Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
FARMACOLOGIA E ONCOLOGIA MOLECOLARE

CICLO XXVIII°

COORDINATORE Prof. Antonio Cuneo

DIFFERENTIAL EFFECTS OF PALMITOYLETHANOLAMIDE AGAINST AMYLOID-β INDUCED TOXICITY IN CORTICAL NEURONAL AND ASTROCYTIC PRIMARY CULTURES FROM WILD-TYPE AND 3xTG-AD MICE.

Settore Scientifico Disciplinare BIO/14

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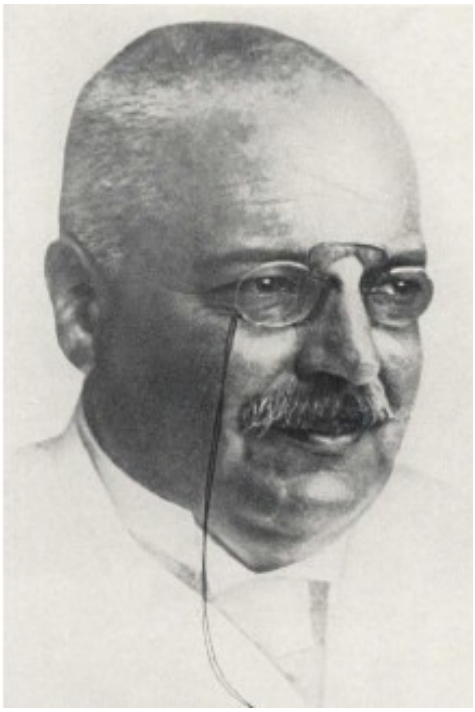
List of abbreviations.

ABH4: alpha/beta-hydrolase 4;
Aβ: beta amyloid proteins;
AchE: acetylcholinesterase;
AChEIs: acetylcholinesterase inhibitors;
AD: Alzheimer's disease;
AEA: anandamide, N-arachidonyl-ethanolamine;
AEs: acylethanolamides;
APP: amyloid precursor protein;
BACE1: β-site APP converting enzyme 1;
BBB: blood-brain barrier;
CBD: cannabidiol;
chAT: cholinergic acetyltransferase;
CNS: central nervous system;
CSF: cerebrospinal fluid;
CT: computed tomography;
DAMPs: damage associated molecular patterns;
DIV: days in vitro;
EAAT2: excitatory amino acid transporter;
ECS: endogenous cannabinoid system;
FAD: familial Alzheimer's Disease;
fMRI: functional MRI;
GPR5: orphan receptor G-protein coupling receptor;
GSH: glutathione;
IL-1β: interleukin-1β;
iNOS: inducible nitric oxide synthase;
IP: intraperitoneal;
KO: knockout;
KPI: Kunitz Protease Inhibitor;
LTP: long-term potentiation;
MAPK: mitogen-activated protein kinase;
mGlu: metabotropic glutamate receptor;
MRI: magnetic resonance imaging;
NAAA: N-acylethanolamine-hydrolyzing acid amidase;
NAEs: N-acylethanolamines
NAPE: N-acyl-phosphatidiyl-ethanolamines;
NAPE-PLD: NAPE-selective phospholipase D;
NFκB: nuclear factor κB;
NMDA: N-metyl-D-aspartate;
NO: nitric oxide;
NSAIDs: nonsteroidal anti-inflammatory drug;
NTFs: neurofibrillary tangles;
OEA: N-oleoyl-ethanolamine;
PAMPs: pathogen associated molecular patterns;
PEA: Palmitoylethanolamide;
PET: positron emission tomography;
PS: presenilin;
PTPN22: protein tyrosine phosphatase N22;
RNS: reactive nitrogen species;
ROS: reactive oxygen species;
SAD: sporadic Alzheimer's Disease;
SCI: spinal cord injury;
SP: senile plaques;
sPLA2: secretory phospholipase-2;
TGN: trans-Golgi-network;
TLR: toll-like receptor;
TNF-α: tumor necrosis factor-alpha;
TRPV1: transient receptor potential vannilloid-type 1
T2D: type 2 diabetes;
Δ^9-THC: Δ^9-tetrahydrocannabinol

ALOIS ALZHEIMER



The psychiatrist and neuropathologist Alois Alzheimer was born on the 14th of June 1864 in Marktbreit am Main (Germany) and died in Breslau (now Wroclaw, Poland) on December 19, 1915.

