present study we used h-apoE TR mice to investigate the effect of the strong genetic AD risk factor apoE4 on the gene expression of selected proteins involved in the regulation of neurodegenerative processes. In particular, the interaction between HCD and apoE3 and apoE4 genotype on TRX1/GRX1 antioxidant protein levels has been investigated in the hippocampus of 6 months old mice. The gene expression of PIN1, SIRT1, PSEN1 and pro-BDNF was measured in apoE 18 old months mice fed with normal diet. The modulation of these genes by the apoE4 genotype was assessed in different brain regions known to be impaired in the first stages of AD, such as

hippocampus, and entorhinal, frontal and parietal cortices. Data from the present research demonstrated selective changes of gene expression in the different examined brain areas. The apoE TR mice are particularly useful to compare the effects of apoE isoforms on molecular pathways involved in AD onset before that the pathological brain lesions became evident. In fact, several *in vivo* studies suggest that the cognitive impairment can occur in the absence of Aβ and neurofibrillary tangles deposition. The human apoE TR mice used in the present study is a validated transgenic model to investigate the pathogenesis of apoE-related cognitive deficits in the earliest phases of dementia.

5.2 PIN1 GENE EXPRESSION

In vitro studies

The *in vitro* data show that Aβ 25-35 aggregates exposure determines a significant decrease of PIN1 gene expression at 5 and 24 hours in SH-SY5Y human neuroblastoma cells, whereas no changes are observed at 48 hours. At present, no experimental studies concerning the effects of Aβ on the PIN1 mRNA levels have been performed.

A recent study demonstrated that human hippocampal cultured neurons exposed to Aβ (1–42) oligomers show a PIN1 protein up-regulation and activation associated with decreased p-tau, suggesting that Aβ might transiently induce PIN1 expression and prevent tau hyperphosphorylation (*Bulbarelli A et al., 2009*). It has been also suggested that, in physiological conditions, PIN1 may be protective against Aβ toxicity, promoting the trans conformation of APP and increasing its non-amyloidogenic processing. However, the role of PIN1 in the protection or promotion of neuronal degeneration it is not completely elucidated. PIN1 protein was significantly down-regulated and oxidized in AD hippocampus (*Sultana et al., 2006*). This oxidation led to the loss of PIN1 isomerase activity supporting a direct link between oxidative damage to PIN1 and the pathogenesis of AD. A recent study also showed a compensatory activation/up-regulation of PIN1 protein in later stages of AD pathogenesis, whereas its reduction seems to play a role in early

stages of the disease (*Wang et al., 2007 b*). These results suggest a pivotal role of PIN1 protein in the protection against p-tau and Aβ deposition during neuronal degenerative processes.

Therefore, the down-regulation of PIN1 gene expression observed in the present *in vitro* study may be considered an early phenomenon induced by the Aβ peptide neuronal toxicity. On the other hand, PIN1 could be involved in mechanisms of neuronal protection against Aβ toxicity that probably occur late in the pathogenetic AD-related processes. PIN1 gene expression is in fact down-regulated in a transient manner after the early exposure to Aβ at 5 and 24 hours, to then revert to a physiological rate following a more prolonged contact with Aβ peptides (48 hours). We may then hypothesize that this shift of PIN1 mRNA levels might be a compensatory mechanism to counteract the Aβ toxic insults.

***In vivo* studies**

The data obtained from 18 months old apoE4 mice showed an increase of PIN1 gene expression in the hippocampus and PIN1 down-regulation in the entorhinal and parietal cortices compared to apoE3 controls. No changes were observed in the frontal cortex.

This is the first study that investigates, in the h-apoE TR mice model, the possible modulation of PIN1 gene by the apoE4 genotype in brain areas impaired in AD. An immunohistochemical study in the hippocampus and in the parietal cortex of normal and AD human brains showed that the regions with lower PIN1 protein levels are the same prone to the degeneration. These data suggest an inverse correlation between the PIN1 expression and the vulnerability to neuronal cell death (*Liou et al., 2003*). However, it has also been reported that hippocampal neurons of AD patients show granular accumulation of PIN1 without neurofibrillary tangles (*Holzer M et al., 2002*). These lesions could be considered alternative pathological alterations, maybe aiming to counteract the neuronal degeneration. PIN1 is also the first protein whose deletion seems to cause age-dependent neurodegeneration and tau pathologies. PIN1-knockout mice show hyperphosphorylated tau, motor-behavioral deficits and neuronal loss, as well as described in AD patients (*Liou et al. 2003*). However, *in vivo* studies on PIN1-knockout mice showed opposite data

