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*Behavioral and neurochemical effects
of Palmitoylethanolamide in a murine model of
Alzheimer’s Disease*

Tutor

Prof. Tommaso Cassano

PhD Student

Dr. Lorenzo Pace

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia affecting elderly people. AD is a multifaceted pathology characterized by accumulation of extracellular neuritic plaques, intracellular neurofibrillary tangles (NFTs) and neuronal loss mainly in the cortex and hippocampus. AD etiology appears to be linked to a multitude of mechanisms that have not been yet completely elucidated. At present, no therapies in clinical use are able to effectively impact the disease course. Therefore, new drugs able to simultaneously ameliorate the numerous pathogenic mechanism involved in AD are therapeutically promising. To this regard, the aim of this research project was to investigate whether the Palmitoylethanolamide (PEA), an endogenous fatty acid amide, might modulate the symptoms of the AD.

To this aim, the triple transgenic mouse model of AD (3xTg-AD) and wild type littermate (NonTg) have been used. The 3xTg-AD mice harbor three mutant human genes (APP^{swe}, PS1^{M146V}, and tau^{P301L}) and are one of the most thoroughly characterized model of AD. The 3xTg-AD mice develop amyloid plaques and NTF pathology in a hierarchical manner in AD-relevant brain regions, and closely mimic the disease progression in humans.

The mice at 3-months and 9-months of age have been treated with PEA for 90 days and, at the end of treatment, they were subjected to different behavioral tests in order to investigate their mood and learning/memory domains. At the end of behavioral tests the animals were sacrificed to determine, by biochemical analyses, the effect of treatment on neuropathological and neuroinflammatory hallmarks.

Interestingly PEA is able to improve cognitive and non-cognitive functions in 3xTg-AD at 6 months of age, while has only effect on short-term memory in transgenic mice at 12 months of age.

The present work provides also an extensive investigation of the effect of PEA treatment on the onset and progression of A β and tau pathology in 3xTg-AD mice. We showed that PEA significantly reduces the levels of full-length APP in cortex of 6-month-old 3xTg-AD mice and, more interestingly, it decreases also the levels of A β *56, an A β oligomer. Similarly, PEA treatment is able to reduce steady-state levels of full-length APP also in 3xTg-AD mice at 12 months of age, suggesting that it could modulate APP processing in these animals.

Interestingly, PEA treatment is also associated with a significant reduction in tau phosphorylation at residues 202/205. These results suggest that cognitive improvement is probably due to changes in overall A β levels and tau pathology or to a mixture of both hallmarks.

Furthermore, we did not find significant changes in almost all neuroinflammatory markers taken into account, such as COX-2 or in microglial/astrocytic activation markers.

Although further studies are needed to determine the molecular mechanisms underlying the beneficial effects of PEA against AD neuropathology, our data indicate that the compound may be effective in early AD or when A β is accumulating and initiating damage in the central nervous system.

Keywords: *Alzheimer's disease, Palmitoylethanolamide, Behavioral tests, 3xTg-AD*

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CHAPTER I. Introduction

1. Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and it's the most common form of dementia [Blennow *et al.* 2006].

According to estimates published in The World Alzheimer Report 2015, 46.8 million people worldwide are living with dementia. This number will almost double every 20 years, reaching 74.7 million in 2030 and 131.5 million in 2050.

Age is the most significant risk factor, in fact AD affects around 10% of individuals over age 65 and up to 40% of individuals over age 85 [Hebert *et al.* 2013].

AD was described for the first time in 1907 by the German psychiatrist Aloysius Alzheimer, when he analyzed brain tissue from a patient that showed severe disorders of memory and difficulties in everyday life [Alzheimer, 1907].

He observed degenerating cortical neurons with bundles of intracellular fibrils [Alzheimer, 1907] and these neuropathological alterations are still recognized and have become universally accepted as the neuropathologic hallmarks of the disease.

AD is categorized into early onset or familial AD (fAD) and late onset or sporadic AD (sAD) [Vergas *et al.* 2015]. About 2% of cases are familial, and associated with mutations of genes encoding for amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) [Querfurth and LaFerla, 2010]. The etiology underlying sporadic AD, which accounts for more than 98% of AD cases, is complex and multi-factorial [Galimberti *et al.* 2006], but the mutation of the apolipoprotein E (ApoE) gene on chromosome 19 is a risk factor now recognized by the scientific community [Corder *et al.* 1993].

Both sAD and fAD show the same clinical symptoms and neuropathology, but fAD is marked by a more rapid disease progression and can be transmitted to offspring in a Mendelian manner [Ashby *et al.* 2015; Grimm *et al.* 2015].

Despite numerous scientific research, currently only a post-mortem analysis of brain tissue allows for a quick and safe diagnosis by pathological evidence that can confirm an earlier clinical evaluation. In fact, it is possible

to observe both macroscopic and microscopic changes that characterize this disease.

From a macroscopic point of view, the most obvious feature of the brain of an AD patient is a marked cerebral atrophy which leads to a reduction of the weight of the brain and an increased amplitude of the brain grooves and the increase in the ventricular volume. A constriction of the cerebral convolutions is also evident, especially in the fronto-temporal and parietal areas, the ventral surface of the temporal lobe and the parahippocampal gyrus [Waldemar *et al.* 2007] (Fig. 1).

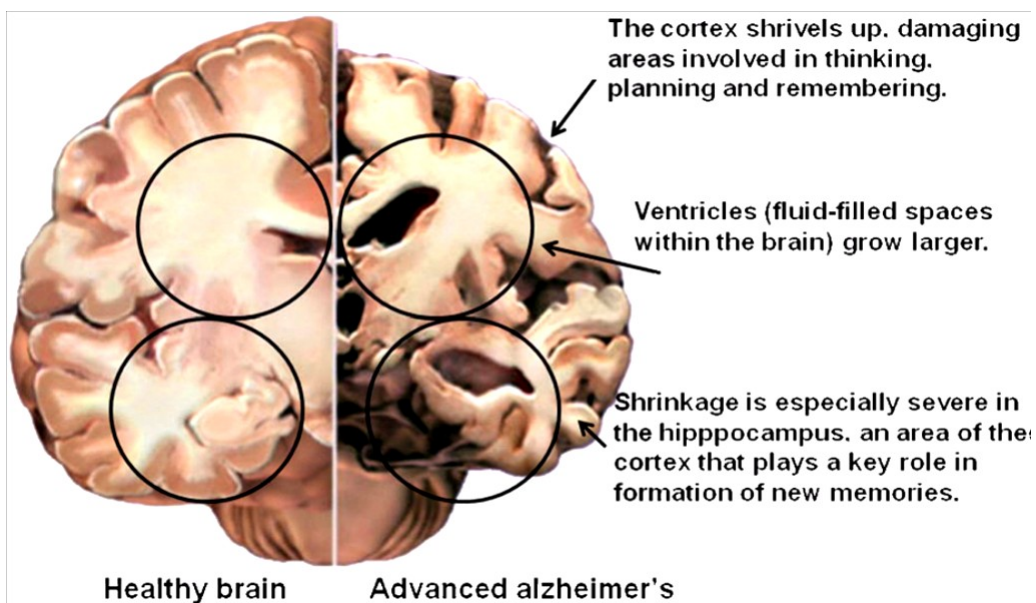


Fig. 1: Macroscopic alteration in AD

At the microscopic level, the neuropathology is characterized by neuronal and synaptic loss in many parts of the central nervous system, and by the presence in the brain of senile plaques composed primarily of amyloid- β peptide ($A\beta$) and neurofibrillary tangles (NFTs), containing hyperphosphorylated tau protein [Querfurth and LaFerla, 2010] (Fig. 2).

In AD the senile plaques and neurofibrillary tangles are mainly detected in specific brain areas such as the cortex, hippocampus, basal forebrain and amygdala [Mattson, 2004]. Moreover, several post-mortem studies of the brain of AD patients showed also loss of synapses, reactive gliosis, microglial activation and neuroinflammation in brain regions that regulate memory and acquired skills [Lee *et al.*, 2010].

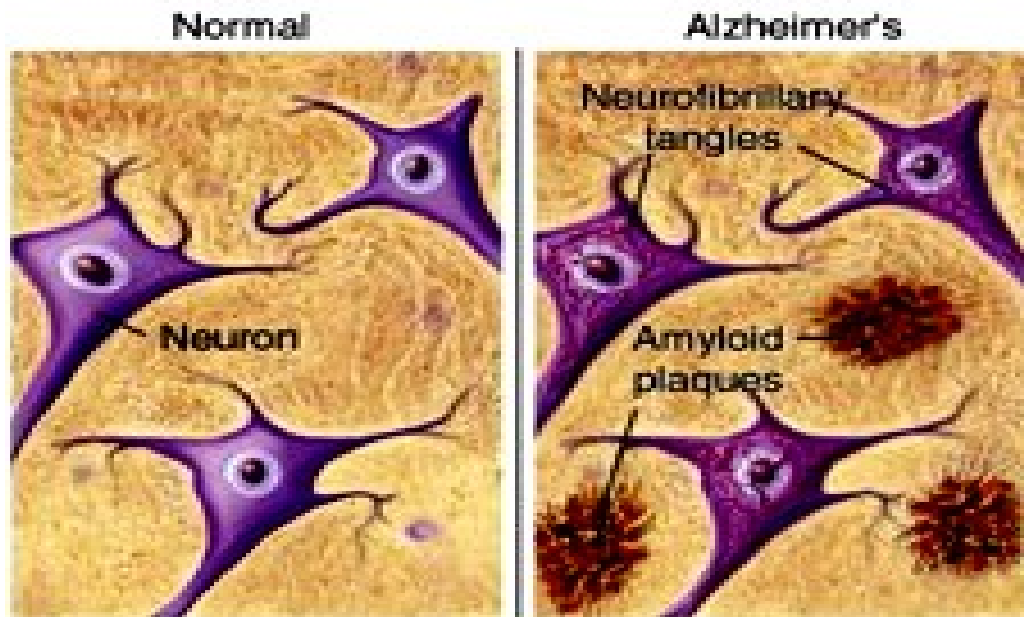


Fig. 2: Microscopic alteration in AD

2. *Amyloid cascade hypothesis*

Hardy and colleagues proposed for the first time "the amyloid cascade hypothesis", highlighting as neurodegeneration in AD was caused by an abnormal accumulation of β -amyloid plaques in various areas of the brain [Hardy and Higgins, 1992]. According to this hypothesis the $A\beta$ accumulation damages neurons through different mechanisms. The $A\beta$ deposition and diffused plaque formation lead to local microglial activation, cytokine release, reactive astrogliosis and a multi-protein inflammatory response [Barage and Sonawane, 2015]. Moreover, $A\beta$ interacts with components of the cell membrane and directly damages neurons, increasing susceptibility to excitotoxicity and mitochondria damage (Fig. 3).



Fig. 3: Amyloid cascade hypothesis

Amyloid is a peptide generated intracellularly by proteolytic cleavage of amyloid precursor protein (APP), a type I membrane glycoprotein encoded by APP gene, located on chromosome 21 [Yoshikai *et al.* 1990].

APP endoproteolysis can be achieved from a sequential cleavage by groups of enzymes or enzyme complexes termed α -, β - and γ -secretases. Three enzymes with α -secretase activity have been identified, all belonging to the disintegrin- and metalloproteinase-family enzyme (ADAM): ADAM9, ADAM10 and ADAM17 (also known as tumour necrosis factor converting enzyme). Conversely, β -secretase, also called β -site cleaving APP enzyme 1 (BACE-1), was identified by several groups as a type I.

There are two proteolytic processing pathways of APP (Fig. 4).

In the non-amyloidogenic pathway, APP is cleaved by α and γ secretases, resulting in the production of a soluble form of APP (sAPP α) and p3 peptide, that prevents the formation of β -amyloid plaques and has several neuroprotective properties [Barage and Sonawanw, 2015].

In the amyloidogenic pathway, APP is cleaved by β -secretase, generating a membrane bound C-terminal fragment (C99), which is cleaved by γ -secretase leading to an amyloid- β peptide of 40-42 amino acids [Walter *et al.* 2001].

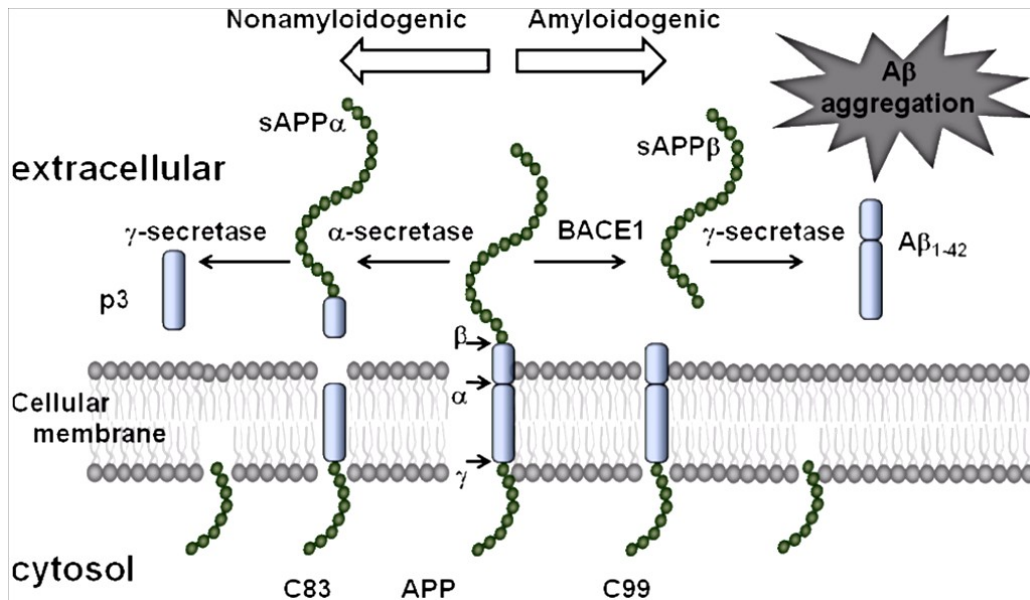


Fig. 4: Proteolytic processing pathways of APP

Another important role in AD pathogenesis is given by presilins. Presenilin 1 and presilin 2 proteins are predominantly present in neurons, and are encoded by PSEN 1 and PSEN 2 genes (located on chromosome 14 and chromosome 1, respectively). Further research showed that PSEN1 and PSEN2 are part of the γ -secretase complex, which cleaves APP at several points. All PSEN mutations lead to an increase in the A β ₄₂:A β ₄₀ ratio, although the total quantity of A β produced remains constant [Šimić *et al.* 2016].

Under normal physiological conditions, the A β ₁₋₄₀ isoform is the most common isoform, contrary to the AD pathology in which the A β ₁₋₄₂ isoform is the most abundant [Kuo *et al.* 1996], having the highest propensity for aggregation and leading to their accumulation in senile plaques [LaFerla and Oddo, 2005; Lista *et al.* 2014].

In recent years numerous studies have challenged the validity of the hypothesis formulated by Hardy, where cognitive deficits of AD are due to the effect of the A β in fibrillar form.

These observations indicate that small soluble oligomeric species of A β (A β ₁₋₄₂), rather than amyloid plaques, may be neurotoxic and responsible for synaptic

and network dysfunctions leading to the physiopathologic consequences of AD [Faucher *et al.* 2016].

In fact, Oddo and colleagues, through the use of transgenic animal models, had been shown that the intracellular accumulation of A β o well correlates with the onset of cognitive impairment [Oddo *et al.* 2003a].

3. *Tau protein hyperphosphorylation and neurofilament accumulation*

Tau (tubulin-associated unit) is a low molecular weight protein, isolated for the first time by Weingarten, which showed the capacity to promote microtubule (MT) assembly [Weingarten *et al.* 1975].

Tau is a product of the microtubule-associated protein (MAPT) gene, located on chromosome 17q21.1, and has become known also for other functions, such as the maintenance of axonal transport and providing linkage for signal trasduction [Martin *et al.* 2011; Andreadis, 2005]. In the adult brain six isoforms of tau proteins have been identified, which derive from alternative splicing of one single gene located on the long arm of chromosome 17 [Martin *et al.* 2011; Andreadis *et al.* 1992].

Structurally, tau is subdivided into two main functional domains: (i) the basic MT binding domain (MBD), that consists of four sequence repeats (R1–R4) of serine (S) and threonine (T) followed by proline (P) and regulates the rate of MT polymerization; (ii) the acid projection domain, that is involved in cell signaling and, in synapses, interacts with protein kinase Fyn, postsynaptic density protein 95 and N-methyl-D-aptate receptors (NMDAr) [Barage and Sonowane, 2015; Šimić *et al.* 2016].

As demostred by an immunohostochemical study using the tau-1 antiboby, tau protein is most abundantly expressed in axons of central nervous system neurons, but can also be found in the somatodendritic compartment of neurons, oligodendrocytes, and non-neural tissues [Binder *et al.* 1985; Couchie *et al.* 1988].