His most widely contribution to the Neurosciences is the description of the disease that was named after him by Emil Kraepelin, 100 years ago, when he gave a lecture at a congress in Tübingen, Germany.

In 1906, doctor Alois Alzheimer reported the first description of this dementing illness, with detailed clinical characteristics. He documented the progression of symptoms that beset a farmer's wife, Auguste D., as her mental status deteriorated through a complex series of behavioral, delusional and unable to remember recent events.

After her death, Alzheimer drew on his interest in the emerging tecniques of histochemistry. He stained sections from the autopsied brain and discovered the presence of "miliar foci, which are caused by deposition of peculiar substance in the cortex" (now recognized as neuritic or senile plaques). He also reported "very peculiar changes in the neurofibrils" (now recognized as paired helical filaments or tangles). By concerning himself with the structure of the diseased brain and the abnormal deposits that he found, he was among the early pioneers whose studies linked brain structure to function. Then, it also noteworthy for its era the inclusion of the neuropathological examination and the proposal that the abnormal behavior of the patient was the consequence of the abnormal deposits in her brain.

Alzheimer is rightly considered a founding father of neuropathology and his life's achievements exemplify the strong roots this discipline has, and needs to have, in the clinical neurosciences (Blennow et al., 2006; Herrup, 2010).

1. ALZHEIMER'S DISEASE.

1.1 Introduction.

Alzheimer's disease (AD) is the most common cause of dementia (accounting for 80% of all cases) in elderly people (Sun et al., 2012; Steardo Jr et al,. 2015). It is a chronic, irreversible, progressive neurodegenerative, and age-dependent pathology, in fact, the prevalence rises with age, doubling every 5 years between the ages 60 and 90, from 1% till 20% respectively (Kumar et al., 2015; Ballard et al., 2011; Li et al., 2011; Li et al., 2011).

Among regional populations of 60 year-olds, those from North America and Western Europe are believed to exhibit the highest prevalence and incidence rate of dementia, followed by those from Latin America and China and its western-Pacific neighbors (Fig. 1) (Reitz and Mayeux, 2014).

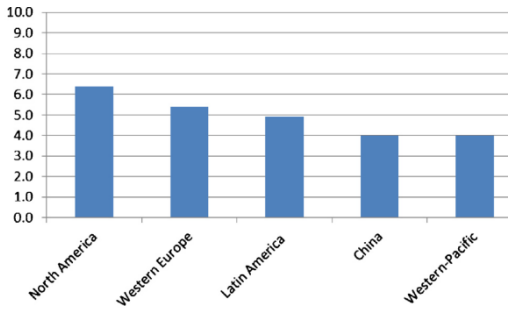


Fig. 1 Global prevalence of dementia (%).

Currently, the worldwide number of people suffering from AD is estimated at 36 million but it's expected that it will triple by 2050 with nearly a million of new cases per year because of the increase in life expectancy (Khachaturian and Khachaturian, 2015).

Memory loss, particularly of recent events during the initial phases, is one of the earliest symptoms of AD, along with confusion, impaired judgment, disorientation, till also impairments in other cognitive domains which interfere with personality and behavior, reason, and language. Eventually, even the simple tasks, such as maintaining personal hygiene, cannot be performed and patients become completely socially dependent (LaFerla and Oddo, 2005; Sezgin and Dincer, 2014).

For these reasons, actually, AD is also the major public-health problem and poses a considerable economic burden to family and society in developed countries because of the lack of treatments to delay or halt the progression of the disease, the insidious course, since,

typically, a decade passes before the illness has taken its course (LaFerla and Oddo, 2005; Sun et al., 2012), and the prolonged duration of disability that requires labor-intensive care for the caregiver, often the spouse or a relative, with pressures that are wide-ranging, involving social, psychological, physical and economic elements of their life (Bedse et al., 2015; Huang and Muckle, 2012; Reitz and Mayeux, 2014).