regarding the effect of PIN1 on Aβ production (*Akiyama et al. 2005*; *Pastorino et al. 2006*). The mechanisms underlying the PIN1 modulation of tau phosphorylation and Aβ deposition during AD progression are still undefined as well as the role of PIN1 in the regulation of normal neuronal function. Genetic studies on humans suggest that PIN1 promoter polymorphisms are associated with an increased risk of late-onset AD (*Segat et al. 2006*). A recent study also showed an increased PIN1 gene expression in PBMCs of late onset AD patients, especially if carrying at least one ε4 allele (*Arosio et al., 2012*).

The hippocampus and entorhinal cortex are brain structures involved in memory formation and firstly impaired during early neurodegenerative processes of AD (*Braak and Braak, 1997*). The increase of PIN1 mRNA levels in the hippocampus of apoE4 mice reported in the present research might be a compensatory/protective mechanism to counteract the detrimental effects of the apoE4 genotype on neurons. Alternatively, the up-regulation of PIN1 could represent a reactive biochemical mechanism to counteract the decreased PIN1 protein levels as observed in AD patients (*Liou et al., 2003*).

The down-regulation of PIN1 in the other brain regions such as entorhinal and parietal cortices may be considered a consequence of a higher vulnerability of these neurons to the apoE4 genotype. Otherwise, it is possible that in these regions an increase of PIN1 gene expression also occurs to counteract the cell damage, but may became evident later on the apoE4 mice life.

The frontal cortex is a brain region generally impaired during the mild/late stages of AD, and gives a general description of the full AD pathological features. In the frontal cortex of apoE4 mice PIN1 gene expression levels are unchanged. It can be hypothesized that PIN1 plays a marginal protective role in this area or that the frontal cortex is overall more able to counteract the detrimental apoE4-related processes.

It is possible to conclude that, in h-apoE TR mice model used in the present study, PIN1 plays different and specific roles against neurodegenerative processes in the brain regions involved in AD. Therefore, in AD patient the apoE4 genotype could contribute to the onset of neurodegeneration also through the modulation of PIN1 gene expression. PIN1 thus represents a novel potential diagnostic and therapeutic target in AD. However, clinical

studies need to confirm and elucidate the role of PIN1 in human neurodegenerative diseases.

5.3 SIRT1 GENE EXPRESSION

In vitro studies

The *in vitro* studies show that Aβ 25-35 aggregates exposure induces, in SH-SY5Y human neuroblastoma cells, a significantly decrease of SIRT1 gene expression at 5 and 24 hours, whereas no changes are observed at 48 hours. No *in vitro* studies regarding the modulation of SIRT1 mRNA levels by Aβ aggregated peptides are currently reported in literature.
SIRT1 is a histone deacetylase (HDAC) protein highly expressed in neurons and involved in the regulation of several functions, including antioxidant, anti-inflammatory and anti-apoptotic processes. SIRT1 is implicated in pathways related to synaptic plasticity, learning and memory. Several studies suggested that SIRT1 protects against AD degenerative mechanisms, in particular decreasing Aβ accumulation and reducing the tau-related pathology (*Donmez et al., 2010*).

The decrease of SIRT1 mRNA levels in the present *in vitro* study may be the expression of the Aβ peptide-induced neuronal toxicity. The SIRT1 down-regulation here reported is in agreement with data coming from humans showing that SIRT1 mRNA and protein levels are decreased in the parietal cortex of AD patients (*Julien et al., 2009*). However, no association between SIRT1 gene polymorphism and AD risk is described.

The present findings showed that SIRT1 gene is down-regulated in a transient manner after the acute exposure to Aβ at 5 and 24 hours, since it reverts to a physiological level after 48 hours. This time-course modulation of SIRT1 gene expression may be considered a molecular compensatory mechanism counteracting the Aβ-induced toxicity.
Previous *in vitro* studies already demonstrated the protective role of SIRT1. SIRT1 has been shown to protect against microglia-dependent Aβ toxicity through the inhibition of

inflammatory signaling (*Chen et al., 2005*). Moreover, the antioxidant natural compound resveratrol protects neuronal cells against Aβ also through the activation of SIRT1 protein expression and activity (*Albani et al., 2010*).