The most common post-translational modifications of tau proteins are phosphorylation and O-glycosylation. Phosphorylation changes the shape of tau molecule and regulates its biological activity [Buée *et al.* 2000]. Normally tau is an abundant soluble protein in axon and promotes the assembly and stability of micrutubules [Lee *et al.* 2010].

On the contrary, Tau hyperphosphorylation is associated with tau assembly into paired helical filaments (PHF) which are deposited in neurofibrillary tangles

NFTs), which, together with amyloid plaques, are the principal hallmarks of AD [Kopke *et al.* 1993] (Fig. 5).

It is believed that the causes that lead to the hyperphosphorylation of tau are many, such as up-regulation or aberrant activation of tau kinases, downregulation of phosphatases, mutations of the tau gene, covalent modifications of tau proteins, and indirect events such as A β -mediated toxicity, oxidative stress and inflammation [Ballatore *et al.* 2007].

Accumulating evidence suggest that the amino acids of MBD of tau are hyperphosphorylated by various enzymes such as glycogen synthase kinase 3 β (GSK-3 β), cyclin-dependent protein kinase5 (cdk5) mitogen-activated protein kinase (MAPK), protein kinase A (PKA) [Iqbal *et al.* 2005].

The hyperphosphorylation of tau reduces its affinity towards microtubules [Mazanetz and Fischer, 2007].

The loss of normal tau function leads to a pathological disturbance in structural and regulatory functions of the cytoskeleton. When the axonal transport is altered neurons degenerate and the neural network is interrupted, causing synaptic dysfunction and neurodegeneration [Oddo *et al.* 2003].

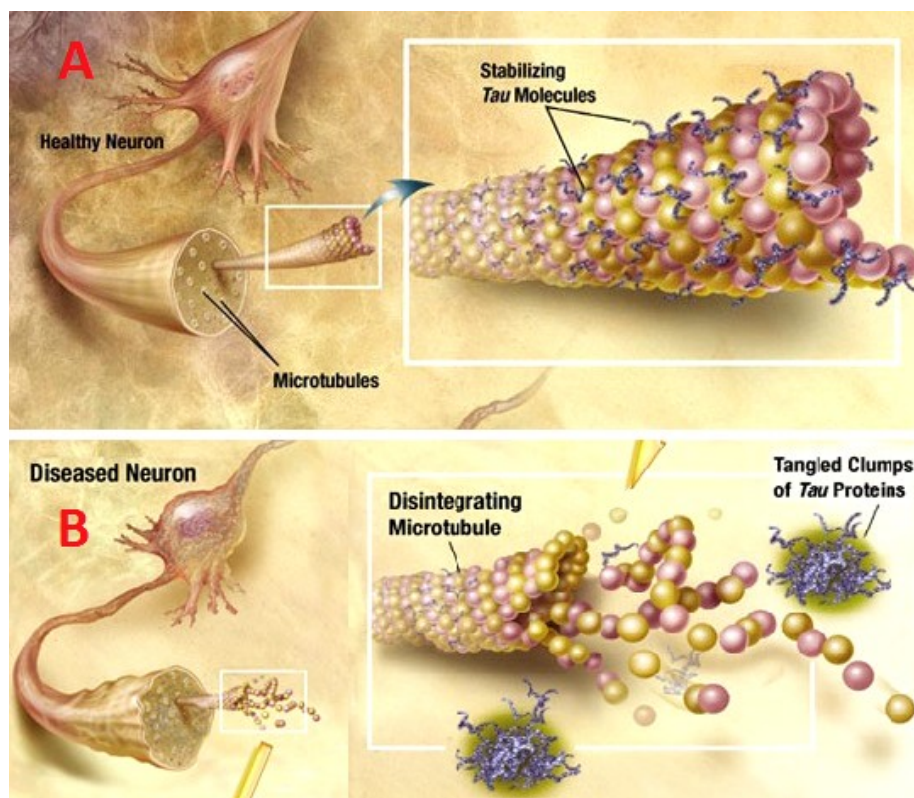


Fig. 5: Representation of Tau protein in healthy and AD neuron

4. *Glutamatergic alteration in Alzheimer's disease*

Glutamate (Glu) is the most common excitatory neurotransmitter in the central nervous system (CNS). Today it is well established that some of the most important brain functions such as learning, memory, and control of motor function occurs through the release of glutamate at the synaptic level [Cassano *et al.* 2015].

Glutamate receptors are categorized into two main groups, ionotropic and metabotropic [Stayte and Vissel, 2014].

The ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels and, once activated, allow the ion flow (mainly Na⁺ and/or Ca²⁺) through the membrane. These receptors are mainly involved in excitatory synaptic transmission throughout the CNS and are important for numerous brain functions.

To this family of iGluRs belong N-methyl-D-aspartate (NMDA), α -amino-3-hydroxyl-4-isoxazolepropionic acid (AMPA) and Kainate subtypes. [Hollmann and Heinemann, 1994]

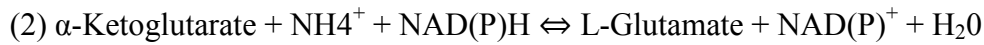
On the contrary, metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors. Once bound to the ligand, they initiate a series of intracellular reactions, mediated by second messengers involved in signal transduction [Conn *et al.* 2005].

Intracellular brain levels of glutamate are very high (10 mM), but in the extracellular spaces of the brain the concentrations of this amino acid are almost ten thousand times lower (1-5 μ M), due to the action of three major carriers: (i) EAAC1 (Excitatory Amino Acid Carrier 1), that is present in the brain and reuptakes glutamate in the neuron; (ii) GLT1 (Glutamate Transporter 1), that is abundantly expressed in astrocytes and is the most important carrier to maintain low concentrations of glutamate in the extracellular spaces; (iii) GLAST (Glutamate/Aspartate Transporter), that is expressed in glia and is activated by high concentrations of glutamate.

During a glutamatergic transmission, the action potential causes a depolarization of the membrane, with consequent opening of Ca²⁺ channels. The glutamate binds to its receptor and the Glu released from neurons can be transported to astrocytes via glutamate transporters, where it is amidated to Glutamine (Gln). In neurons Gln is metabolized to Glu, generating indirectly γ -

aminobutyric acid (GABA) as well as the tricarboxylic acid (TCA) cycle intermediate, α -ketoglutarate (α -KG) [Dienel, 2013; Magistretti, 2006].

The reactions are:



High extracellular glutamate levels can cause neuronal death. This phenomenon is called “excitotoxicity” and it is involved in many neurological diseases in CNS. [Yang *et al.* 2011; Atlante *et al.* 2001] (Fig. 6).

Glutamate neurotoxicity is characterized by time-dependent damage, where ROS are generated in different cell compartments due to several reaction. Multiple lines of evidence suggest that Glu dysregulation plays a central role in AD [Mattson, 2003].

In AD there is a progressive loss of cortical and hippocampal glutamate receptors [Greenamyre, 1985], and this alteration of the glutamatergic system could be responsible for some clinical manifestation of AD, like memory and learning deficit [Greenamyre *et al.* 1986].

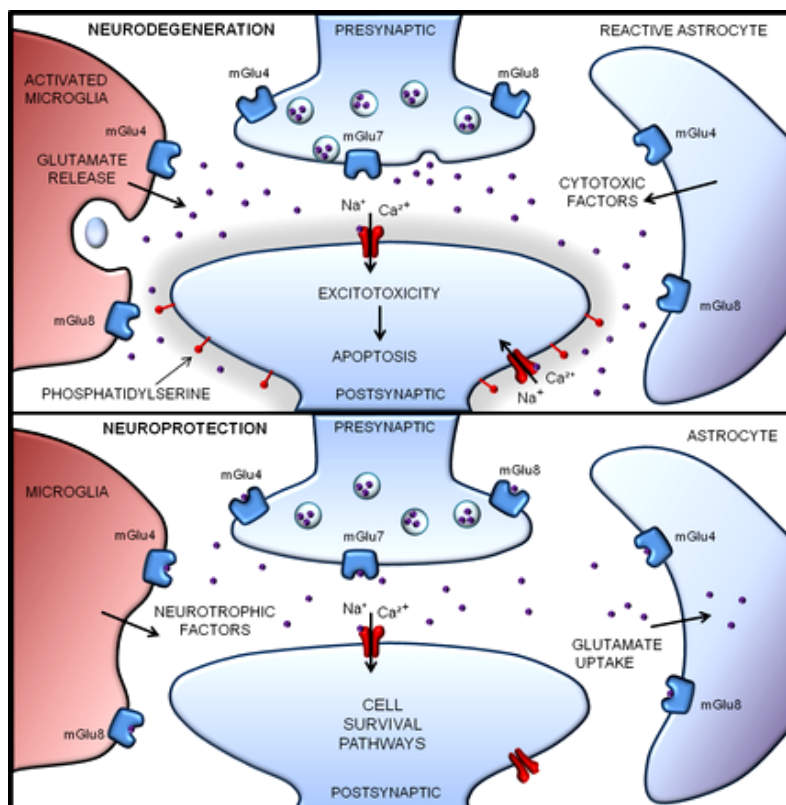


Fig. 6: Excitotoxicity

Several studies have reported reduced levels of glutamate in the tissue samples taken from frontal cortex and hippocampus of AD patients, and reduced glutamate uptake activity in the frontal and temporal cortices of AD brains. These alterations are thought to be responsible for the elevation of extracellular glutamate concentration, which, in turn, renders neurons for excitotoxicity. Thus, while the theory that chronic excitotoxicity contributes to multiple neurodegenerative diseases is supported by many layers of scientific evidence, it is still not clear that therapeutic re-interventions establish glutamatergic during homeostasis ongoing neurodegeneration will be effective tools for stopping the disease process. Future research will focus in this regard.

5. *Neuroinflammation in Alzheimer's disease*

Inflammation is a complex cellular and molecular response to insults, an attempt to defend against these insults. The hypothesis that inflammation was a risk factor for Alzheimer's disease was initially based on autopsies, in which it was possible to find inflammatory changes associated with the lesions of the disease, and on the epidemiological evidence of the protective effect of anti-inflammatory drugs.

There is strong evidence that A β toxicity could be mediated through the induction of inflammatory events in the brain. Over the past decade it has been speculated that the inflammatory response associated with the presence of neuritic plaques could be involved in neuronal damage and contribute to the progression of the disease (Fig. 7).

Today we know that glial cells activation play a central role in the inflammatory pathogenesis of AD.

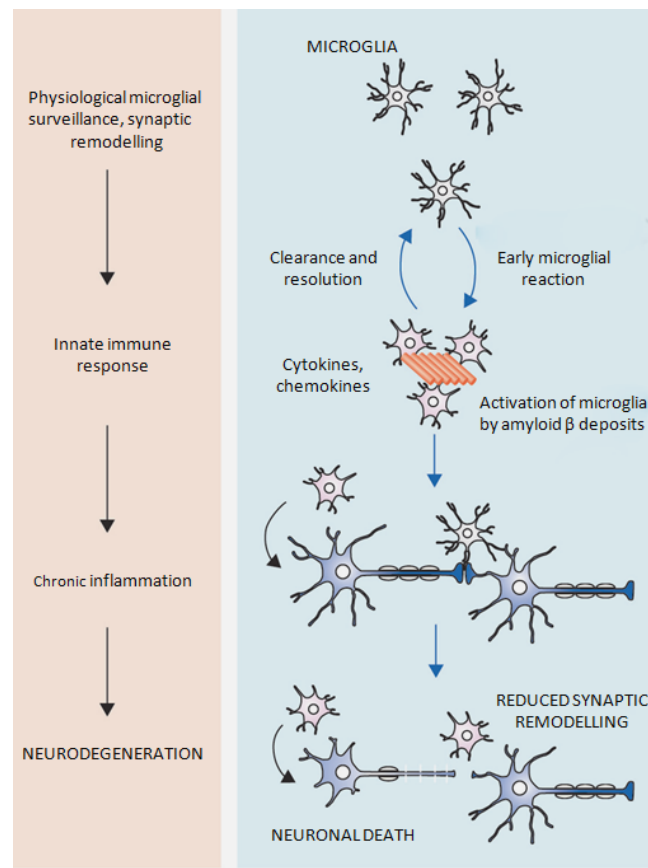


Fig. 7: Inflammatory response associated with the presence of neuritic plaques

5.1 Microglia

An important role in inflammatory processes associated with neurodegenerative diseases is attributed to microglia, which represents the first defense of neuronal tissue against all kinds of environmental insult [Lee *et al.* 2010].

In Alzheimer's disease, microglial cells are able to bind to soluble amyloid β oligomers and A β fibrils, thanks to the link with some cell-surface receptors, such as SCARA1, CD36, CD14, α 6 β 1 integrin, CD47, and Toll-like receptors [Bamberger *et al.* 2003]. A β can attract and activate microglia, leading to clustering of microglia around A β deposits sites in the brain.

Activated microglia show also an increase in cell surface expression of MCH II protein, along with increased secretion of proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α , the chemokines IL-8, macrophage inflammatory

protein-1 α (MIP-1 α), and monocyte chemoattractant protein-1 [Rogers and Lue, 2001].

Under normal circumstances, after exposure to a danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs), the acute microglial reaction aims to remove quickly the pathological changes with an immediate benefit [Heneka *et al.* 2015]. Normally, activated microglia can reduce A β accumulation by increasing its phagocytosis or extracellular degradation.

In the case of Alzheimer's disease, this type of inflammatory reaction is sterile because several mechanism compromise cessation of inflammation.

In fact microglia not only have neuroprotective function, but also have neurotoxic effect in the brain. Neurotoxic mechanism involving continuous activation of microglia and toxic factor released by microglia, may lead to neuroinflammation [Streit *et al.* 1999].

In vitro studies have demonstrated the ability of microglia to secrete a large amounts of hydrogen peroxide (H₂O₂) and nitric oxide (NO). Both of these chemicals can directly damage cells and lead to neuronal cell death through mitochondrial depolarization [Lynch and Lynch, 2002]. Proteases secreted by microglia catabolize specific proteins causing direct cellular damage, while cytokines like IL-1 promote demyelination of neuronal axons.

Other researches showed that IL-1 β enhances activation of caspase-3, an enzyme implicated in hippocampal neuron apoptotic death in aged rats [Solenski *et al.* 2003].

Finally, microglia can injure neurons through NMDA receptor-mediated processes by secreting glutamate, aspartate and quinolinic acid [Gehrmann *et al.* 1995].

These evidences suggest that the continuous activation of microglia may act as trigger for the progression of AD pathology [Lee *et al.* 2010].

5.2 Astrocytes

Astrocytes represent the most abundant cell in human brain, and are involved in numerous and essential function of the central nervous system (CNS) [Scuderi and Steardo 2013]. They, together with microglia, are the cellular component of the resident innate immune system in the CNS and act as crucial effectors of the neuroinflammatory response. Accumulating data shows the relevant role of astrocytes in A β pathogenesis of AD.

They express the receptor for advanced glycation end product (RAGE) which binds A β , and possess a complex apparatus able to take up A β [Wyss-Coray *et al.* 2003].

Numerous studies conducted both in humans and in experimental models of AD demonstrated that astrocytes cluster around A β deposits, forming a barrier between healthy and injured tissue [Steardo Jr *et al.* 2015]. The aggregation of astrocytes around A β deposits in AD suggest that these lesions produce chemotactic molecules that induce astrocyte recruitment.

Similar to microglia, astrocytes activated by A β can secrete various proinflammatory molecules such as chemokines, cytokines, and [Johnstone *et al.* 1999].

In a recent paper Scuderi and colleagues investigated the transcription and expression of the GFAP protein, that is considered one of the most important markers of astrocyte activation and also plays a role in mediating most of the signals involved in morphology and functional alterations observed during astrocyte activation. In A β -inoculated rats, they observed an evident increase in the GFAP intensity, and astrocytes show a star-shape with multiple branched processes, which are typical of activated astrocytes [Scuderi *et al.* 2014].

Studies in the Tg2576 animal models of AD showed, in the reactive astrocytes surrounding A β deposits, high levels of interferon- γ , IL-12 mRNA and over-expression of cytokine S100B in neuronal plaques [Mori *et al.* 2009; Scuderi *et al.* 2014]. S100B protein is a very important mediator involved in the reactive gliosis. Infact, in case of brain damage and in Alzheimer's disease, large amounts of S100B are being passively released mainly from astrocytes to diffuse into CSF and blood brain, participating in the amplification of the inflammatory response by further activating microglia and astrocytes [Steiner *et al.* 2011].

In addition, it was demonstrated that astrocytic NF- κ B is activated after A β exposure, causing an increased expression and release of IL-1 β and IL-6 [Bales *et al.* 1998].

Finally, astrocytes may contribute to neuronal damage observed AD as well. For example Simic and colleagues found, in AD brain, high levels of nitric oxide synthase (NOS)-positive astrocytes compared to controls [Šimić *et al.* 2000]. In addition, astrocytes activated by IL-1 α and IL-1 β were able to produce NO, which results in neuronal damage [Chao *et al.* 1996].

So, targeting neuroinflammation might be an effective therapeutic strategy in AD, so that astrocytes may be reasonably regarded as a promising new target for innovative treatments [Bélanger and Magistretti, 2009].