1.2 Familial and sporadic AD.

AD is a heterogeneous disorder but, based on its age of onset, it can be classified into early onset AD (onset < 65 years) accounting for 1-5% of all cases, and late-onset AD (onset > 65 years) accounting for > 95% of affected. From a clinically point of view, these two forms are indistinguishable (Iqbal and Grundke-Iqbal, 2011). However, the early onset is characteristic of the "familial" Alzheimer's Disease (FAD) which is hereditary (Mendelian pattern of inheritance) and marked by Alzheimer disease symptoms that appear at an unusually early age with a more rapid rate of progression. It is associated with the mutated genes for amyloid precursor protein (APP) on chromosome 21, presenilin 1 (PS1) on chromosome 14, and presenilin 2 (PS2) on chromosome 1, and tau. Clinical mutations in each of these genes alter the metabolism of APP processing, leading to either increased levels of total Aβ or a selective augmentation of the longer more amyloidogenic $A\beta_{42}$ species (Oddo et al., 2003; Verkhratsky et al., 2015).

The late-onset AD is also definied sporadic (SAD) (i.e. idiopathic), meaning that they are not inherited (Reitz and Mayeux, 2014).

1.3 Pathogenesis.

AD is characterized by a series of neuropathologic changes, observed in both FAD and sporadic AD brain, including cerebral atrophy, cerebral senile plaques containing extracellular deposits of β-amyloid peptide (Aβ), intraneuronal neurofibrillary tangles (NFTs) containing hyperphosphorylated tau protein, (NFTs) (Fig. 2) in vulnerable brain regions, such as the hippocampus and cortex, and astrogliosis (Bedse et al., 2015; Ballard et al., 2011; Li et al., 2011). Briefly, it is currently thought that aberrant processing of the Amyloid Precursor Protein (APP) leads to the formation of Aβ deposits which, in conjuction with other factors, stresses nearby neurons, resulting in tau hyperphosphorylation, neuronal loss, synaptic

dysfunction and additionally initiates an inflammatory response in which astrocytes and microglia play a critical role (Pazos et al., 2004).

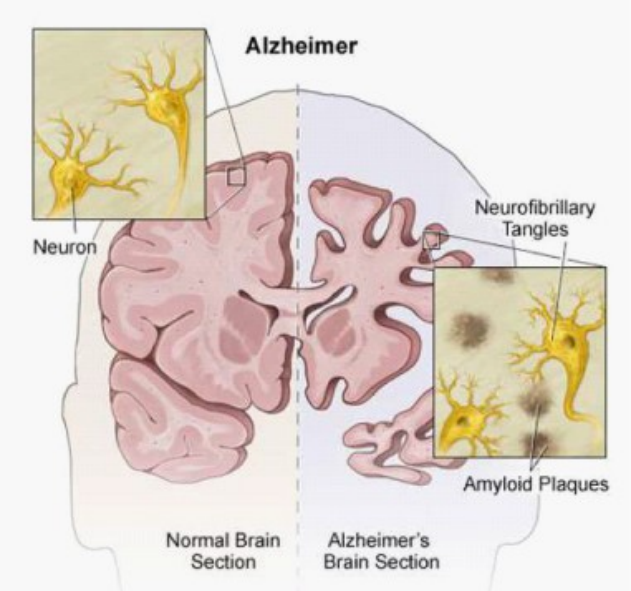


Fig. 2 An illustration comparing a normal brain and a brain affected by Alzheimer's disease with manifested cerebral atrophy. Plaques are extracellular deposits of Aβ surrounded by dystrophic neurites, reactive astrocytes, and microglia, whereas tangles are intracellular aggregates composed of a hyperphosphorylated form of the microtubule-associated protein.

1.3.1 Senile plaques.

The first hallmarks of AD is the presence of senile plaques (SP), extracellular Aβ deposits in the brain parenchyma and in the cerebral blood vessels, of differently-sized and non-soluble fragments of beta amyloid (Aβ) (Oddo et al., 2003). These proteins derive via sequential proteolytic cleavages of the β-amyloid precursor protein (APP). The accumulation of the protein is due to an imbalance between Aβ production, aggregation and clearance. After excessive production, Aβ self-aggregates into Aβ oligomer and then it further aggregates into insoluble extracellular senile plaques (Bedse et al., 2015).