In vivo studies

The *in vivo* data from 18 months old apoE4 mice showed a decrease of SIRT1 gene expression in the frontal cortex compared to apoE3 controls, whereas no changes were observed in the other brain regions examined. These data could be explained taking into account the role of SIRT1 in normal and AD brains. SIRT1 plays a crucial role in cognitive functions and synaptic plasticity in normal mice (*Michán et al., 2010*). Moreover, a recent study showed that transgenic mice model lacking of SIRT1 catalytic activity in the brain show impairment of memory and synaptic plasticity (*Gao et al., 2010*). On the contrary, the over-expression of SIRT1 in the hippocampus of AD transgenic mice protects against neuronal degeneration (*Kim et al., 2007*). Therefore, the SIRT1 down-regulation we observed in the frontal cortex of apoE4 mice might be considered as consequence of the detrimental effects of apoE4 on the SIRT1-related protective functions.
Taking together the *in vitro* and *in vivo* results, we can state that Aβ peptides and apoE4 genotype probably contribute to the neuronal impairment also through the reduction of SIRT1 gene expression.

5.4 PSEN1 GENE EXPRESSION

In vitro and *in vivo* studies

The *in vitro* data show that Aβ 25-35 aggregates exposure determines a significantly decrease of PSEN1 gene expression levels at 5, 24 and 48 hours in SH-SY5Y human neuroblastoma cells. In particular, the decrease of PSEN1 mRNA levels was more

pronounced at the intermediate exposure of 24 hours. Aβ peptides may induce neuronal toxicity also through the decrease of PSEN1 gene expression in this *in vitro* model. The *in vivo* data in 18 months old apoE4 mice showed a decreased PSEN1 gene expression in the hippocampus, frontal and entorhinal cortices compared to apoE3 control group. No changes were observed in the parietal cortex of apoE4 mice.

PSEN1 is a genetic risk factor in the early-onset familial AD. The PSEN1 protein is the catalytic unit of the enzyme γ-secretase leading to amyloidogenic process of APP. However, PSEN1 protein is also involved in the regulation of memory formation and synaptic plasticity. The loss of PSEN1 activity results in hippocampal-dependent spatial and memory impairments, with inflammation and neuronal degeneration. It has also been reported that PSEN1 protein levels are reduced in the association neocortex and hippocampus of AD brains (*Davidsson et al., 2001*). The functional loss of PSEN1 leads to increased p-tau levels and impairment of neuronal functions (*Pigino et al., 2003*). The decrease of PSEN1 mRNA levels in the brain areas involved in AD degeneration suggests that the effect of apoE4 also involves PSEN1-related mechanisms.

The *in vivo* data are consistent with the *in vitro* PSEN1 gene expression results, suggesting that the PSEN1 modulation induced by Aβ peptides and apoE4 genotype could in part contribute to the neuronal loss and impairments in AD processes. However, further elucidations of the roles played by presenilins in the brain are necessary for the development of effective therapeutic strategies in neurodegenerative disorders.

5.5 BDNF GENE EXPRESSION

In vitro studies

The *in vitro* data show that the exposure of SH-SY5Y human neuroblastoma cells to Aβ 25-35 aggregates induces a significant decrease of BDNF gene expression at 5 hours followed by an increased mRNA levels at 24 and 48 hours.

BDNF is implicated in several brain functions such as synaptic transmission, neuronal differentiation, protection and survival. It is considered one of the key molecules in the pathology of neurodegenerative disorders such as AD. *In vitro* studies recently reported that long-term Aβ 42 or Aβ 25–35 exposure strongly down-regulates BDNF gene expression in rat cortical neurons; however, these reduction is preceded by an increase of BDNF mRNA levels after short-term exposure to amyloid peptides (*Aliaga et al., 2010*). Oligomeric, but not fibrillar Aβ 42, significantly decreases BDNF mRNA levels also in differentiated SH-SY5Y cells (*Garzon et al., 2007*). However, other studies showed opposite results. BDNF gene expression is reported up-regulated in astrocytes exposed to Aβ 42 (*Kimura et al., 2006*). A recent *in vitro* study showed increased BDNF mRNA levels in the early stage of Aβ toxicity in differentiated neuroblastoma cells. In the same study the BDNF exposure rescues the cells from neuritic degeneration caused by Aβ (*Zhang et al., 2012*). In SH-SY5Y cells Aβ also produce an increased release of BDNF protein, maybe reflecting a compensatory cellular adaptation by enhancing neurotrophic support (*Olivieri et al., 2003*). These studies suggest that Aβ triggers distinct effects on the BDNF expression and production depending on the neuronal cellular lineage and experimental conditions.