5.3 Peroxisome-proliferator activated receptor alpha (PPAR- α)

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily. There are three PPAR isotypes that have been characterized: PPAR- α , PPAR β/δ , and PPAR- γ [Chung *et al.* 2008]. Functionally, PPAR- α and PPAR- γ have been identified as key regulators of cellular differentiation processes and antiinflammatory regulation, whereas PPAR β/δ has also been associated with modulation of immune response [Wanger and Wanger, 2010]. PPARs are expressed in the brain at low levels under physiological conditions. Researches have revealed that agonists for PPARs are effective in many CNS disease models, and this has enthused investigations over exploring the physiological actions of these receptors in the brain [Sodhi *et al.* 2011]. PPARs are activated when bound by endogenous lipid/lipid metabolite ligands or synthetic xenobiotic ligands. Once activated, PPARs heterodimerize with the Retinoid X Receptor (RXR) and bind to the PPAR Response Elements (PPREs) in the promoter regions of target genes involved in diverse processes such as energy metabolism, oxidative stress, inflammation, circadian rhythm, immune response, and cell differentiation [Moran and Ma, 2015] (Fig. 8).

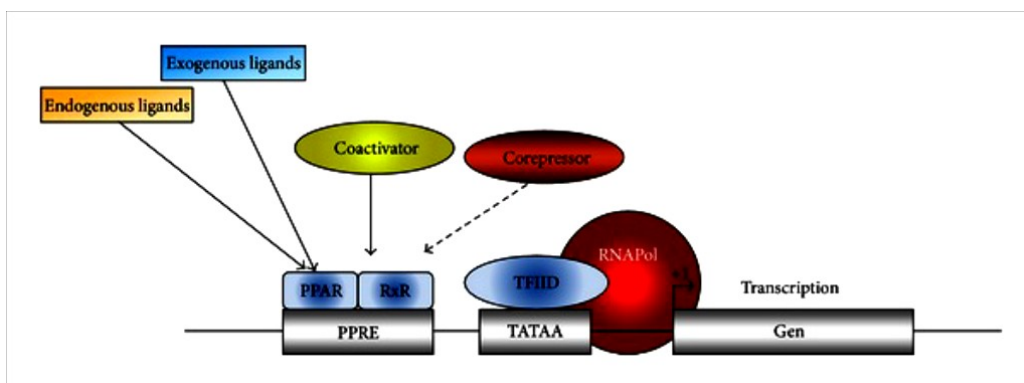


Fig. 8: Mechanism of gene transcription mediated by PPARs

PPAR has neuroprotective effect in several neurodegenerative disease, and may have a therapeutic effects in Alzheimer's disease [Landreth, 2007] (Fig. 9).

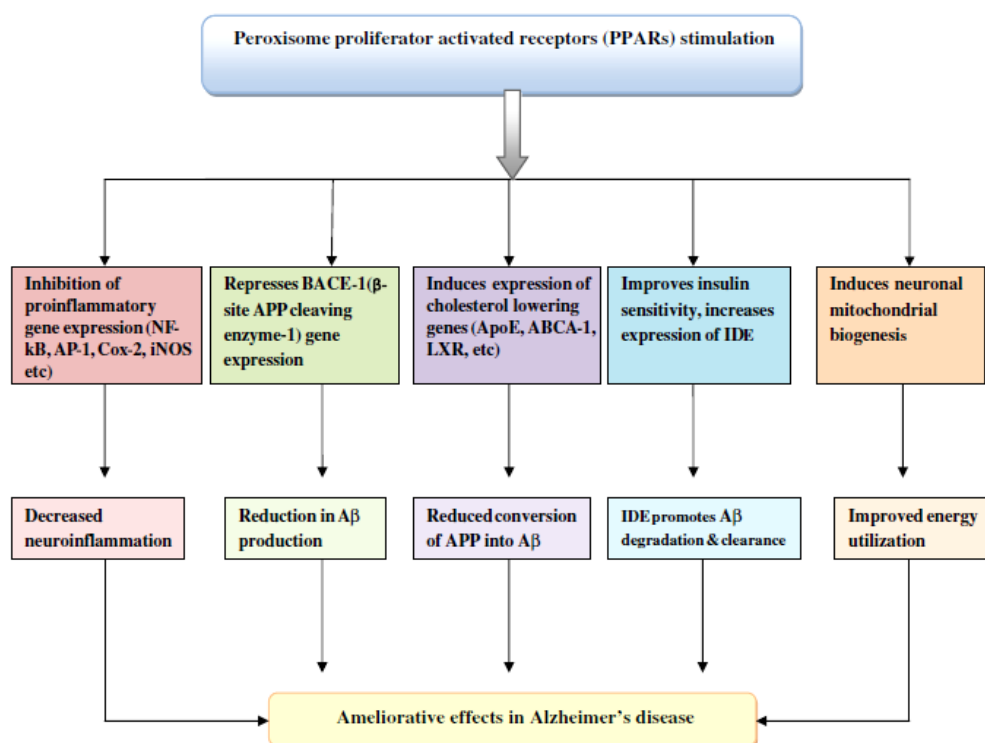


Fig. 9: Therapeutic effects of PPARs in Alzheimer's disease

Studies have revealed that PPAR- α inhibit the expression of inflammatory genes and the activation of these receptors modulates oxidative stress-sensitive pathways, redoxresponsive nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1) [Huang *et al.* 2008]. PPAR- α agonist-induced neuroprotective effect is associated also with a decrease in cerebral oxidative stress depending on the increase in activity of numerous antioxidant enzymes. Another study has been demonstrated that a PPAR- α agonist inhibited A β -stimulate expression of tumour necrosis factor α and interleukin-6 reporter genes in a dose-dependent manner [Bordet *et al.* 2006].

So these findings suggest that the neuroprotective effect of PPAR- α are due in part to PPAR- α 's anti-inflammatory and antioxidant proprieties. These central effects of PPAR- α may contribute to the onset and progression of AD and it could become an interesting target for the development of new therapeutic agents.

6. Palmitoylethanolamide (PEA)

As discussed previously, the inappropriate and prolonged inflammatory process may contribute independently to neuronal dysfunction, cell death and AD progression. At present, no therapies in clinical use are able to effectively impact the disease course, but targeting neuroinflammation might be an effective therapeutic strategy in AD.

At this regard PEA has attracted much attention for its anti-inflammatory and neuroprotective proprieties reported in many neuropathological conditions, including AD [Scuderi *et al.* 2014].

PEA, the naturally occurring amide between ethanolamine and palmitic acid, is an endogenous lipid and belongs to the family of the fatty acid ethanolamides (FAEs). PEA is abundant in mammalian brain and, as in the case of other FAEs, the production of PEA occurs through an *on-demand* synthesis within lipid bilayer [Cadas *et al.* 1996].

Specifically, the synthesis of PEA is performed in two reactions: first there is the transfer of palmitic acid from phosphatidylcholine (PC) to the phosphatidylethanolamine (PE), by the N-acyl-transferase, with the N-acylated phosphatidylethanolamine formation (NAPE). This reaction is cAMP and Ca^{2+} -dependent. Subsequently a specific NAPE-phospholipase D (NAPE-PLD) intervenes, releasing the PEA in the cell. [Okamoto *et al.* 2004].

Unlike other brain chemical mediators, the acylethanolamides are not produced and stored in secretory vesicles, but, completed their function, are rapidly deactivated.

The inactivation of the PEA consists mainly in its intracellular hydrolysis by hydrolase lipid. The enzyme responsible for this activity is the fatty acid amide hydrolase (FAAH), a serine hydrolase bound to the intracellular side of the phospholipid bilayer, which is capable of hydrolyzing the PEA in palmitic acid and ethanolamine. Recent studies have identified a second enzyme, the PEA preferring amidase acid (PAA), which would be involved in the hydrolysis process of the PEA [Ueda *et al.* 2001] (Fig.10).

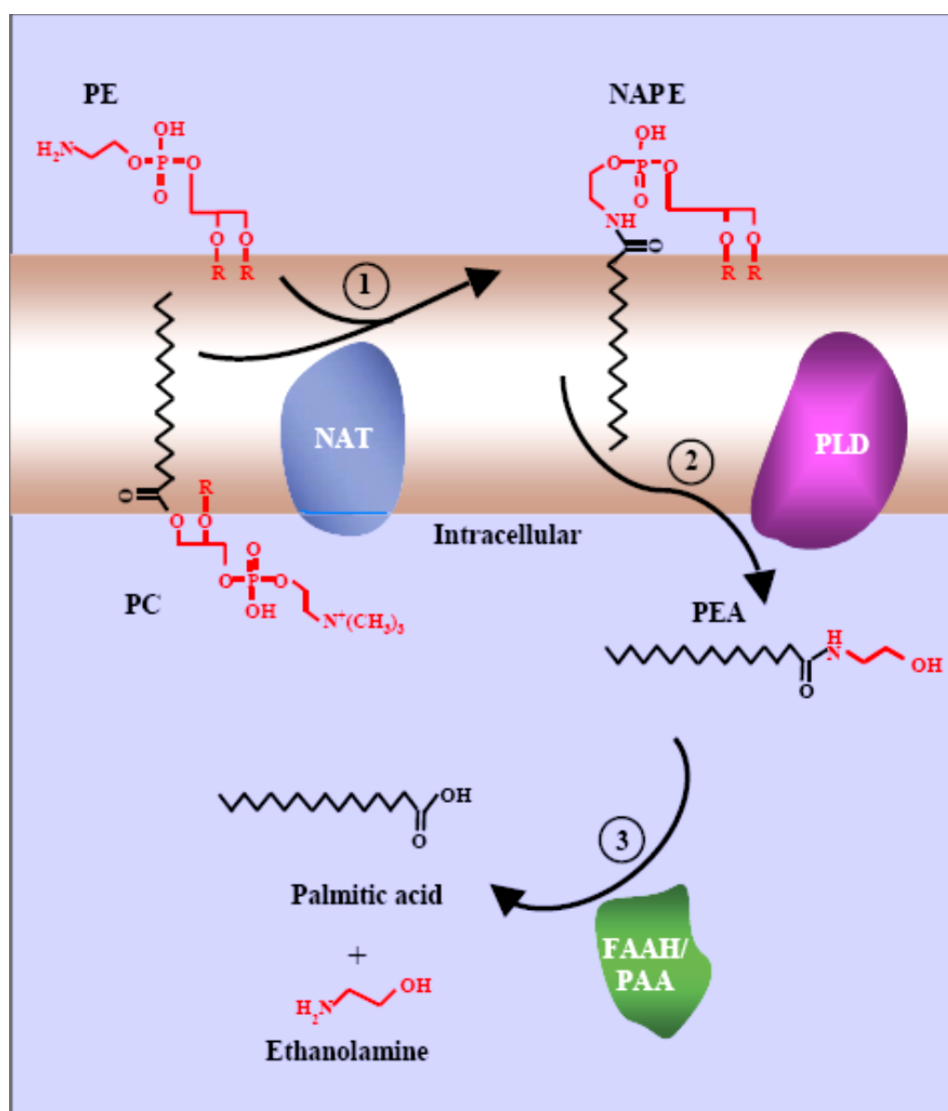


Fig.10: Biosynthesis and inactivation of the PEA

PEA shows a considerable pharmacological potential when used as a drug, or when its endogenous levels are enhanced by the inhibition of its catabolism [Lo Verme *et al.* 2005, Solorzano *et al.* 2009].

In the central nervous system, PEA, produced by neurons, microglia, and astrocytes, exerts a local anti-injury function through a down-modulation of mast cells and by protecting neurons from excitotoxicity, but its exact biological roles remain elusive [Tomasini *et al.* 2015].

Initially, the structural similarity of the PEA with anandamide (AEA) has suggested that both of these lipid mediators target the cannabinoid type 2 receptor (CB2R). However using SR144528, a selective cannabinoid antagonist

for CB2R, it inhibited the anti-nociceptive activity of the PEA, but not its anti-inflammatory prolonged activity [Costa *et al.* 2002]. This effect has been called “*entourage effect*” and it consists on enhancing endocannabinoid activity at their receptors and/or inhibiting endocannabinoid degradation [Tomasini *et al.* 2015]. In fact PEA, even if does not possess the characteristics of endocannabinoids and does not bind the CB1 and CB2 receptors, is able to bind to GPR55 and GPR119, defined orphan receptors, considered of additional cannabinoid receptors.

But many of the beneficial properties of PEA have been considered to be dependent on the activation of PPAR- α . Both PPAR- α and PEA are clearly detected in the CNS and their expression may largely change in many pathological conditions, including AD. It has been observed that A β significantly blunts PPAR- α expression in primary rat astrocyte cell culture, suggesting the possibility that the downregulation of this receptor may represent one of the molecular mechanisms by which A β induces astrocyte activation and possibly exerts toxicity.

Scuderi and colleagues showed in a recent work, that PEA counteracts A β -induced reactive gliosis. PEA was able to blunt the A β -induced upregulation of GFAP and S100B, two important astrocyte-related proteins, through the involvement of PPAR- α [Scuderi and Steardo 2011]. Another study conducted on experimental models of rat demonstrated that A β 1-42 injection induced the upregulation of iNOS and COX-2 compared to vehicle. They found also a marked release of IL-1 β and TNF α . PEA, once again, blunted A β 1-42 effect and controlled the release of pro-inflammatory cytokines. The administration of the selective PPAR- α antagonist, GW6471, completely abolished PEA's effects, indicating the significant involvement of this receptor [Scuderi *et al.* 2014].

PEA is able to reduce BACE 1 and APP, proteins involved in the amyloidogenic pathway, and also to reduce hyperphosphorylation of tau protein. These findings indicate an important neuroprotective function and identify PEA as a potential agent that is able to stop the detrimental cycle in which neuroinflammation and amyloidogenesis cooperate in sustaining the pathological state and the activation process of astrocytes [Scuderi *et al.* 2014].

CHAPTER II. Aim of the study

Alzheimer's disease (AD) is a human neurodegenerative disorder, which causes a deterioration of memory and other cognitive domains leading to death within 3 to 9 years after diagnosis [Querfurth and LaFerla 2010]. AD is characterized pathologically by the presence of senile plaques composed mainly of β -Amyloid (A β) peptide and intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau protein [Flood *et al.* 1999].

The pivotal role that the amyloid-beta peptide has in the development of Alzheimer's disease (AD) is now almost universally accepted. Emerging evidence from transgenic mice and patients indicate that intraneuronal accumulation of A β is an early, pathological biomarker for the onset of AD and associated with cognitive deficits. How intracellular A β might cause cellular dysfunction remains unclear, although some site of action have been identified. Besides plaques and tangles in AD brain numerous other structural and functional alterations ensue, including neurotransmitter alterations, mitochondrial injury and inflammatory processes [Heneka *et al.* 2007; Lin *et al.* 2006].

In fact, AD is multi factorial in nature and is linked to different multiple mechanisms in the brain. The ideal treatment to AD should also modulate the disease through multiple mechanisms rather than targeting a single dysregulated mechanism. Unfortunately, no new drugs have been approved in more than a decade for AD treatment and current pharmacotherapies can only help to alleviate the symptoms of the disease.

Recent evidence suggests that inflammatory mechanisms represent a component which, once initiated by degeneration, may significantly contribute to disease progression and chronicity. Various neuroinflammatory mediators including complement activators and inhibitors, chemokines, cytokines and inflammatory enzymes are generated and released by glia, astrocytes and neurons. Despite the fact that at first inflammation exert protective effects to bystander neurons, sustained and excessive process results in tissue pathology so that an antiinflammatory treatment strategies should therefore be considered. In this regard, Palmitoylethanolamide (PEA), the naturally occurring amide of ethanolamine and palmitic acid, is an endogenous lipid that modulates inflammation acting as a peroxisome proliferator activated receptors (PPAR)- α agonist. In addition to its known anti-inflammatory activity, PEA protected

cultured mouse cerebellar granule cells from glutamate toxicity and reduced histamine-induced cell death in hippocampal cultures.

Furthermore, a research group of Rome, have recently demonstrated that PEA exerts in vitro a combination of neuroprotective and anti-inflammatory effects in Abeta-induced toxicity by interacting at PPAR- α nuclear site [Scuderi *et al.* 2014]. Whether such actions of PEA are maintained in the AD brain remains to be established, and experiments to test the effect of PEA in the various transgenic animal models of AD are eagerly awaited.

On the basis of the above considerations, the aim of the present work is to exploit the availability of a novel triple transgenic model of AD (3xTg-AD) harboring three mutant human genes (betaAPP^{Swe}, PS1M146V, and tauP301L) to directly test the hypothesis that chronic treatment with PEA might modulate the onset and the progression of AD.

To meet our aim, pellets of placebo or PEA at 10 mg/kg/die dose were implanted subcutaneously to 3 month-old and 9-month-old 3xTg-AD and non-Tg mice for 90 days. The dosage was chosen on the basis of previous studies reported in literature [Costa *et al.* 2002].