Amyloid plaques are thought to trigger local inflammatory responses, mediated by astrocytes and microglia (see Amyloid cascade hypothesis). Importantly, an increased level of Aβ in the brain is correlated with AD-typical cognitive decline. Aβ-induced neurodegeneration also causes long-term disruptions to various neurotransmitter systems: a) elevated levels of glutamate have been detected in the cerebrospinal fluid (CSF) of AD

patients and b) a loss of cholinergic neurons in brain areas relevant for memory processing (i.e., amygdala, hippocampus and frontal cortex) and the accompanying decrease in acetylcholine (ACh) are major neurochemical deficits in AD pathology (Karl et al., 2012).

In addition to these pathological hallmarks, multiple alterations converge in the pathogenesis of AD, including genetic and environmental factors. Vascular factors and concomitant pathologies worsen disease symptoms. Mitochondrial functional defects, increased production of reactive oxygen and nitrogen species (ROS and RNS), and damage to enzymes involved in energy metabolism are causative of nerve cell exhaustion. Altered lipid composition of membranes particularly lipid rafts, inflammatory responses, and altered production of trophic factors, neurotransmitter and neuromodulators, together with impaired function of degradation pathways such as those related to cytoplasmic proteolysis, autophagy, and ubiquitin–proteasome system play, crucial roles as well. Synaptic loss, reduced dendritic arbors, progressive isolation of remaining neurons and nerve cell loss occur with disease progression, and affect multiple brain regions not only the cerebral cortex but also the amygdala, nuclei of the forebrain including Meynert nucleus, striatum, thalamus, and selected nuclei of the brain stem thus involving multiple neurotransmitter systems (Aso et al., 2014).

1.3.2 Neurofibrillary tangles.

The intracellular NFTs are composed largely of paired helical filaments of hyperphosphorylated microtubule-associated protein tau (Blennow et al., 2006; Reitz and Mayeux, 2014; LaFerla and Oddo, 2005). Four distinct stages of neurofibrillary tangle formation were identified: neurons with granular perikaryal tau immunoreactivity (stage 0); fibrillar neuronal inclusions (stage 1); dense neuronal soma-fillimg inclusions (stage 2); and fibrillar deposits (stage 3) (Fuller et al., 2010).

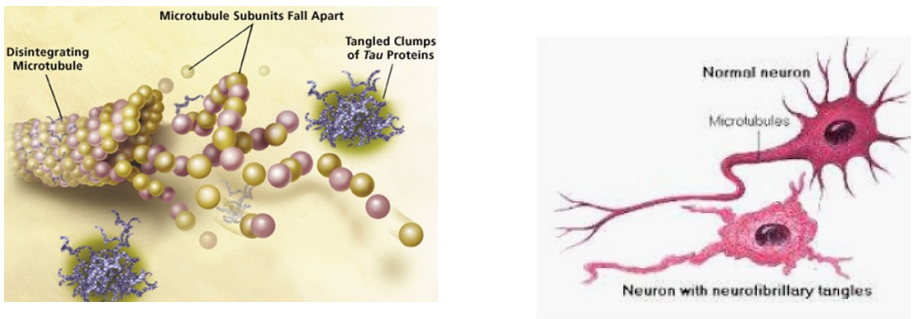


Fig. 3 :Difference between normal and Alzheimer's neurons.

1.3.3 Neuroinflammation in AD.

Next to the classical neuropathological features of AD, namely Aβ deposition and neurofibrillary tangle formation, neuroinflammatory changes have been identified as the third important component of the disease. The inflammatory reactions of microglia and astroglia are intimately associated with the pathogenesis and progress of AD.

The glial involvement in the pathogenesis of Alzheimer's disease (AD) was initially suggested by Alois Alzheimer himself. He had demonstrated that in AD brains the neuritic plaques include glial cells (Fig. 4) (Serrano-Pozo et al., 2013; LaFerla and Oddo, 2005; Bedse et al., 2015; Karl et al., 2015; Heneka et al., 2010).