The decrease of BDNF mRNA levels at 5 hours may be explained as the result of Aβ-mediated acute toxicity. The following increased gene expression following a more prolonged exposure to Aβ (24 and 48 hours) might constitute a neuroprotective BDNF-mediated mechanism that allows the cells to counteract the Aβ toxic effects.

***In vivo* studies**

The *in vivo* data from 18 months old apoE4 mice showed an increse of pro-BDNF gene expression in the hippocampus compared to apoE3 controls, whereas decreased levels in entorhinal cortex were observed. No changes were observed in the frontal and parietal cortices.

BDNF is largely expressed in brain areas characterized by a high plasticity potential such as hippocampus and cortex; several studies suggest that BDNF is required for the hippocampus-mediated learning (*Tapia-Arancibia et al. 2008*). In fact, knock-out mice for

BDNF or its TrkB receptor show reduced dendritic and axonal arborisation, associated to learning and memory impairment (*Poo, 2001*).

BDNF is synthesized as a precursor protein pro-BDNF which is proteolitically cleaved to generate the mature form BDNF. In h-apoE TR mice model, we measured the levels of pro-BDNF mRNA since most of the BDNF secreted by the hippocampal neurons is in the precursor form.

Several clinical evidences showed that the expression of BDNF and its receptor trkB are decreased in *post-mortem* brain regions such as hippocampus, and temporal, parietal and frontal cortices of AD and MCI patients (*Connor et al., 1997; Peng et al., 2005*). The pro-BDNF is also decreased in the hippocampus and parietal cortex even in pre-clinical stages of AD. In our experimental model, the increased pro-BDNF gene expression in the hippocampus does not match with data reported from human studies; it is possible that, in h-apoE TR mice, apoE4 induce a first reactive increase of pro-BDNF aiming to react to the onset of the neurodegenerative process and that, when the disease establishes and progresses, it possibly decreases as occurs in AD patients. This is even more plausible in the hippocampus that is one of the first brain area that is involved in the first stages of AD neurodegenerative process.

In contrast, other studies have reported increased serum BDNF levels in patients with early AD (*Angelucci et al., 2010*). *In vivo* studies regarding mice models of AD also showed an alteration in BDNF protein and gene expression levels. APP and PS1 double transgenic mice model of AD have increased hippocampal BDNF protein expression that is consistent with the pro-BDNF up-regulation we observed in hippocampus (*Szapacs et al., 2004*). The up-regulation of BDNF mRNA expression and protein was also found in microglia and astrocytes of other AD transgenic mice models (*Burbach et al., 2004*). Some authors demonstrated increased hippocampal BDNF mRNA expression following intracranial administration of Aβ peptides (*Tang et al., 2000*). These findings corroborate the hypothesis that the increase of BDNF levels might be a compensatory mechanism against the amyloid toxicity in early stages of AD.

In contrast, in the apoE4 mice, pro-BDNF gene expression is decreased; probably, the entorhinal cortex is not able to activate this neurotrophic support and results more vulnerable to the neuronal impairment induced by apoE4.

Although the therapy with neurotrophins has been suggested as a treatment for neurodegenerative disorders, the precise role of BDNF signaling in AD remains to be clarified.

5.6 TRX1 AND GRX1 PROTEINS MODULATION

In vitro studies

The *in vitro* data show that the exposure of BE(2)-M17 human neuroblastoma cells to human recombinant apoE4 induced a significant decrease of the antioxidant protein TRX1 and GRX1 levels following short-term exposure (5 hours). After a more prolonged-term exposure to apoE4 (24 and 48 hours) both protein levels were increased compared to untreated control cells. The apoE3 protein induced no changes in TRX1 levels, although it up-regulates GRX1 at 48 hours. The concentration of apoE3 and apoE4 proteins (100 nM) used in these experimental procedures is considered physiologically present in CSF of healthy individuals (*Qiu et al., 2003; Riemenschneider, et al., 2002*). Based on the evidence that the acute exposure to apoE4, but not apoE3, reduces TRX1 and GRX1 protein levels, it is plausible to state that apoE4-related toxicity also impairs these neuronal antioxidant systems. The neuronal cells then respond to a more prolonged exposure to apoE4 increasing the expression of TRX1 and GRX1, perhaps as a protective mechanism against the apoE4-induced damage. Accordingly, an *in vitro* study showed that, in human SH-SY5Y neuroblastoma cells, Aβ peptides caused a strong and early oxidation of both TRX1 and GRX1 proteins, whereas their over-expression completely protected cells from the Aβ toxicity (*Akterin et al., 2006*). These evidences indicate that TRX1 and GRX1 antioxidant proteins are implicated in the protection of neurons against oxidative stress induced by apoE4 and Aβ.