Animals were used for the study of the behavior and subsequently to determine, by biochemical tests on brain areas, the effect of treatment on neuropathological and neuroinflammatory hallmarks. At the end of the chronic treatment mice were subjected to a behavioral characterization in tasks like Morris water maze test (MWM), novel object recognition test (NORT) and inhibitory passive avoidance (IA) in order to determine cognitive behavior and tail suspension test (TST), forced swim test (FST) and sucrose preference test (SPT) to determine non cognitive behavior.

After all behavioral tests mice were sacrificed either by decapitation or perfusion to collect brains in order to perform western blot analyses or immunofluorescence, respectively.

In particular we investigated, by western blot analyses, the impact of PEA treatment on the expression of the two neuropathological hallmarks of AD, A β and tau, as well as on the expression of neuroinflammatory molecules, such as COX-2, and markers of microglial (Iba-1) or astrocytes (GFAP) activation.

Western blot experiments were performed on both cortex and hippocampus, two brain regions strongly affected by AD neuropathology and highly involved in the regulation of emotionality and mood tone.

CHAPTER III. Material and Methods

1. *Animals and treatment*

In this study, we used a new murine model of AD (3xTg-AD) and wild type littermates (non-Tg). The 3xTg-AD mice harboring APP_{swe}, PS1_{M146v}, and tau_{P301L} transgenes have been genetically engineered by LaFerla and colleagues at the Department of Neurobiology and Behavior, University of California, Irvine [Oddo *et al.* 2003b].

The 3xTg-AD mimics many critical aspects of AD: (i) dysfunction in synaptic plasticity and cognitive impairments occur in conjunction with the early, intraneuronal accumulation of Aβ; (ii) amyloid plaques and neurofibrillary pathology develop in a hierarchical manner in AD-relevant brain regions, mainly the hippocampus, cortex and amygdala; (iii) plaque pathology precedes tangle formation, and plaques consist of the longer, more amyloidogenic Aβ₄₂; (iv) tau protein undergoes conformational and phosphorylation changes following a pattern that parallels the sequence in the human AD; (v) the 3xTg-AD mice show selective loss of nicotinic α7 receptors in the hippocampus and cortex [Oddo *et al.* 2003b; Billings *et al.* 2005].

Genotypes were being confirmed by polymerase chain reaction (PCR) after tail biopsies [Oddo *et al.* 2003b].

Both genotypes were established at the animal facilities of the Puglia and Basilicata Experimental Zooprophyllactic Institute.

The housing condition are being controlled (temperature 22°C, light from 07:00–19:00, humidity 50%-60%), and fresh food and water are being freely available.

All the experiments will be conducted in accordance with Italian National Laws (DL 116/92), with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and regulations on the use of animals for research.

In our experimental protocol pellets of placebo or PEA (*Innovative Research of America, Model NF-141, Sarasota, Florida*) at 10 mg/kg/die dose were implanted subcutaneously to 3 month-old and 9-month-old 3xTg-AD and non-Tg mice for 90 days (Fig 1 and 2). The dosage was chosen on the basis of previous studies reported in literature [Costa *et al.* 2002]. The animals were used for the study of the behavior and subsequently to determine, by biochemical

analyses, the effect of treatment on neuropathological and neuroinflammatory hallmarks.

The pellets were placed on the lateral side of the neck between the ear and the shoulder. This site offers three advantages:

- Maximal space between the skin and the muscle so that the pellet sits comfortably;
- The animal cannot play with the pellet in that side;
- Other animals cannot play with the pellet in that side.

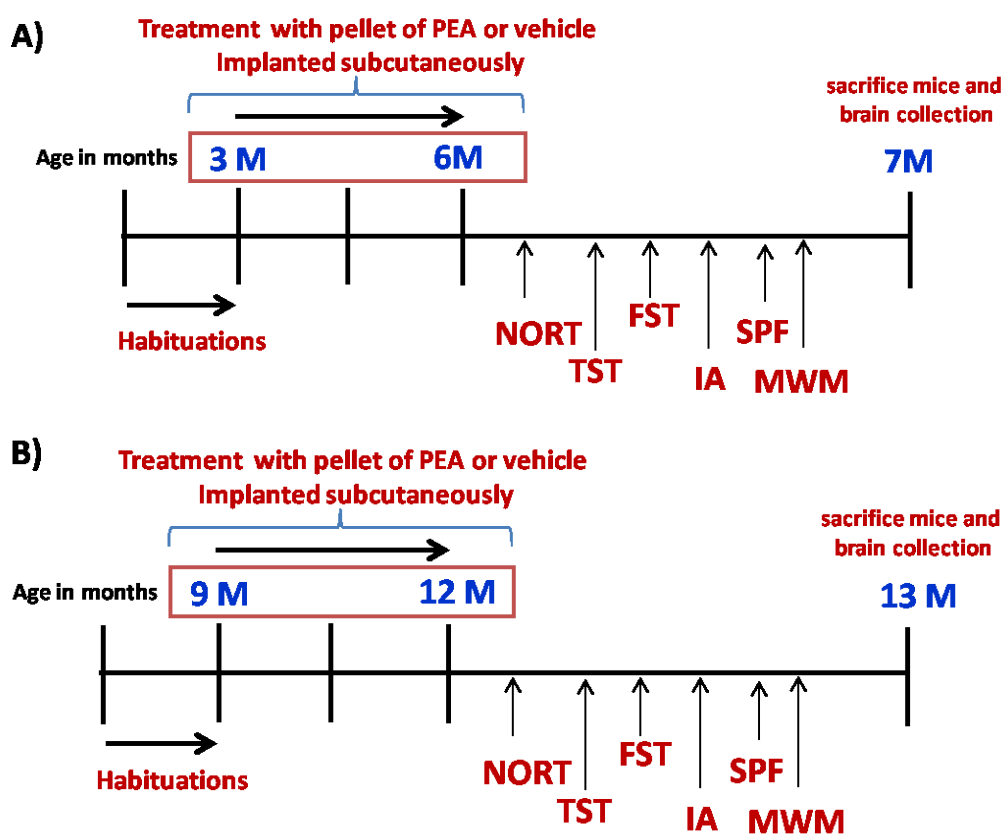


Fig. 1 Timeline of experiments:

A) Effect of PEA treatment on onset of disease;

B) Effect of PEA treatment on progression of disease;

NORT= Novel Object Recognition Test; TST= Tail Suspension Test; FST= Forced Swim Test; SPT= Sucrose Preference Test; MWM= Morris Water Maze test

2. Behavioral assay

Behavioral testing took place at the end of drug/vehicle treatment.

We used a serial behavioral testing procedure that has been validated and compared with single testing procedures in our laboratory, and by other investigators [Kats RJ, 1982; Martinez-Coria *et al.* 2010; Bambico *et al.* 2010; Romano *et al.* 2014].

The Morrison water maze test (MWT), the inhibitory passive avoidance (IA) and the novel object recognition test (NORT) were used to explore the cognitive behaviours, whereas the tail suspension test (TST), the Porsolt forced swim test (FST) and the sucrose preference test (SPT) for antidepressant/depression-like coping behaviors.

A 1- to 2-day interim period was interlaced between tests. All tests were performed between 8:00 a.m. and 3:00 p.m., in a dimly lit condition. On the day of testing, the mice were acclimated for about 60 min in the behavioral room before the procedures were initiated. The apparatus was cleaned with 70% alcohol and water after each run. The behaviors were recorded, stored, and manually analysed.

After all behavioural tests mice were killed to collect brains and determine whether the treatment was effective in preventing or slowing down the occurrence of the neuro-functional alterations in the 3xTg-AD mice.

2.1 Novel object recognition test

The object recognition task is based on the spontaneous tendency of rodents to explore a novel object longer than a familiar one. Each mouse was habituated to an empty plexiglas arena (45×25×20 cm) for 3 consecutive days. On training (day 4), the mice were exposed to two identical objects (A+A) placed at opposite ends of the arena for 5 minutes. 30 min and 24 hours later, the animal were subjected to a 5 min retention session where they were exposed to one object A and to a novel object B (after 30 min) and object C (after 24 h). Exploration was considered as pointing the head toward an object at a distance of < 2.5 cm from the object, with its neck extended and vibrissae moving. Turning around, chewing, and sitting on the objects were not considered exploratory behaviors. The time of exploration were recorded, and an object recognition index (ORI) were calculated, such that $ORI = (TN - TF) / (TN + TF)$,

where TN and TF represent times of exploring the familiar and novel object, respectively [Martinez-Coria *et al.* 2010].

Mice that did not explore both objects during training were discarded from further analysis. Objects used in this task were carefully selected to prevent preference or phobic behaviors. To avoid olfactory cues, the objects were thoroughly cleaned with 70% ethanol and the sawdust was stirred after each trial.

2.2 *Morris water maze*

The test was conducted in a circular tank of 1.2 meters in diameter, located in a room with several extra maze cues as previously described [Billings *et al.* 2005, Medina *et al.* 2011, Martinez-Coria *et al.* 2010]. Briefly, mice were trained to swim to a 14-cm-diameter circular Plexiglas platform submerged 1.5 cm beneath the surface of the water and invisible to the mouse while swimming. The water temperature was kept 25°C throughout the duration of the testing. The platform was fixed in place, equidistant from the center of the tank and its walls. Mice were subjected to 4 training trials per day and were alternated among 4 random starting points for 5 consecutive days. Mice were allowed to find and escape onto the submerged platform. If the mice failed to find the platform within 60 seconds, they were manually guided to the platform and were allowed to remain on it for 10 seconds. After this, each mouse was placed into a holding cage under a warming lamp for 25 s until the start the next trial. Retention of the spatial training (the probe trial) was assessed 1.5 and 24 hours after the last training session and consisted of a 60-second trial without the platform. Mice were monitored by a camera mounted in the ceiling directly above the pool, and all trials were stored on videotape for subsequent analysis. The parameters measured during the probe trial included (i) initial latency to cross the platform location, (ii) number of platform location crosses, and (iii) time spent in the target quadrant [Billings *et al.* 2005, Medina *et al.* 2011, Martinez-Coria *et al.* 2010].

2.3 *Inhibitory passive avoidance*

In this test the experimental subjects learn to avoid an environment in which is provided an aversive stimulus (foot-shock). The IA task was performed as previously described [Martinez-Coria *et al.* 2010; Billings *et al.*

2005]. Briefly, testing began with a training trial in which each mouse was placed in the illuminated compartment and was recorded the time for it to enter the dark compartment (baseline time). Upon entering the dark compartment, the door between the two compartments was closed and after 2 second of delay, the mouse received an electric foot shock (0.1 mA, 1 second). Animals were returned to the home cage 30 seconds after foot shock. The animals were subsequently tested 24 h and 7 days after the training phase to assess the short- and long-term memory. During this phase, the mouse was again placed in the illuminated compartment and was analyzed the latency to return to the dark compartment (previously associated with shock). The retention trial was interrupted if the animal took more than 300 seconds to cross into the dark compartment.

2.4 Forced swim test

Originally developed by Porsolt *et al.* [Porsolt *et al.* 1977] the Forced Swim Test (FST) is to date one of the most used behavioral tests to study the depressive-like behavior and the effect of antidepressant drugs in rats or mice. When rodents were forced to swim in a confined space from which there is no escape, they will adopt a characteristic immobile posture after a brief period of vigorous activity.

The FST examines the dynamics of transition from an active (swimming) to a passive (immobility) mode of coping in an inescapable water-filled pool. Enhancement of immobility normally ensues after exposure; a phenomenon argued to reflect learned behavioral despair [Porsolt *et al.* 1977] and prevented by antidepressant treatment.

The Forced Swim test was performed as previously described [Bambico *et al.* 2010; Romano *et al.* 2014]. Briefly, mice were placed individually into Plexiglas cylindrical bins (20 cm diameter, 50 cm high) filled with water (25–27°C water temperature) to a depth of 20cm. This depth did not allow the tail and hindpaws to touch the floor of the bin. The mice were allowed to swim for 6min. After recording, the mice were rescued using a plastic grid and caged near a heat source (lamp). The mouse was considered immobile when making only those movements necessary to keep its head above water. The total duration of activity was determined during the last 4 min.

2.5 Tail suspension test

The Tail Suspension Test (TST) is the most widely used model for studying antidepressant-like activity in mice. In the TST, as in the FST, immobility reflecting a behavioral despair is used as ethological relevant parameter to evaluate the depressive-like phenotype in the animals. In this paradigm the animals subjected to an inescapable stress of being suspended by tail, will initially try to escape by engaging vigorous movements and then will develop an immobile posture. Immobility is defined as the absence of initiated movements including passive swaying. Similarly to the FST, sensitivity of the TST to all major classes of antidepressant drugs has been reported.

In our procedure, as previously described [Romano *et al.* 2014], animals were individually suspended by the tail from a lever (distance to floor = 30 cm) in an apparatus consist of a plastic white enclosures box (30×30×30 cm) using an adhesive tape (distance from tip of tail = 2 cm). Mice were suspended for a 6 minutes session, while only the last 4 minutes were used for analysis. The number of seconds spent in a completely immobile posture, termed immobility, was the behavioral parameter recorded.

2.6 Sucrose preference test

One of the main symptoms of depression is anhedonia, defined as the inability to experience any kind of pleasure. This function can be evaluated in rodents as a decrease in the capacity of response to the rewards, such as a sucrose solution [Kats, 1982]. The SPT was performed as previously described [Romano *et al.* 2014]. Briefly, the animals were isolated in small cages (22.5×14×16,7 cm). Each of them had free access to two drinking bottles, the first filled with 100 ml of tap water, while the other with a solution of sucrose to 2%. Before the test, there was a period of adaptation lasted 48 hours, in order to accustom the animals to the different types of fluids. The animals were then deprived of food and liquids for 3 hours.

During the next 24 h, free consumption of water and 2% sucrose solution took place, in the presence of ad libitum food. Fluid intake was measured afterwards by weighing the drinking bottles. Sucrose preference was calculated from the amount of sucrose solution consumed, expressed as a percentage of the total amount of liquid drunk.

3. Quantitative western blot analyses

Mice (n=6 per group) were sacrificed, their brains were rapidly excised and freshly dissected to isolate cortex and hippocampus from 3×Tg-AD and wild type control mice. Brain areas were frozen in dry ice and stored at -80°C until needed. Each portion was thawed in ice-cold RIPA buffer (pH 7.4) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1mM EDTA, 0,1% SDS, supplemented with the proper protease inhibitor cocktail (phenylmethylsulfonyl fluoride 1mM, aprotinin 10 µg/ml, leupeptin 0,1 mM and sodium orthovanadate 1 mM, *all from Sigma-Aldrich*). Brain areas were homogenized by sonication (*UP50H, Hielscher, USA*) and the resulting homogenates were centrifuged at 14,000 rpm for 15 min at 4°C to remove cellular debris. The supernatant was extracted to determine the total protein concentration by the Bradford assay and then stored at -80°C until use. Equivalent amounts (30 µg) of each sample calculated by Bradford assay were resolved on 12% precast polyacrylamide gels (*12% Mini-PROTEAN[®] TGXT[™], BioRad, Hercules, CA, USA*). Proteins were transferred into a nitrocellulose membrane. Membranes were blocked for 1h in 5% w/v nonfat dry milk powder in Tris-buffered saline-Tween 20 0,1% (TBS-T). Following blocking, detection was made using a panel of primary antibodies diluted in 5% w/v nonfat dry milk powder in TBS-T: rabbit anti-β-actin (1:10000, *Abcam plc, Cambridge, UK*), mouse anti-β-amyloid 6E10 (1:1000, *Signet Laboratories-Covance, Emeryville, CA, USA*), mouse anti-phospho-tau AT8 and AT180 (1:1000, MN1020 and MN1040, *Thermo Scientific, Waltham, MA, USA*), rabbit anti-BACE1 (1:1000, *Abcam plc, Cambridge, UK*), rabbit anti-Iba1 (1:3000, *Abcam plc, Cambridge, UK*), rabbit anti-COX-2 (*Abcam plc, Cambridge, UK*), rabbit anti-GFAP (*Abcam plc, Cambridge, UK*). After being extensively washed in TBS-T, membranes were incubated for 1 h in the proper secondary horseradish peroxidase-conjugated antibodies (HRP-conjugated goat anti-rabbit or goat anti-mouse IgG, 1:5000, *Jackson ImmunoResearch, Europe, Suffolk, UK*). Membranes were developed with a chemiluminescent HRP Substrate kit (*WesternBright[™] ECL, Advansta Corporation, Menlo Park, CA, USA*), acquired with Chemi-Doc MP (*Bio-Rad, Hercules, CA, USA*) and analyzed using Image Lab software (*Bio-Rad, Hercules, CA, USA*). Size approximations were taken by comparing the stained bands to that of the marker. As mentioned above, the process was repeated for a structural protein, the β-actin that should not change between the samples. All data were

normalized to the amount of β -actin. Results were expressed as percentage of vehicle-treated animals.