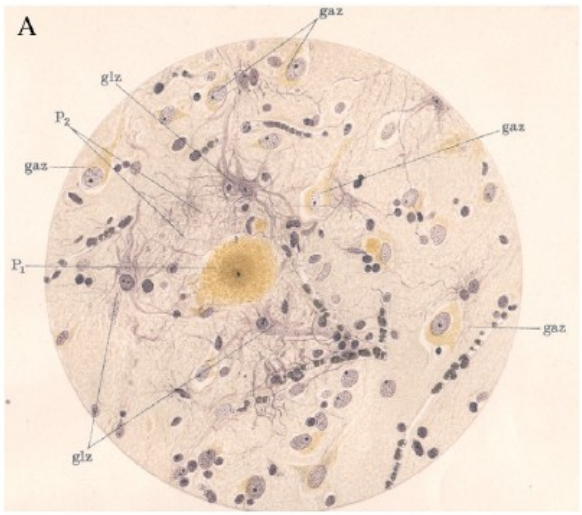


Fig. 4 Activation of glial cells at sites of β-amyloid deposition in human brain and in APP transgenic mice. Neuritic plaques as seen and drawn by Alois Alzheimer (Alzheimer, 1910). The plaque is surrounded by activated astrocytes; and activated microglia is present at the peripheral region. Abbreviations: P1—the central part of the plaque (amyloid deposition); P2—periphery of the plaque; gaz—neurone; glz—glial cell (Heneka et al., 2010).

Microglial cells are the resident macrophages of CNS. In the resting, quiescent state, they have small somatas and multiple fine processes which are constantly moving scanning the microenvironment in their anatomical domain, and produce neurotrophic and anti-inflammatory factors (Heneka et al., 2010). But, insults to the nervous system trigger a

complex and multi-stage activation of microglia, which results in both phenotypic and functional changes; in fact they change from a ramified phenotype to motile activated amoeboid cells. In pathological condition, such as neurodegenerative diseases, these cells migrate to and surround damaged or dead cells, and subsequently clear cellular debris from the area. Activated microglia up-regulate a variety of surface receptors, including major histocompatibility complex and complement receptors. Once they are immunostimulated in response to neurodegenerative events, these microglia cells release a variety of proinflammatory mediators including cytokines, reactive oxygen species, complement factors, neurotoxic secretory products, free radical species and NO, all of which can contribute to neuronal dysfunction and cell death, ultimately creating a vicious cycle (Heneka et al., 2010).

Under normal conditions, the presence of Aβ activates microglia, which accumulates at the site of Aβ deposition and actively engulfs and clears Aβ deposits, secreting also pro-inflammatory enzymes for the removal of Aβ. In AD, the clusters of activated microglia seem to be incapable of completely removing Aβ, which is thought to be caused by an impaired phagocytic or clearance ability. This in turn allows Aβ plaques to develop along with the build-up of inflammatory cytokines that contribute to the pronounced inflammation, which is also an important source of oxidative stress, neurotoxicity and local tissue damage in the proximity of pathological structures seen in AD. Ironically, this enhances the production of Aβ even further (Sezgin and Dincer, 2014; Fuller et al., 2010).

Additionally, also astrocytes are essential in the control of brain homeostatasis. However, several brain injuries, including Aβ deposition, modify their physiological functioning and astrocytes acquire a reactive phenotype. Similarly, their activation is fundamentally a protective response aimed at removing injurious stimuli. However, uncontrolled and prolonged activaction goes beyond physiological control and detrimental effects override the beneficial ones. Transformations of astrocytes begin at the early phases of the AD (Verkhratsky et al., 2015).

In this condition, astrocytes foster neuroinflammatory response, accounting for the synthesis of different cytokines and proinflammatory mediators (Steardo Jr. et al., 2015) In AD, inflammation, astroglial activation is primarily triggered by amyloid deposits in the extracellular space, as demonstrated in cultured glial cells isolated from human AD brains. But also *in vitro* mixed culture model, Aβ induced functional and reactive changes in astrocytes because it led to spontaneous $[Ca^{2+}]_i$ elevations and oscillations which lasted for

many hours and were linked to neuronal death. Then, Aβ was shown to induce mitochondrial depolarization, oxidative stress in astrocytes, release of ROS from stressed astrocytes which caused neuronal death, and down-regulation of glutamate transporter expression (Fuller et al., 2010; Heneka et al., 2010; Verkhratsky et al., 2015).

Importantly, production of pro-inflammatory cytokines (e.g. IL-1β, IFN-γ and TNF-α) and chemokines and the activation of the complement cascade have been observed in AD patients and post-mortem analysis of inflammatory markers is correlated also with synaptic loss in AD brain tissue. Also, IL-1β released from glia activates MAPK and NFκB signaling cascades in astrocytes and neurons, resulting in excessive inflammation and tau phosphorylation (Karl et al., 2012; Fagan and Campbell, 2014).