***In vivo* studies**

In this study, TRX1 and GRX1 protein levels have been also measured in the hippocampus of apoE3 and apoE4 ND and HCD mice. Data showed that TRX1 protein expression was decreased in apoE4 mice, almost in the same extent independently of the diet, and also in apoE3 HCD mice compared to control group. In the modulation of TRX1 protein levels in apoE h-TR mice fed on different diets, the main effect was played by the apoE4 genotype. GRX1 levels were instead unaffected by both apoE4 genotype and high carbohydrate diet in this *in vivo* model. Although the variations in GRX1 expression was not significant, the immunoblotting showed a trend of protein increasing in apoE HCD mice, suggesting that in this model the diet plays a major role in the regulation of GRX1.

In this model, it is possible that the apoE4 genotype and HC diet can impair the antioxidant defense of neuronal cells decreasing TRX1 levels through the indirect activation of degrading enzymes. The increased gene expression of TRX1 observed in apoE4 mice could be a compensatory mechanism towards the decrease of the protein levels. ApoE4 and HC diet affect TRX1/GRX1 cellular antioxidant system, making the hippocampal neurons more vulnerable to the oxidative stress insults. The hypothesis that the deregulation of these antioxidant proteins, especially TRX1, could play a role in the early pathogenesis of AD is supported by previous studies reported in literature. Brain tissues of AD patients showed decreased TRX1 protein levels, suggesting that the TRX1 alteration can contribute to the neuronal impairment. Furthermore, TRX1 has a protective effect against the neurotoxicity of Aβ in hippocampal cell culture (*Lovell et al., 2000*). The loss of TRX1 activity was also found in transgenic mice model over-expressing Aβ. A study reported an increased GRX1 and decreased TRX1 neuronal levels in the hippocampus and frontal cortex of AD brains (*Akterin et al., 2006*). Furthermore, the gradual loss of antioxidant functions during normal aging may contribute to the oxidative stress and neuronal impairment of hippocampus and frontal cortex (*Venkateshappa et al., 2012*). Several studies in cell lines, in animal models and humans support the association between apoE4 genotype and the increase of oxidative stress and inflammation (*Jofre-Monseny et al., 2008 a*). A recent study showed that the serum concentration and activities of the enzymes involved in the antioxidant defense are

decrease in AD patients carrying apoE4 genotype (*Kharrazi et al., 2008*). However, in the brains of healthy apoE3 and apoE4 h-TR mice no significant differences of activity and levels of antioxidant enzymes were found (*Ophir et al., 2005; Jofre-Monseny et al., 2008 b*). Further studies are needed to clarify the molecular basis of antioxidant imbalance, and also to establish the apoE genotype-mediated effects on oxidative status in the pathogenesis of AD.

TRX1/GRX1 may represent new biomarkers in early neurodegenerative processes; pharmacological strategies to increase the levels or activity of these antioxidant proteins in neurons may have future therapeutic applications for AD treatment.

5.7 CONCLUDING REMARKS

The molecular mechanisms underlying the pathogenesis of AD are largely unknown probably due to the multifactorial etiology of this heterogeneous disease. Among the different factors that contribute to the pathogenesis of late-onset AD, Aβ peptides and the genetic risk factor apoE4 are prominent on the basis of genetic evidence and strong experimental data. Although the roles of apoE4 Aβ-dependent have been widely studied, apoE4 also has detrimental effects on neurons that are independent of Aβ.

Trying to provide new insights into cellular processes of neurodegeneration, this research evaluated the role of Aβ peptides and apoE4 genotype on the modulation of genes and proteins differently involved in aging, using *in vitro* and *in vivo* models. In particular, the effects on the gene and protein expression contributed to clarify selected aspect of the complex molecular pathways leading to the AD onset. All the genes and proteins studied in the present investigation are modulated by Aβ and apoE4 in different ways, suggesting their involvement in the early neurodegenerative mechanisms. Thus, these proteins might represent novel potential diagnostic and therapeutic targets in AD.

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