4. Immunofluorescence

Mice (n=4 per genotype) were intra-cardioventricularly perfused with saline followed by fixation solution (4% paraformaldehyde in 0.1 M phosphate buffer, PB, pH 7.4) at a flow rate of 36 ml min⁻¹ [Cassano *et al.* 2011]. Brains were post-fixed in the fixation solution for 1 day and then transferred in 0.02% sodium azide in PB. After removal of excess sucrose, brains were frozen in 2-methylbutane (-50°C). Brain coronal sections of 20 μ m thickness were cut on a cryostat (-20°C) and thaw-mounted on positively charged slides. Slides were washed with PBS 0.1 M, pH 7.4. The section were blocked in 5% normal donkey serum/PBS with 0.3% Triton X-100 and then incubated with the primary antibody (anti- β -Amyloid 6E10 Signet, dilution 1:1500) overnight at 4°C. After washing excess of antibody, slices were incubated with the secondary antibody (Alexa Fluor 488 goat anti-mouse, dilution 1:250) for 1h30' at room temperature. After final washes section were treated with Bisbenzimidazole (Hoestch, 1:5000) in order to stain nuclei.

5. Statistical analyses

All data were expressed as mean $\pm$ SEM. After testing for assumptions of normality of data distribution and of homogeneity of variance, the behavioral data were submitted to two-way analyses of variance (ANOVA) with genotype (3 $\times$ Tg-AD vs non-Tg) and PEA treatment (PEA vs vehicle) as between-subject factors. Tukey's honestly significant difference (HSD) test was used for multiple post hoc comparisons when required. Results from western blot were expressed as percentage of controls and were analyzed by unpaired Student's t-test. The threshold for statistical significance was set at $p < 0.05$.

CHAPTER IV. Results

1. Effects of PEA treatment on the depressive-like behavior in 3×Tg-AD mice

We tested the depressive-like phenotype of mice in two models of stress-coping behavior (FST and TST) and with an anhedonia test (SPT).

Figure 1 presents the emotional profile of 3×Tg-AD and Non-Tg mice treated either with PEA or with vehicle at 6 months of age.

Figure 1A shows the behavioral profile in the FST comparing differences in immobility time (sec). Data were analysed by two-way analyses of variance (ANOVA) with genotype (3×Tg-AD vs non-Tg) and treatment (PEA vs vehicle) as between-subject factors.

All the results from the statistical analyses are reported in the table 1.

Two-way ANOVA revealed a significant main effect of genotype [$F_{(1,39)} = 28.559$, $p < 0.001$], treatment [$F_{(1,39)} = 5.437$, $p < 0.05$] and genotype by treatment interaction [$F_{(1,39)} = 11.442$, $p < 0.01$].

We next analysed the data with Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons. Post hoc comparisons showed that immobility time was higher in vehicle-treated 3×Tg-AD mice than in transgenic mice treated with PEA ($p < 0.001$) and with respect to vehicle-treated Non-Tg mice ($p < 0.001$) (Fig. 1A).

Similarly to FST, also in the TST we found a significant genotype [$F_{(1,39)} = 44.062$, $p < 0.001$] treatment [$F_{(1,39)} = 7.203$, $p < 0.05$] and genotype by treatment interaction effect [$F_{(1,39)} = 9.308$, $p < 0.01$].

Subsequent post hoc comparisons revealed a significantly higher immobility time in 3×Tg-AD mice treated with vehicle compared with those treated with PEA ($p < 0.001$) and with respect to vehicle-treated Non-Tg mice ($p < 0.001$) (Fig.1B).

Figures 1C and 1D present the behavioral profile of 3×Tg-AD and Non-Tg mice in the SPT. As reported in Table 1, we found a significant effect of genotype [$F_{(1,39)} = 22.547$, $p < 0.001$], treatment [$F_{(1,39)} = 3.608$, $p < 0.05$] and genotype by treatment interaction [$F_{(1,39)} = 8.933$, $p < 0.01$].

Multiple post hoc comparisons showed a significant higher preference for the sucrose solution in the transgenic group treated with PEA compared to vehicle-treated transgenic mice ($p < 0.01$).

Interestingly, PEA treatment restored the normal preference for the sweet solution, since 3×Tg-AD mice treated with vehicle did not show any preference (Fig. 1D).

This effect was not accounted for by a difference in total fluid intake (Fig. 1C). We next examined the emotional behavior of the 4 experimental groups at 12 months of age. All the results from the statistical analyses are reported in the table 2.

Figure 2A presents the behavioral profile in the FST. Results from two way ANOVA revealed a significant genotype [$F_{(1,29)} = 39.686$, $p < 0.001$], and genotype by treatment interaction effect [$F_{(1,29)} = 4.690$, $p < 0.05$].

Post hoc comparisons showed only a significant higher immobility time in transgenic mice treated with vehicle with respect to vehicle-treated Non-Tg mice ($p < 0.001$).

Similar results were obtained in the TST, in which we found only a significant main effect of genotype [$F_{(1,28)} = 11.873$, $p < 0.001$]. Also the post doc analyses showed a significant higher immobility in transgenic mice respect non-Tg (Fig. 2B).

Figure 2C and 2D report the behavioral profile of 3×Tg-AD and Non-Tg mice in the SPT. Two-way ANOVA revealed a significant genotype by treatment interaction effect [$F_{(1,39)} = 4.563$, $p < 0.05$].

Multiple post hoc comparisons revealed a significant greater preference for the sucrose solution in the PEA-treated group of transgenic mice compared to the vehicle-treated group ($p < 0.05$) (Fig. 2D).

Also in these groups there was no difference in the total fluid intake (Fig. 2C).

All together these results suggest that 3×Tg-AD mice showed a depressive-like and anhedonia-like phenotype that appeared at 6 months of age and persisted at 12 months of age, respectively the symptomatic and pathological phases of the disease in this transgenic mouse model.

Interestingly, 10 mg/kg/die of PEA treatment was able to revert this phenotype in 6 month-old mice but 12- month-old mice suggesting a possible effect of the PEA on the onset of the disease in the 3×Tg-AD mice.

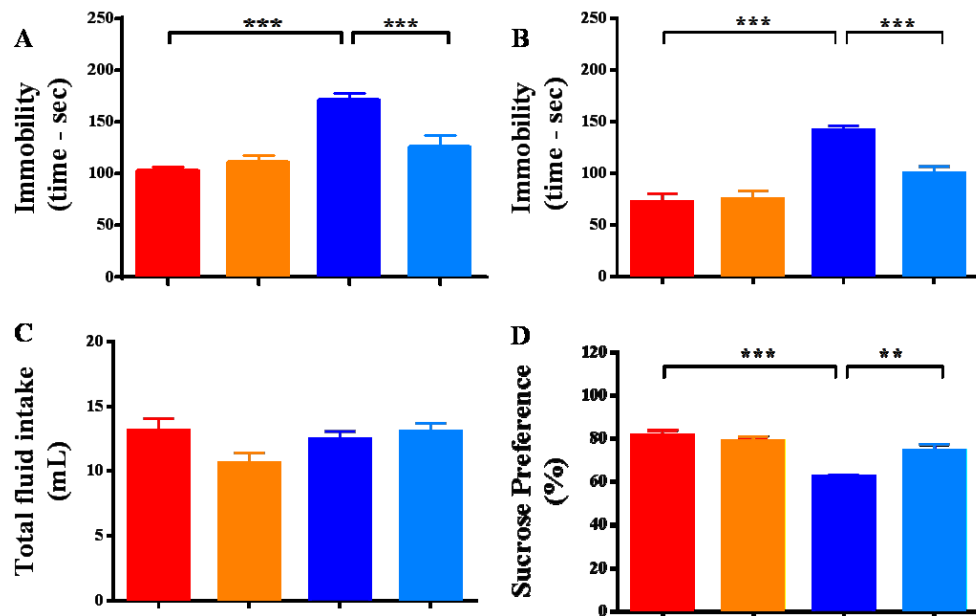


Fig.1: Behavioral tests performed by 6-month-old mice: forced swimming test (A), tail suspension test (B) and sucrose preference test (total amount of fluid drunk and percentage of preference for the sucrose solution, C and D respectively). Vehicle-treated 3×Tg-AD mice (blue bars) showed a significantly higher immobility time with respect to PEA-treated transgenic mice (light blue bars) or compared to Non-Tg groups treated with vehicle (red bars) or PEA (orange bars) in both FST and TST. In the SPT no differences were observed in the total fluid intake between all groups (C). Conversely, 3×Tg-AD mice treated with PEA and both groups of Non-Tg mice showed a significantly higher preference for the sucrose solution compared to vehicle-treated transgenic mice (D). Data are expressed as mean ± SEM (n=10). Behavioral data were submitted to two-way analyses of variance (ANOVA). Tukey's honestly significant difference (HSD) test was used for multiple post hoc comparisons when required. The threshold for statistical significance was set at $p < 0.05$.

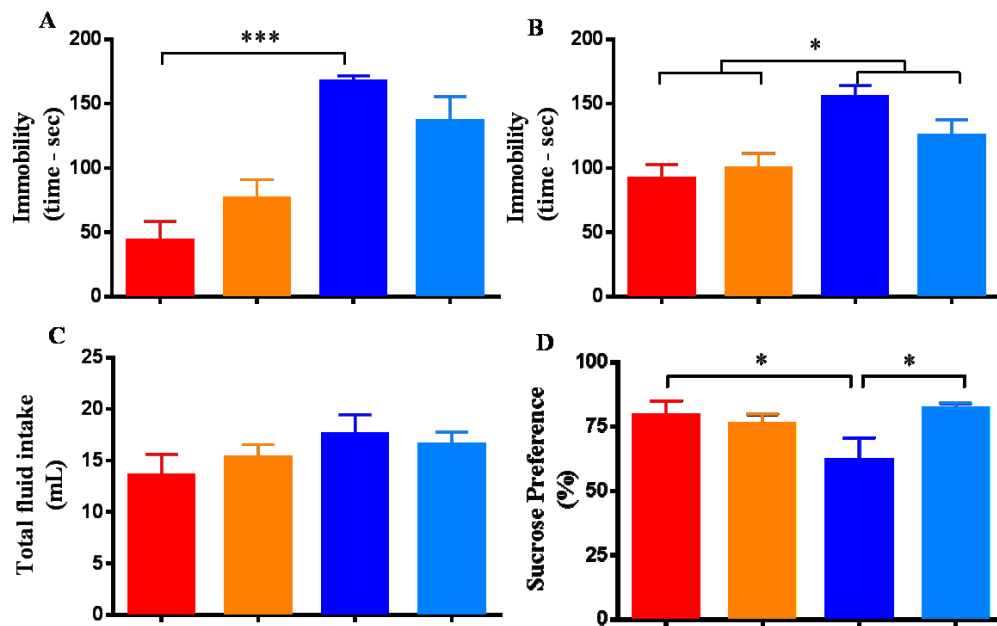


Fig.2 Behavioral tests performed by 12-month-old mice: forced swimming test (A), tail suspension test (B) and sucrose preference test (total amount of fluid drunk and percentage of preference for the sucrose solution, C and D respectively). Vehicle-treated 3×Tg-AD mice (blu bars) showed a significantly higher immobility time with respect to PEA-treated transgenic mice (light blu bars) or compared to Non-Tg groups treated with vehicle (red bars) or PEA (orange bars) in both FST and TST. In the SPT no differences were observed in the total fluid intake between all groups (C). Conversely, 3×Tg-AD mice treated with PEA and both groups of Non-Tg mice showed a significantly higher preference for the sucrose solution compared to vehicle-treated transgenic mice (D). Data are expressed as mean $\pm$ SEM (n=10). Behavioral data were submitted to two-way analyses of variance (ANOVA). Tukey's honestly significant difference (HSD) test was used for multiple post hoc comparisons when required. The threshold for statistical significance was set at $p < 0.05$.

Behavioral tests	Parameter	Genotype	Treatment	Interaction G × T
<i>NORT</i>	Object recognition index – 30 min	$F(1,38) = 0.377$, n.s.	$F(1,38) = 1.389$, n.s.	$F(1,38) = 6.406$ $p < 0.05$
	Object recognition index – 24 hours	$F(1,37) = 11.993$, $p < 0.01$	$F(1,37) = 2.617$, n.s.	$F(1,39) = 9.214$, $p < 0.01$
<i>TS</i>	Immobility	$F(1,39) = 44.062$, $p < 0.001$	$F(1,39) = 7.203$, $p < 0.05$	$F(1,39) = 9.308$, $p < 0.01$
<i>FS</i>	Immobility	$F(1,39) = 28.558$, $p < 0.001$	$F(1,39) = 5.473$, $p < 0.05$	$F(1,39) = 11.442$, $p < 0.01$
<i>SPF</i>	Total fluid intake	$F(1,39) = 0.349$, n.s.	$F(1,39) = 0.382$, n.s.	$F(1,39) = 1.991$, n.s.
	Sucrose preference	$F(1,39) = 22.547$, $p < 0.001$	$F(1,39) = 3.608$, $p < 0.05$	$F(1,39) = 8.933$, $p < 0.01$
<i>IA</i>	Latency to enter dark compartment – 24 hours	$F(1,33) = 16.499$, $p < 0.001$	$F(1,33) = 5.513$, $p < 0.05$	$F(1,33) = 15.509$, $p < 0.001$
	Latency to enter dark compartment – 7 days	$F(1,34) = 21.239$, $p < 0.001$	$F(1,34) = 29.431$, $p < 0.001$	$F(1,34) = 10.556$, $p < 0.01$
<i>MWM</i>	Latency to cross platform location – 1.5 hours	$F(1,39) = 6.842$, $p < 0.05$	$F(1,39) = 6.481$, $p < 0.05$	$F(1,39) = 8.757$, $p < 0.01$
	Latency to cross platform location – 24 hours	$F(1,39) = 4.698$, $p < 0.05$	$F(1,39) = 4.325$, $p < 0.05$	$F(1,39) = 7.879$, $p < 0.01$
	Time in the target quadrant – 1.5 hours	$F(1,39) = 4.441$, $p < 0.05$	$F(1,39) = 4.474$, $p < 0.05$	$F(1,39) = 6.050$, $p < 0.05$
	Time in the target quadrant – 24 hours	$F(1,39) = 4.836$, $p < 0.05$	$F(1,39) = 3.812$, n.s.	$F(1,39) = 4.994$, $p < 0.05$
	Number of platf location crosses – 1.5 hours	$F(1,39) = 4.835$, $p < 0.05$	$F(1,39) = 7.845$, $p < 0.01$	$F(1,39) = 5.634$, $p < 0.05$
	Number of platf location crosses – 24 hours	$F(1,39) = 3.454$, n.s.	$F(1,39) = 3.660$, n.s.	$F(1,39) = 4.633$, $p < 0.05$

Table 1 Results from the statistical analysis of data obtained from the behavioral tests of 6-month-old mice . Two-way analyses of variance (ANOVA) with genotype (3×Tg-AD vs non-Tg) and PEA treatment (PEA vs vehicle) as between-subject factors. G = genotype, T = treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, (n = 8-10 per group). Details are reported in the text.

Behavioral tests	Parameter	Genotype	Treatment	Interaction G × T
<i>NORT</i>	Object recognition index – 30 min	$F(1,30) = 0.154$, n.s.	$F(1,30) = 1.251$, n.s.	$F(1,30) = 9.460$ $p < 0.01$
	Object recognition index – 24 hours	$F(1,31) = 9.310$, $p < 0.01$	$F(1,31) = 0.112$, n.s.	$F(1,31) = 3.431$, n.s.
<i>TST</i>	Immobility	$F(1,28) = 11.873$, $p < 0.01$	$F(1,28) = 0.363$, n.s.	$F(1,28) = 1.681$, n.s.
<i>FST</i>	Immobility	$F(1,29) = 39.686$, $p < 0.001$	$F(1,29) = 0.00321$, n.s.	$F(1,29) = 4.690$, $p < 0.05$
<i>SPF</i>	Total fluid intake	$F(1,29) = 2.638$, n.s.	$F(1,29) = 0.0558$, n.s.	$F(1,29) = 0.733$, n.s.
	Sucrose preference	$F(1,29) = 1.126$, n.s.	$F(1,29) = 2.319$, n.s.	$F(1,39) = 4.563$, $p < 0.05$
<i>LA</i>	Latency to enter dark compartment – 24 hours	$F(1,33) = 4.799$, $p < 0.05$	$F(1,33) = 0.261$, n.s.	$F(1,33) = 2.189$, n.s.
	Latency to enter dark compartment – 7 days	$F(1,29) = 1.653$, n.s.	$F(1,29) = 0.179$, n.s.	$F(1,29) = 1.723$, n.s.
<i>MWM</i>	Latency to cross platform location – 1.5 hours	$F(1,29) = 1.273$, n.s.	$F(1,29) = 3.760$, n.s.	$F(1,29) = 4.326$, $p < 0.05$
	Latency to cross platform location – 24 hours	$F(1,29) = 5.725$, $p < 0.05$	$F(1,29) = 0.388$, n.s.	$F(1,29) = 0.464$, n.s.
	Time in the target quadrant – 1.5 hours	$F(1,29) = 3.715$, n.s.	$F(1,29) = 3.022$, n.s.	$F(1,29) = 3.437$, n.s.
	Time in the target quadrant – 24 hours	$F(1,29) = 2.107$, n.s.	$F(1,29) = 1.303$, n.s.	$F(1,29) = 1.277$, $p < 0.05$
	Number of platf location crosses – 1.5 hours	$F(1,29) = 1.783$, n.s.	$F(1,29) = 1.030$, $p < 0.01$	$F(1,29) = 6.303$, $p < 0.05$
	Number of platf location crosses – 24 hours	$F(1,29) = 4.474$, $p < 0.05$	$F(1,29) = 0.261$, n.s.	$F(1,29) = 0.225$, n.s.