After their release, pro-inflammatory signal molecules act, in an autocrine way, to self-perpetuate reactive gliosis and, in a paracrine way to kill neighboring neurons expanding the neuropathological damage. So it is important to highlight that inflammatory process, once initiated, may contribute independently to neural dysfunction and cell death, thereby establishing a self-fostering vicious cycle, by which the inflammation represents a substantial cause of further neurodegeneration (Sun et al., 2012; Steardo Jr. et al., 2015). Moreover, astrocytes outnumber microglia in the brain and its reactivation has been believed to last longer than microglia, suggesting that astrocytes may have a more important and sustained role than microglia in the enduring neuroinflammatory process in AD (Li et al., 2011).

1.3.4 Excitotoxicity in AD.

The amino acid L-glutamate is considered to be the major mediator of excitatory signals in the mammalian CNS and is probably involved in most aspects of normal brain function including cognition, memory and learning. It also plays major roles in the development of the CNS, including synapse induction and elimination, and cell migration, differentiation and death.

Glutamate is released by approximately 90% of neurons during excitation, after which it diffuses across the synaptic cleft and is recognized by receptors on the post-synaptic neuron. Then, it must be rapidly removed from the synapse (Fuller et al., 2010).

Glutamate is also a potent neurotoxin, and excitotoxicity is the pathological process of damaging and killing neuronal cells as a result of excessive stimulation and activation of ionotrophic receptors by glutamate and similar substances (Fagan and Campbell, 2014).

Glutamate excitotoxicity has been implicated in a range of neurological diseases, including AD. Glutamate-induced cell death may follow a rapid path, involving a massive influx of Na^+ and Cl^- and cell swelling, followed by massive Ca^{2+} influx and activation of Ca^{2+}-mediated downstream cascades leading to neuronal injury and death. These include activation of phospholipase A2, calpain, and Ca^{2+}-dependent NO synthases, leading to (nitro)-oxidative stress, disruption of cell membranes and cytoskeleton, mitochondrial failure, and cell death by necrosis. Glutamate-induced injury also may follow a slow pathway by triggering apoptosis, and this has been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease.

Furthermore, high levels of extracellular glutamate may inhibit the import of cystine cystine/ glutamate antiporter, resulting in the depletion of glutathione and then in a form of cell injury called oxidative glutamate toxicity. This phenomenon can also involve neurons lacking functional NMDA receptors (Fuller et al., 2010).

1.3.5 Oxidative stress in AD.

Oxidative stress is a cytotoxic condition brought on by the increased intracellular production or accumulation of reactive species of oxygen (ROS) and reactive nitrogen species (RNS). The predominant free radicals are superoxide and hydroxyl radicals but also other molecules contribute to the cellular redox state, such as hydrogen peroxide and peroxynitrite. (Fuller et al., 2010).

ROS are normal products of the mitochondrial respiratory chain and oxidative stress results when cellular antioxidant defenses are insufficient to keep the levels of free radicals below a toxic threshold. This may be due to excessive production of free radicals, loss of antioxidant defenses, or both.

Nitric oxide (NO) is a signaling molecule in the CNS, acting as a neurotransmitter and a vasodilator. NO reacts with superoxide (O_2^-) to form peroxynitrite, which, following protonation, generates cytotoxic species that oxidize and nitrate proteins and other cell constituents (Fuller et al., 2010).

Regulation of ROS and RNS is vital to cell survival as a net increase in free radicals can produce damage to lipids, proteins, carbohydrates and nucleic acids, resulting in a significant disruption of cellular function and induce necrosis or apoptosis.

Enzymes that are responsible for the production of toxic free radicals include NADPH oxidase and nitric oxide synthase (NOS). NADPH oxidase is a plasma membrane associated enzyme, which catalyses the production of superoxide ($O2^{-}$) by the one-electron reduction of oxygen, using NADPH as the electron donor. NOS catalyses the synthesis of NO from $_{L}$-arginine. There are 2 forms of NOS—the constitutive form and the inducible form (iNOS), the latter being expressed in activated astrocytes. iNOS is regulated at the transcriptional level in response to a variety of stimuli, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and results in the production of NO by reactive astrocytes.