Table 2. Results from the statistical analysis of data obtained from the behavioral tests of 12-month-old mice . Two-way analyses of variance (ANOVA) with genotype (3×Tg-AD vs non-Tg) and PEA treatment (PEA vs vehicle) as between-subject factors. G = genotype, T = treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, (n = 8-10 per group). Details are reported in the text.

2. *Effects of PEA treatment on the learning and memory in 3×Tg-AD mice*

To determine the effects of PEA on learning and memory, at the end of treatment, mice were tested using: the spatial version of the Morris water maze (MWM), a hippocampal-dependent task, the novel object recognition test (NORT), a behavioral tasks mainly dependent on multiple cortical areas and inhibitory passive avoidance (IA), which is mainly dependent on the amygdala [McGaugh *et al.* 2002].

During the MWM, mice received 4 training trials per day for 5 consecutive days to find a hidden platform.

When the probe trial was performed, 1.5 and 24 hour after the last training session, we analyzed three parameters for this test: (i) initial latency to cross the platform location, (ii) number of platform location crosses, and (iii) the time spent in the target quadrant.

Mice performance was analyzed by two-way analyses of variance (ANOVA) with genotype (3×Tg-AD vs non-Tg) and PEA treatment (PEA vs vehicle) as between-subject factors.

At 6 months of age, after 1.5 hours from the last probe trial, two-way ANOVA revealed a significant main effect of genotype, treatment and genotype by treatment interaction effect in all parameters misured (see Table 1 for statistic).

After using a Tukey's honestly significant difference (HSD) post hoc test, we found that 3×Tg-AD mice treated with PEA performed at the level of Non-Tg mice and, more interestingly, they performed significantly better than vehicle-treated transgenic mice (Fig. 3A, B, C).

Similar results were obtained when the retention of spatial training was assessed 24 hours after the last training session with a significant, treatment and genotype by treatment interaction effect for all parameters misured (see Table 1 for statistic).

Multiple post hoc comparisons showed that transgenic mice treated with PEA performed significantly better with respect to 3×Tg-AD mice treated with vehicle. (Fig 3D, E, F)

Results from 12-month-old mice showed only a significant treatment [$F_{(1,29)} = 1.030$, $p < 0.01$] and genotype by treatment interaction effect [$F_{(1,29)} = 6.303$, $p < 0.05$] in the number of platfor location crosses and also a significative genotype

by treatment interaction effect [$F_{(1,38)} = 4.326$, $p < 0.05$] for latency to cross platform location, after 1.5 hours test.

No effect was found in time spent in the target quadrant (Table 2).

Subsequent post hoc analyses showed that PEA-treated transgenic mice performed the task significantly better than 3×Tg-AD mice treated with vehicle for the latency to cross platform location ($p < 0.05$) (Fig 4A) and number of platform location crosses ($p < 0.05$) (Fig 4B).

In contrast, when tested 24 hours after the last training session, we found only a significant genotype effect in latency to cross platform location [$F_{(1,29)} = 5.725$, $p < 0.05$] and number of platform location crosses [$F_{(1,29)} = 4.474$, $p < 0.05$], and also a significant genotype by treatment interaction effect [$F_{(1,29)} = 1.277$, $p < 0.05$] for time spent in the target quadrant (Table 2).

As expected from these results we did not find any difference in the performance between vehicle- and PEA-treated 3×Tg-AD mice in latency to cross platform location and number of platform crosses (Fig. 4D and E), but only a difference in time spent in target quadrant ($p < 0.05$) (Fig. 4F).

To assess cortical function, mice were also tested in NORT. This task relies mostly on cortical areas [Brown and Aggleton, 2001] and exploits the natural tendency of mice to explore objects perceived as novel, resulting less stressful than the MWM. As previously described, also for NORT the retention session was performed at two different time points (30 minutes and 24 hours) after the exploration session in order to assess both short- and long-term memory, respectively.

Two way ANOVA indicated significant changes in the time mice spent exploring the new object across the 4 different groups at 6 months of age. In particular, at the time point of 30 minutes we found significant genotype by treatment interaction effect [$F_{(1,38)} = 6.406$, $p < 0.05$], while no treatment [$F_{(1,38)} = 1.389$, n.s.] and genotype [$F_{(1,38)} = 0.377$, n.s.] effects were found (Table 1).

Multiple post hoc comparisons showed a significant higher object recognition index (ORI) for 3×Tg-AD mice treated with PEA respect to transgenic mice treated with vehicle ($p < 0.05$) (Fig. 5A)

When the probe trial was performed 24 hours later the exploration session we observed a significant genotype [$F_{(1,37)} = 11.993$, $p < 0.01$] and genotype by treatment interaction effects [$F_{(1,39)} = 9.214$, $p < 0.01$] in time spending in the exploration of the novel object between the groups. No treatment [$F_{(1,37)} = 2.617$, n.s.] effect was found (Table 1).

The following post hoc test showed that transgenic mice performed significantly better than the vehicle-treated transgenic group ($p < 0.001$) (Fig. 5B).

Furthermore, we found only a significant genotype by treatment interaction effect [$F_{(1,30)} = 9.460$, $p < 0.01$] between the 4 groups of 12-month-old mice at the time point of 30 minutes (Table 2).

Tukey's post hoc test indicated that PEA-treated transgenic mice performed significantly better than 3×Tg-AD mice treated with vehicle ($p < 0.05$) (Fig. 6A).

In contrast, we found a only significant genotype [$F_{(1,30)} = 9.310$, $p < 0.01$] effects for the object recognition index when the task was performed at the time point of 24 hours (Table 2).

Multiple post hoc comparisons did not showed a greater recognition index for 3×Tg-AD mice treated with PEA with respect to those treated with vehicle ($p < 0.01$) (Fig. 6B).

At the end we evaluated the mice on a contextual learning and memory task using passive inhibitory avoidance.

As descussed above, animals were tested 24 h and 7 days after the training phase to assess the short- and long-term memory. During this phase, the mouse was again placed in the illuminated compartment and was analyzed the latency to return to the dark compartment (previously associated with shock).

Mice performance was analyzed by two-way analyses of variance (ANOVA) with genotype (3×Tg-AD vs non-Tg) and PEA treatment (PEA vs vehicle) as between-subject factors.

At 6 months of age, after 24 hours from the training phase, two-way ANOVA revealed a significant significant main effect of genotype [$F_{(1,33)} = 16.499$, $p < 0.001$], treatment [$F_{(1,33)} = 5.513$, $p < 0.05$] and genotype by treatment interaction [$F_{(1,33)} = 15.509$, $p < 0.01$] (Table 1).

Multiple post hoc comparisons showed a significant lower latency to enter in dark compartment for 3×Tg-AD mice treated with vehicle respect to transgenic mice treated with PEA ($p < 0.001$) and non-Tg ($p < 0.001$) (Fig. 5C).

Similar results were obtained when we valuted the long-term memory after seven days from aversive stimulus with a significant genotype [$F_{(1,34)} = 21.239$, $p < 0.001$], treatment [$F_{(1,34)} = 29.431$, $p < 0.001$] and genotype by treatment interaction effect [$F_{(1,34)} = 10556$, $p < 0.01$].

Tukey's post hoc test indicated that PEA-treated transgenic mice performed significantly better than 3×Tg-AD mice treated with vehicle ($p < 0.001$) (Fig. 6B).

In contrast with the data showed for 6-months-old mice, we found only a significant genotype effect [$F_{(1,33)} = 4.799$, $p < 0.05$] between the 4 groups of 12-months-old mice at the time point of 24 hours, while no effects were found after seven days (Tab.2).

As expected from these results we did not find any difference in the performance between vehicle- and PEA-treated 3×Tg-AD mice in latency to enter dark compartemen at both time points (Fig. 6C and D)

Taken together these results showed that PEA treatment ameliorates spatial learning and improves recognition memory in 3×Tg-AD at 6 months of age, while has only effect on short memory in transgenic mice at 12 months of age.

It's important to add that PEA have not pharmacological effect on non-Tg mice.

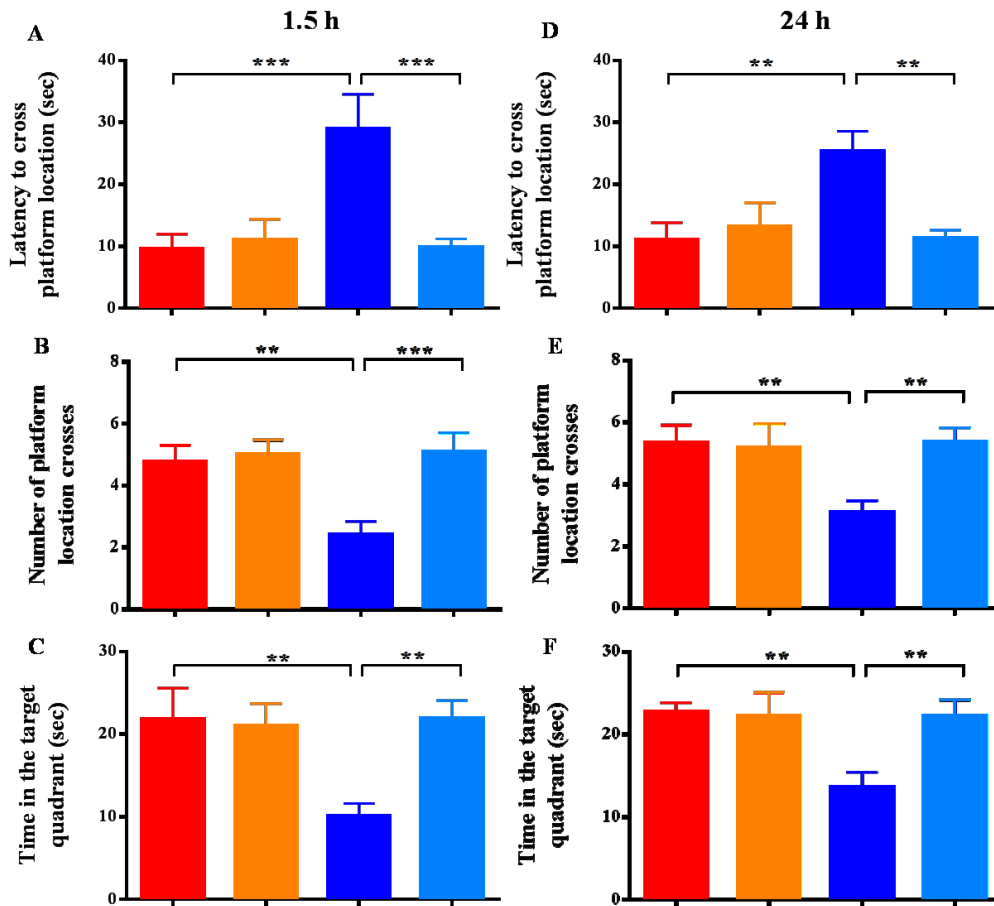


Fig.3 Morris water maze test performed 1.5 hours (A, B and C) and 24 hours (D, E, F) after the last training session by 6-month-old mice. At both time points the four groups [Non-Tg vehicle (red bars), Non-Tg PEA (orange bars), 3xTg-AD vehicle (blue bars) and 3xTg-AD PEA (light blue bars)] showed differences in the latency to cross platform location (A, D), in the number of platform location crosses (B, E), and time in the target quadrant (C, F). Data are expressed as mean $\pm$ SEM ($n=8-9$). Behavioral data were submitted to two-way analyses of variance (ANOVA). Tukey's honestly significant difference (HSD) test was used for multiple post hoc comparisons when required. The threshold for statistical significance was set at $p<0.05$.

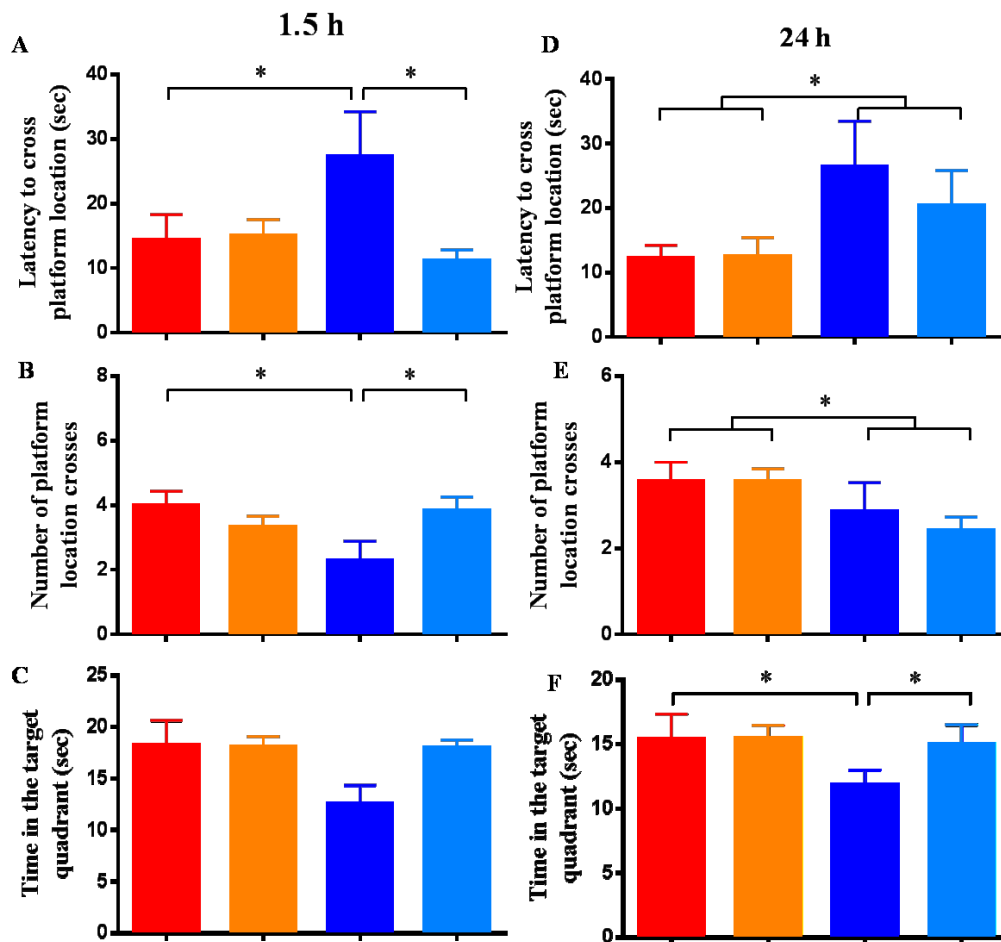


Fig.4 Morris water maze test performed 1.5 hours (A, B and C) and 24 hours (D, E and F) after the last training session by 12-month-old mice. At 1,5 h time points data showed differences between vehicle-treated 3xTg-AD mice (blue bars) and PEA 3xTg-AD mice (light blue bars) in the latency to cross platform location (A) and in the number of platform location crosses (B). At 7 days time points none of the four groups [Non-Tg vehicle (red bars), Non-Tg PEA (orange bars), 3xTg-AD vehicle (blue bars) and 3xTg-AD PEA (light blue bars)] showed any differences in the latency to cross platform location (D) or in the number of platform location crosses (E). In contrast, vehicle-treated 3xTg-AD mice spent significantly less time in the target quadrant compared to the other groups (F). Data are expressed as mean $\pm$ SEM (n=8-9). Behavioral data were submitted to two-way analyses of variance (ANOVA). Tukey's honestly significant difference (HSD) test was used for multiple post hoc comparisons when required. The threshold for statistical significance was set at $p < 0.05$.

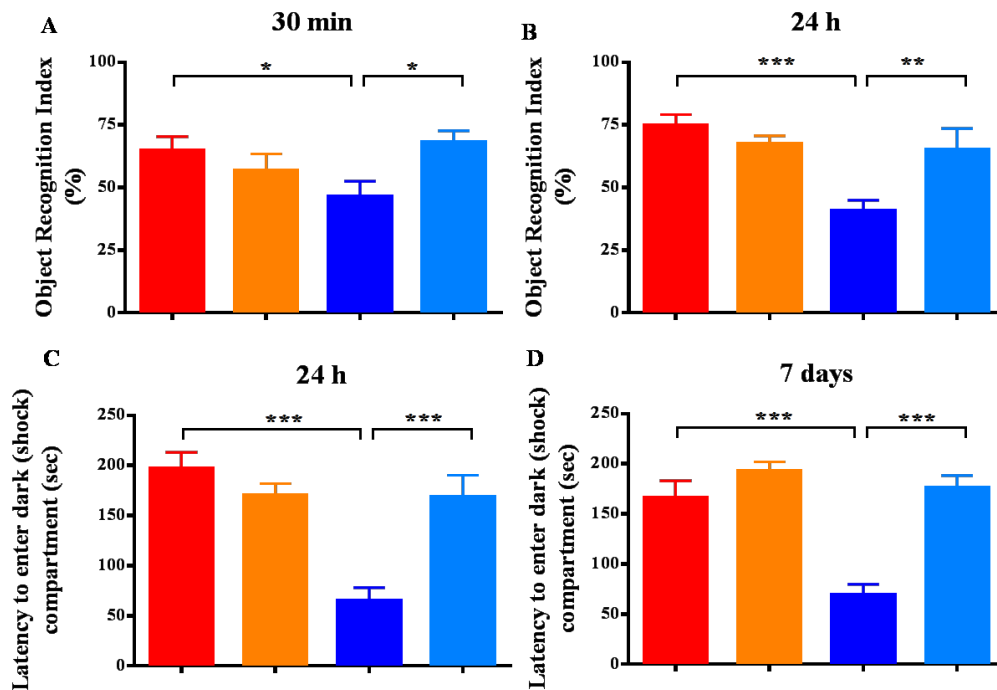


Fig. 5 Novel object recognition test (A,B) and inhibitory passive avoidance (C,D) performed by 6-month-old mice. 3xTg-AD mice treated with PEA (light blue bars) showed a significantly higher object recognition index compared to vehicle-treated transgenic mice (blue bars) at both time points (30 minutes and 24 hours after the exploration session, A and B respectively). Similar results were obtained in inhibitory passive avoidance, in which 3xTg-AD mice treated with PEA showed a latency to enter dark compartment similar to that of Non-Tg mice treated with vehicle (red bars) or PEA (orange bars) and significantly higher than that of transgenic mice treated with vehicle at both time points (C,D). Data are expressed as mean $\pm$ SEM (n=8-10). Behavioral data were submitted to two-way analyses of variance (ANOVA). Tukey's honestly significant difference (HSD) test was used for multiple post hoc comparisons when required. The threshold for statistical significance was set at $p < 0.05$.

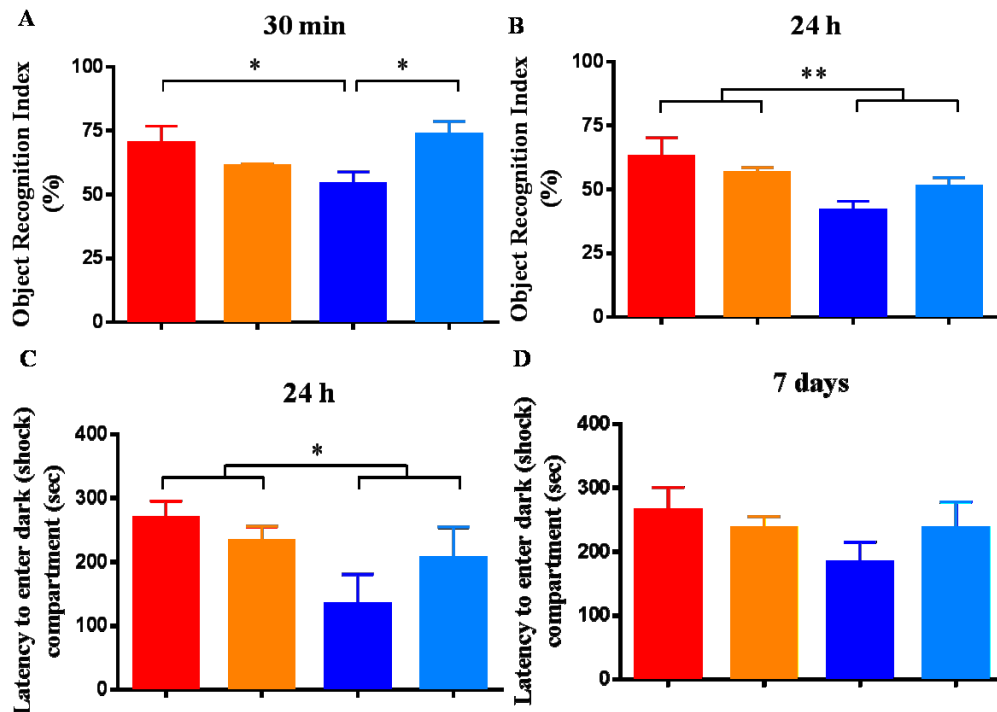


Fig.6: Novel object recognition test (A,B) and inhibitory passive avoidance(C,D) performed by 12-month-old mice. 3xTg-AD mice treated with PEA (light blue bars) showed a significantly higher object recognition index compared to vehicle-treated transgenic mice (blue bars) at 30 minutes (A) time point but not at 24 hours (B) time point after the exploration session. At contrast at both time points none of the four groups [Non-Tg vehicle (red bars), Non-Tg PEA (orange bars), 3xTg-AD vehicle (blue bars) and 3xTg-AD PEA (blue light bars)] showed any differences in the latency to enter dark compartment (C,D). Data are expressed as mean $\pm$ SEM (n=8-10). Behavioral data were submitted to two-way analyses of variance (ANOVA). Tukey's honestly significant difference (HSD) test was used for multiple post hoc comparisons when required. The threshold for statistical significance was set at $p < 0.05$

3. *Effects of PEA treatment on the A β and Tau processing in 3 $\times$ Tg-AD mice*

Western blotting analysis of homogenates obtained from cortex and hippocampus of 6- and 12-month-old 3 $\times$ Tg-AD mice treated with either PEA or vehicle revealed the presence of specific immunoreactive bands for β -amyloid (A β), β -site cleaving APP enzyme 1 (BACE) and phosphorylated tau [Ser202/Thr205 (AT8) and Thr231 (AT180)] (Table 3 and 4).

Densitometric analysis showed significantly decreased levels of both immunoreactive bands for full-length APP and for the A β dodecamer (A β *56) in cortex of PEA-treated mice compared to the vehicle group at 6 months of age. Conversely A β levels did not significantly differ between the two groups in the hippocampus at this age. Likewise, results from both cortex and hippocampus showed that there were not changes after treatment in BACE-1 protein levels (Fig 7A and 7B).

In accordance with the progression of tau pathology in these mice [Oddo *et al.* 2003b], we did not find any difference in pospho-tau level at 6 months of age in the analyzed brain regions (Fig. 7 C and 7D).

At 12 months of age PEA treatment was associated with significantly lower levels of full-length APP and tau phosphorylation at residues 202/205 (AT8) in both brain areas, whereas no differences were found in BACE-1 and phospho-Thr231-tau (AT180) protein content (Fig. 8)

4. *Effects of PEA treatment on the neuroinflammation in 3 $\times$ Tg-AD mice*

Specific immunoreactive bands from westen blot experiments for cyclooxygenase-2 (COX-2), glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba-1) are shown (Table 3 and 4). Densitometric analysis of the immunoreactive bands revealed that there were no differences between PEA- and vehicle-treated 3 $\times$ Tg-AD mice at 6 months of age in both cortex and hippocampus. (Fig 9A and B).

In contrast we found significantly lower levels of GFAP in hippocampal homogenates of 12-month-old mice treated with PEA with respect to the vehicle group. No differences were observed in the levels of the other markers

of neuroinflammation taken into account in both brain areas analyzed at 12 months of age (Fig 9C and 9D).

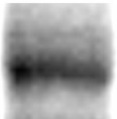
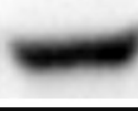
Protein	MW (kDa)	Cortex		Hippocampus	
		Vehicle	PEA	Vehicle	PEA
APP	100				
BACE	70				
COX-2	69				
AT8	60				
AT180	60				
A β *56	56				
GFAP	55				
Iba-1	17				
β -actin	44				

Table 3 Western blot analysis of the expression of β -amyloid, tau phosphorylation level, BACE, COX-2, GFAP and Iba-1. Each lane shows a representative western blotting analysis of specific immunoreactive bands for protein obtained from cortex and hippocampus homogenates of 6-month-old 3 $\times$ Tg-AD mice treated either with vehicle or PEA.

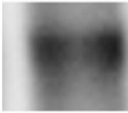
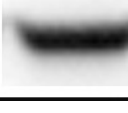
Protein	MW (kDa)	Cortex		Hippocampus	
		Vehicle	PEA	Vehicle	PEA
APP	100				
BACE	70				
COX-2	69				
AT8	60				
AT180	60				
A β *56	56				
GFAP	55				
Iba-1	17				
β -actin	44				

Table 4 Western blot analysis of the expression of β -amyloid, tau phosphorylation level, BACE, COX-2, GFAP and Iba-1. Each lane shows a representative western blotting analysis of specific immunoreactive bands for protein obtained from cortex and hippocampus homogenates of 12-month-old 3 $\times$ Tg-AD mice treated either with vehicle or PEA.

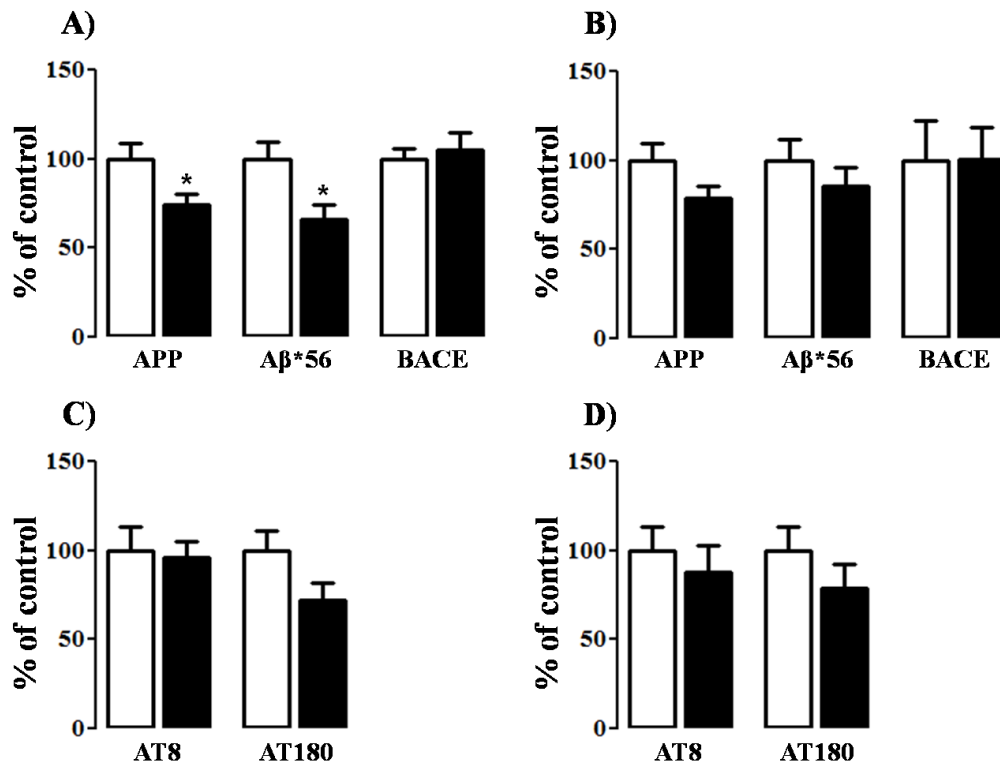


Fig.7 Densitometric analysis of immunoreactive bands for β -amyloid ($A\beta$), β -site cleaving APP enzyme 1 (BACE) and phosphorylated tau [Ser202/Thr205 (AT8) and Thr231 (AT180)] of 6-month-old 3 $\times$ Tg-AD mice treated with vehicle (open bars) or with PEA (black bars). We found a significant reduction in both steady-state levels of full-length APP and for $A\beta$ dodecamer ($A\beta^*56$) in cortex of transgenic mice treated with PEA with respect to vehicle-treated mice, while no differences were observed for BACE protein level (A). In contrast, we did not find any difference in APP, $A\beta^*56$ and BACE protein levels in the hippocampus (B). Likewise, no statistical significant changes were found for tau phosphorylation levels in both cortex (C) and hippocampus (D). Data from western blot were expressed as percentage of controls (vehicle-treated 3 $\times$ Tg-AD mice) and were analyzed by unpaired Student's t-test. Results were expressed as mean $\pm$ SEM (n=3-4). The threshold for statistical significance was set at $p < 0.05$.

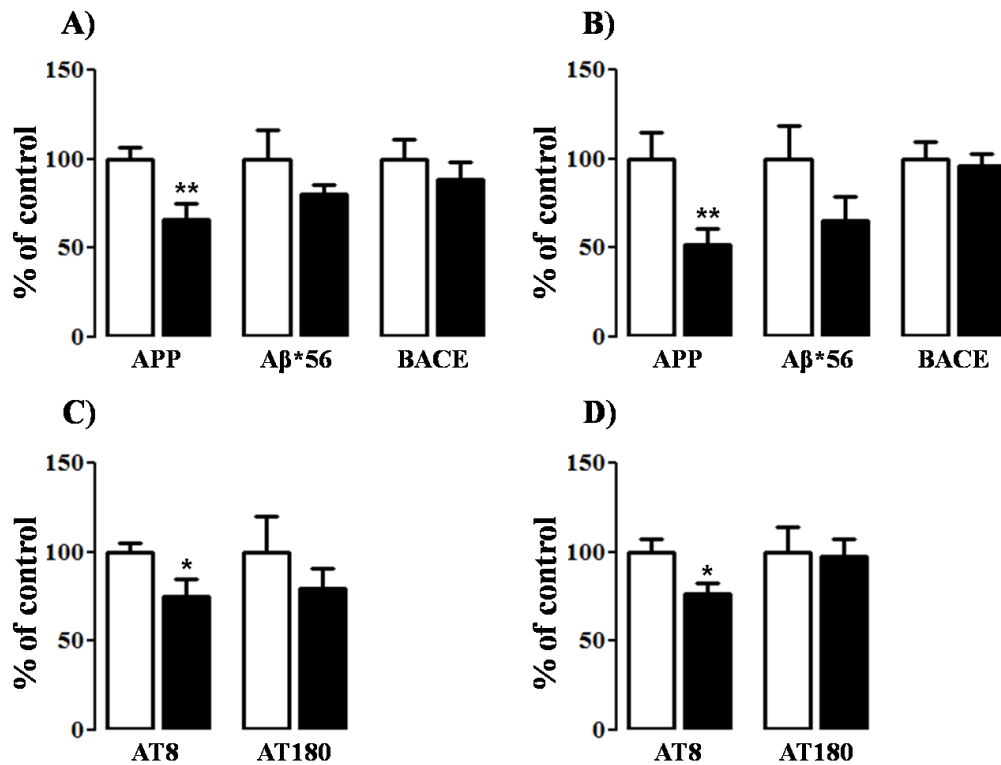


Fig.8 Densitometric analysis of immunoreactive bands for β -amyloid ($A\beta$), β -site cleaving APP enzyme 1 (BACE) and phosphorylated tau [Ser202/Thr205 (AT8) and Thr231 (AT180)] of 12-month-old 3×Tg-AD mice treated with vehicle (open bars) or with PEA (black bars). Statistical analysis revealed a significant reduction of steady-state levels of full-length APP in transgenic mice treated with PEA compared to vehicle-treated mice in both cortex (A) and hippocampus (B) without significant changes in $A\beta$ *56 and BACE levels. Furthermore, PEA treatment was associated with a significant reduction in tau phosphorylation at residues 202/205 (AT8) in both cortex (C) and hippocampus (D) whereas no differences were found in phospho-Thr231-tau (AT180) protein content. Data from western blot were expressed as percentage of controls (vehicle-treated 3×Tg-AD mice) and were analyzed by unpaired Student's t-test. Results were expressed as mean $\pm$ SEM (n=3-4). The threshold for statistical significance was set at $p < 0.05$.