Furthermore, oxidative stress can lead to mitochondrial dysfunction, another early feature of AD. Indeed, disrupted energy metabolism was determined in the post-mortem AD brain. the damage induced by ROS is responsible of the collapse of the *trans*-membrane electrochemical gradient and the release of proapoptotic factors like cytochrome c, procaspases and caspase activeted DNAse (Fagan and Campbell, 2014; Sezgin and Dincer, 2014).

Extracellular ROS are highly neurotoxic thereby inducing oxidative damage, while intracellular ROS are crucial for pro-inflammatory functioning.

In AD brains, different studies have found prominent ROS-mediated injuries, especially in regions with high senile plaque and NFT loads, and increased lipid peroxidation and protein oxidation. Furthermore, upregulation of iNOS has been found in senile plaques of AD brains and in microglia after Aβ administration in vivo. While the oxidation of proteins and lipids can have immediate effects with respect of enzyme activities and membrane integrity, the oxidation of DNA may also lead to long-term mutagenic effects, and therefore, to dramatic alterations of the genetic programs of the neurons (Fuller et al., 2010; Fagan and Campbell, 2014).

1.4 APP: functions and processing.

APP is a type I transmembrane protein which contains Aβ sequence in its extracellular domain (Sezgin and Dincer, 2014). The APP gene is located on chromosome 21 in humans with three major forms arising from alternative splicing. These are APP695, APP751 and APP770 (containing 695, 751 and 770 amino acids respectively). APP751 and APP770 are expressed in the most tissues and contain a 56 amino acids Kunitz Protease Inhibitor (KPI) domain within their extracellular regions, whereas APP695 is predominantly expressed in

neurons and lacks the KPI domain. It is known that the protein and mRNA levels of KPI-containing APP isoforms are elevated in AD brain and associated with increased Aβ deposition. Moreover, prolonged activation of extrasynaptic NMDA receptor in neurons can shift APP expression from APP695 to KPI-containing APP isoforms, accompanied with increased production of Aβ. So also a dysregulated splicing of APP RNA can contribute to disease pathogenesis (Zhang et al, 2011).

Although APP has been the subject of much study since its identification, its function is not yet established: mice lacking the APP gene are viable, fertile and show relatively minor neurological impairments. This is probably due to compensatory effects mediated by two other members of the APP gene family: amyloid-precursor-like protein -1 and -2. This view is supported by evidence showing that the combined ablation of APP and APLP2, both APLP genes or all three family members leads to early postnatal lethality, even if the Aβ domain is unique to the APP protein (LaFerla and Oddo, 2005). However, since this protein is expressed in the CNS and has been found to be concentrated in the synapses between neurons (Li et al., 2011), a role for APP has been suggested in neurite outgrowth and synaptogenesis, neuronal protein trafficking along the axon, transmembrane signal transduction, cell adhesion and calcium metabolism.

Full-length APP protein is synthesized in the endoplasmic reticulum and then transported through the Golgi apparatus to the trans-Golgi-network. It is at these two cellular compartments where the highest concentrations of APP was found in neurons at steady state.

APP undergoes rapid anterograde transport in neurons and it is proteolyzed into various fragments (Fig.5) during its intracellular trafficking and these APP metabolites mediate various and sometimes adverse functions. Therefore, the net effect of full-length APP on cellular activity may be a combination of its metabolites' functions (Zhang et al, 2011).

A group of enzymes are involved in the production of Aβ, starting from APP which is cleaved by three types of proteases, which are termed as α-, β-, and γ- secretases (Fig. 5).

Proteolytic processing of APP occurs by one of two pathways, the amyloidogenic or the non-amyloidogenic. The first one leads to the production of Aβ oligomers.

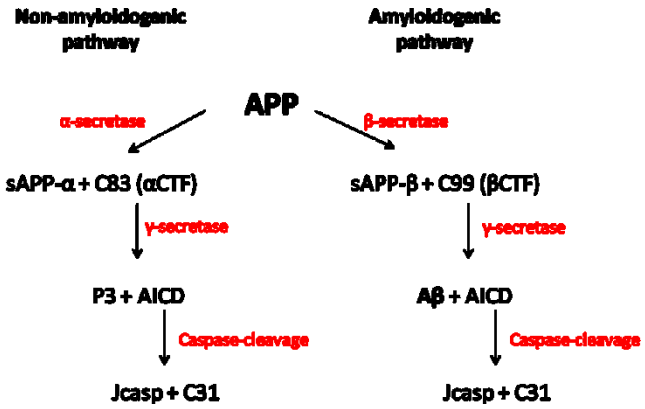


Fig. 5 Processing of amyloid beta precursor protein APP.