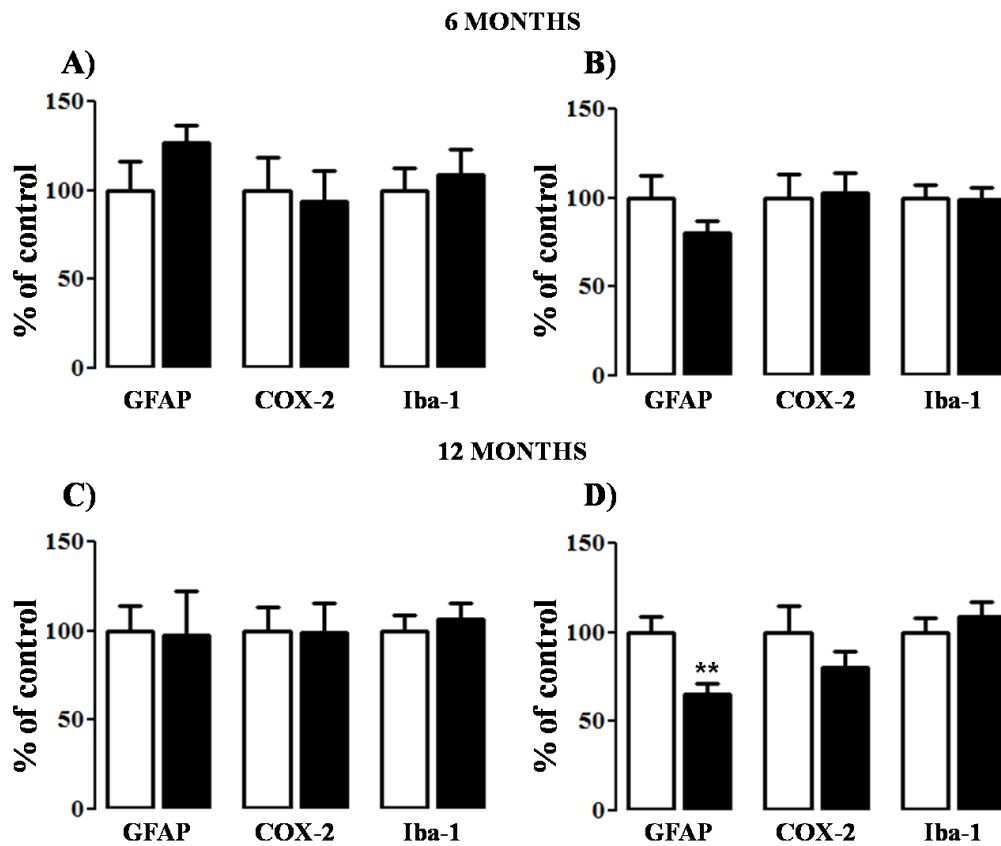


Fig.9 Densitometric analysis of immunoreactive bands for cyclooxygenase-2 (COX-2), glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1(Iba-1) in 6- and 12-month-old transgenic mice treated with vehicle (open bars) or with PEA (black bars). No significant changes were found in GFAP, COX-2 or Iba-1 protein levels between the two groups in both cortex (A) and hippocampus (B) at 6 months of age. Similarly, in 12-month-old mice we did not find any difference in cortex (C), whereas only a significant decrease in GFAP protein content was found in hippocampus of transgenic mice treated with PEA compared to those treated with vehicle (D). Data from western blot were expressed as percentage of controls (vehicle-treated 3×Tg-AD mice) and were analyzed by unpaired Student's t-test. Results were expressed as mean $\pm$ SEM (n=3-4). The threshold for statistical significance was set at $p < 0.05$.

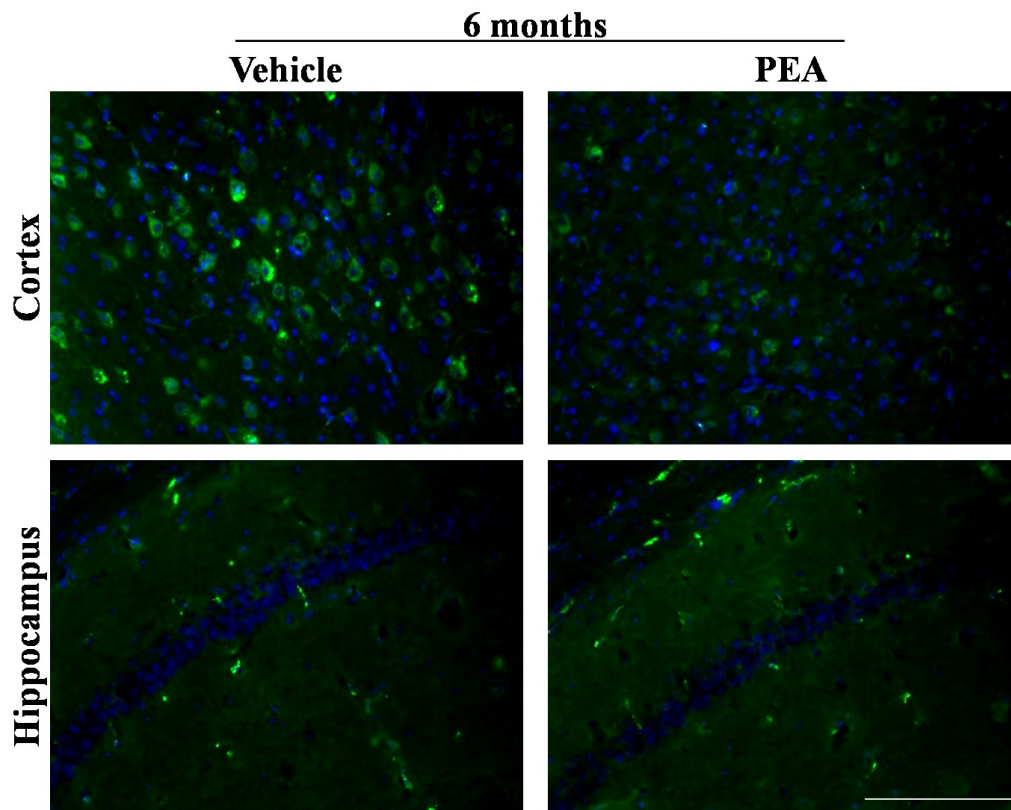


Fig.10 Representative microphotographs of β -Amyloid immunofluorescence (6E10 antibody, green) from brain coronal sections (20 μ m) collected from vehicle- and PEA-treated 3 $\times$ Tg-AD mice. Original magnification 20 $\times$. Scale bar was set at 100 μ m.

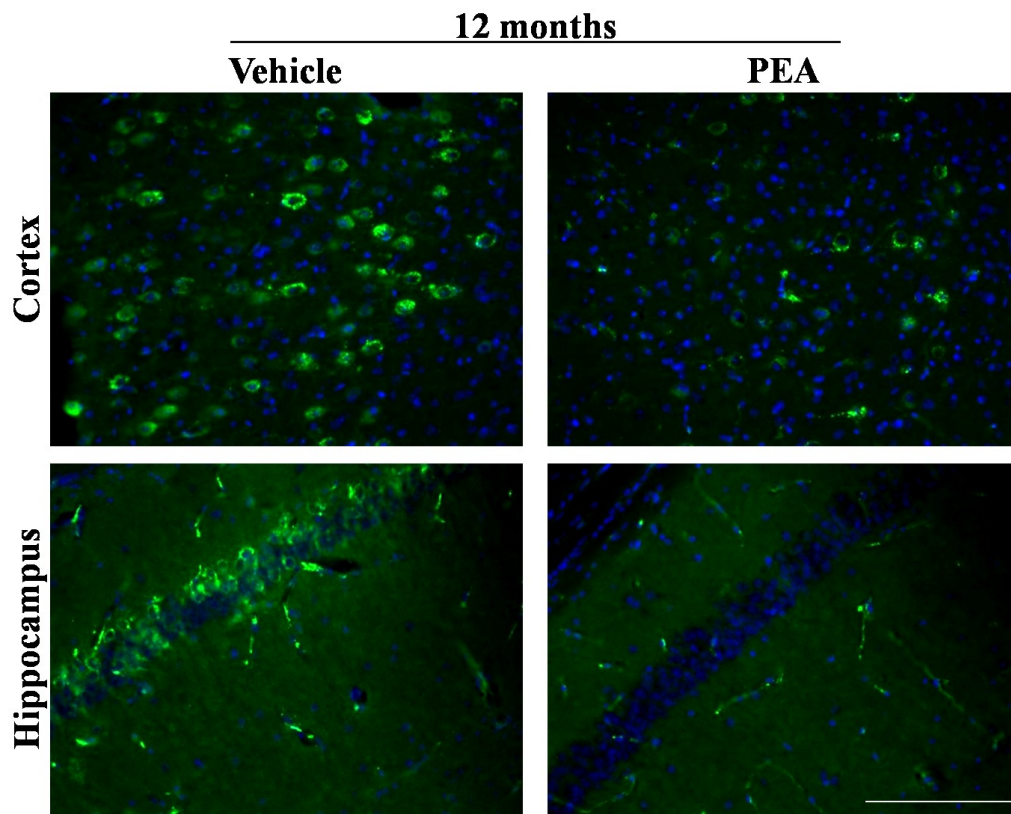


Fig.11 Representative microphotographs of β -Amyloid immunofluorescence (6E10 antibody, green) from brain coronal sections (20 μ m) collected from vehicle- and PEA-treated 3xTg-AD mice. Sections were treated with Bisbenzimidazole (Hoechst, blue) in order to stain nuclei. Original magnification 20 $\times$. Scale bar was set at 100 μ m.

CHAPTER V. Discussion

The present study provides an extensive characterization of the effects on cognitive and non-cognitive behaviors deriving from the treatment with PEA, a lipid messenger that mimics several endocannabinoid-driven actions [Scuderi *et al.* 2014], in the triple transgenic mouse model of Alzheimer disease (3×Tg-AD).

3×Tg-AD mice develop AD-like phenotype and show cognitive decline in an age-dependent manner with respect to wild type mice (Non-Tg) [Oddo *et al.* 2003b], with some histopathological lesions and cognitive alterations that start to be slightly evident by 4 months of age but are prominent at older ages.

Therefore, by the subcutaneous treatment with PEA to 3- and 9-month-old 3×Tg-AD mice it is possible to evaluate the pharmacological effect on both the onset and progression of AD neuropathology.

Recently, it has been demonstrated that PEA exerts *in vitro* a combination of neuroprotective and anti-inflammatory effects in Aβ-induced toxicity, including anti-oxidant and anti-inflammatory effects by interacting at PPAR-α nuclear site.

Although data suggest that PEA markedly attenuated *in vitro* the reactive gliosis induced by Aβ injury, further studies need to focus on the precise relationship between progressive pathological events in AD, in terms of Aβ and tau pathologies, which might be modulated by PEA.

In fact as reported by Hesselink, PEA has a number of other properties, related to its affinity for various receptors. In fact, PEA can bind GPR55 and GPR119, the two orphan cannabinoid receptors. Furthermore PEA may potentiate the effect of anandamide (AEA) through a process called "entourage effect" by inhibiting the enzyme FAAH and/or with an allosteric effect on the transient receptor potential vanilloid 1 (TRPV1) [Hesselink and Kopsky 2015].

Most of the studies in the literature have used animal models where the AD pathology was caused by injection of Aβ. Our work focused on the potential beneficial effect of PEA on behavioral alteration and neuropathological markers in a murine model of AD.

Cognitive deficits, including spatial memory and recognition memory, are among the most striking symptoms of AD. We investigated whether PEA treatment improves spatial learning and recognition memory in 3×Tg-AD mice in the context of Morris water maze test and the novel object recognition test, which allowed testing of both short- and long-term memory and of different

aspects of cognitive functions (i.e. spatial and working memories). In addition the present study explores the role of PEA also on depression-related behavior in this transgenic model of AD.

Depressive-like behaviors in rodents can be evaluated by means of acute stress models. In these, a mouse faces a stressful, inescapable situation that has been suggested to engender a state of behavioral despair, akin to the hopelessness manifest in clinical depression. We used two behavioral despair tests with considerable face value similarity, such as FST and TST. In these paradigms the enhancement of immobility normally ensues after exposure; a phenomenon argued to reflect learned behavioral despair [Porsolt *et al.* 1977] and it is prevented by antidepressant treatment [Steru *et al.* 1985]. Furthermore, we evaluated in 3×Tg-AD mice and wild-type littermates also anhedonia, a central symptoms of depression, by using the SPT. Anhedonia is defined as the decreased capacity to experience pleasure of any sort and this feature can be modelled in rodents as a decrease in responsiveness to rewards, such as a sweet sucrose solution [Katz, 1982].

In our Morris water maze task, 6-month-old 3×Tg-AD mice treated with PEA and non-Tg mice groups show similar latency to cross platform location, number of platform location crosses and time in the target quadrant in the probe trial at both 1.5 h and 24 h after the last training session. Interestingly, they performed better than 3×Tg-AD mice treated with vehicle at both time points.

Conversely, at 12 months of age 3×Tg-AD mice treated with PEA showed a reduction of latency to cross platform location and an increases of number of platform location crosses, compared to vehicle-treated transgenic group, only when the retention trial was performed at the time point of 1.5 h. At the 24h time-point there is a significant difference between 3×Tg-AD mice treated with PEA and vehicle only in the time spent in the target quadrant. These results suggest that, although long-term spatial memory appears to be disrupted in the advanced stages of AD neuropathology, PEA might slow down both short- and long-term memory deterioration in the 3×Tg-AD mice.

In the novel object recognition test, after the training session, mice were given a binary choice between the previously explored object (A), to which they were habituated and a novel object, presented 30 min (B) or 24 h (C) after the training session. Vehicle-treated 3×Tg-AD mice did not show any difference in time spent exploring object A, B or C at both ages, indicating that impairment in recognition memory starts to be evident in the early stage of AD neuropathology in these mice.

6-month-old 3×Tg-AD mice treated with PEA showed a significant increase in time spent in investigation of the novel object at both time points. Conversely, 12-month-old 3×Tg-AD mice treated with PEA showed a significant increase in time spent in investigation of the novel object only at 30 min time-point (short-term memory).

Similar results were obtained from inhibitory passive avoidance test. In this test the short- and long-term memory is evaluated in relation to an aversive stimulus (footshock). Younger 3×Tg-AD mice treated with PEA showed longer latency to enter into the dark compartment, previously associated with the shock, respect to 3×Tg-AD mice treated with vehicle at both 24 h (short-term memory) and 7 days (long-term memory) after the training session. On the contrary, we found no differences in the 12-month-old mice between genotype. These data suggest that the treatment with PEA restores both short- and long-term recognition memories in 6-month-old 3×Tg-AD, whereas only the short-term memory in elderly mice.

In the present study, further evidence for a potential beneficial effect of PEA arises from the depression-related behavioral tests. In particular PEA treatment is able to reduce immobility time in 3×Tg-AD mice compared to the vehicle-treated transgenic group both in the FST and the TST. These results are confirmed only for 6-month-old mice suggesting that FAAH inhibition could counteract the development of non-cognitive disturbances only in the early phase of neuropathology in this murine model of AD and not in the more advanced stages.

In addition, results from SPT suggest that PEA might restore the normal responsiveness to reward, since transgenic mice treated with PEA show a robust preference for the sweet solution at both ages as well as wild-type control mice, whereas vehicle-treated 3×Tg-AD mice did not show any preference. Possible confounding factors in this test can be excluded since there are no differences in the total fluid intake among all treated groups and because the test plans an adaptation period in order to accustom the animals to the different types of fluids. These results are consistent with the antidepressant-like effect observed in FST and TST. Antidepressant effects of PEA treatment could be due to entourage effect of PEA against of the endocannabinoid system.

This explanation is consistent with several previous studies, which have documented that inhibition of the endocannabinoid hydrolysis or reuptake improves the emotional profile of rodents [Hill *et al.* 2009].

The present work provides also an extensive investigation of the effect of PEA treatment on the onset and progression of A β and tau pathology in 3 $\times$ Tg-AD mice. We showed that the treatment of PEA significantly reduces the levels of full-length APP in cortex of 6-month-old 3 $\times$ Tg-AD mice and, more interestingly, it decreases also the levels of A β *56, an A β oligomer. Conversely, we have not found change in hippocampal content of full-length APP or A β *56, accordingly to the notion that the hippocampus appears to be affected later than cortex by A β pathology in this AD murine model [Oddo et al. 2003b]. Similarly, PEA treatment is able to reduce steady-state levels of full-length APP also in 3 $\times$ Tg-AD mice at 12 months of age, suggesting that it could modulate APP processing in these animals. However this effect is unlikely due to a reduction in the expression of the β -secretase, the protease that cleaves APP to form A β , since we did not find any difference in BACE levels at both ages, indicating that other mechanisms probably involved in the regulation of BACE activity might be affected. From this point of view, one possible mechanism is the inflammatory regulation of BACE, which possesses an NF κ B site in its promoter and is upregulated upon inflammatory stimuli [Chen et al 2012; Sastre et al 2008]. Although we cannot rule out this hypothesis, we did not find significant changes in almost all neuroinflammatory markers taken into account, such as COX-2 or in microglial/astrocytic activation markers.

Regarding the reduction in the A β *56 oligomeric form, it is possible that PEA treatment might directly or indirectly affect the clearance or assembly/disassembly of A β species in the brain. One possible explanation for this effect might include prevention of A β monomer aggregation in oligomers, even if the molecular mechanism remains unknown.

Interestingly, PEA treatment is also associated with a significant reduction in tau phosphorylation at residues 202/205. These results suggest that cognitive improvement is probably due to changes in overall A β levels and tau pathology or to a mixture of both.

The novel finding arising from this work is that PEA treatment results in improved cognitive and non-cognitive functions and slowing down of the onset of neuropsychiatric disturbances associated with AD in this transgenic mouse model of the disease, likely through a reduction in A β and tau pathology in cortex and hippocampus.

Although further studies are needed to determine the molecular mechanisms underlying the beneficial effects of PEA against AD neuropathology, our data indicate that the compound may be effective in early AD or when A β is

accumulating and initiating damage in the central nervous system, in according with a recent study [Tomasini *et al.* 2015].

CHAPTER VI. References

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