1.4.1 The non-amyloidogenic pathway.

On the cell surface, α-secretase cleaves APP at the α-site which is located at the N-terminal of the APP, releasing a soluble fragment, sAPPα, into the extracellular space. This is the non-amyloidogenic pathway as the cleavage site is within the Aβ domain and it precludes Aβ generation. sAPPα has been shown to have neurotrophic and neuroprotective properties: it has an important role in neuronal plasticity/survival and is protective against excitotoxicity and oxidative insults. It also regulates neural stem cell proliferation and is important for early CNS development (Sezgin and Dincer, 2014; Zhang et al., 2011; Li et al., 2011). After this first cleavage, the remaining C-terminal fragment which includes 83 amino acid residues (C-83) is directly cleaved by γ-secretase in the intramembrane space. This second cleavage creates two fragments of the APP intracellular domain (AICD) and p3, which are released in the cytosol and extracellular space, respectively. Whereas P3 is rapidly degraded and widely believed to possess no important function, AICD has been shown to possess transactivation activity and can regulate transcription of multiple genes including APP, GSK3β, and BACE1. In addition, free AICD can induce apoptosis and may play a role in sensitizing neurons to toxic stimuli.

1.4.2 The amyloidogenic pathway.

In this case, APP is cleaved by β-secretase in the extracellular space at the β-site, releasing a short soluble APP-β (sAPPβ) fragment, whose physiological role has not been clarified yet (Li et al., 2011).

The major β-secretase is the β-site APP converting enzyme 1 (BACE1), a membrane-bound aspartyl protease and its activity on synthetic APP peptides *in vitro* studies is well documented. The cleavege of APP by β-secretase leads to the formation of sAPPβ, the soluble ectodomain of APP and it was reported to mediate axonal pruning and neuronal cell death. The remaining C-terminal fragment of 99 amino acid residues (C-99) of APP (βCTF) is still membrane bound. C-99 is further cleaved by γ-secretase, releasing two fragments of AICD and an insoluble fragment, Aβ, which contains 38–43 amino acid residues. Overexpression of βCTF was found to be cytotoxic and cause neuronal degeneration, perhaps perturbing APP signal transduction. It is also possible that APP βCTF's cytotoxic effect is actually mediated by the end products of γ- and/or caspase-cleavage including APP intracellular domain (AICD), C31 and Jcasp which are also cytotoxic (Zhang et al, 2011).

1.4.3 γ-secretase.

γ-secretase-mediated cleavage is unique in that the cleavage takes place within the transmembrane domain, though the exact site can vary. γ-cleavage can yield both $A\beta_{40}$, the majority species, and $A\beta_{42}$, the more amyloidogenic species, as well as release the intracellular domain of APP (AICD) (Zhang et al., 2011). Multiple lines of biochemical evidence have shown γ-secretase activity to reside in a high molecular weight complex consisting of at least four components: presenilin (PS, PS1 or PS2), Nicastrin, anterior pharynx-defective-1 (APH-1), and presenilin enhancer-2 (PEN-2) (Fig. 6).

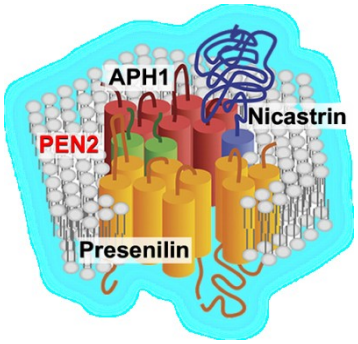


Fig. 6 The gamma-secretase complex.

Presenilins are a family of related multi-pass transmembrane proteins with an unclear number of transmembrane domains. In mammals there are two presenilin homologs, PS1 and PS2. PSs possess two higly conserved aspartate residues indispensable for γ-secretase activity because they represent the catalytic unit of the γ-secretase that liberates Aβ peptides.

Mutations in the genes that encode presenilins, particularly PS1, are causative in the majority of familial early-onset AD (FAD) cases (Sezgin and Dincer, 2014; Zhang et al, 2011; Herrupt, 2010). Nicastrin, the first identified cofactor of PS, is a type I transmembrane glycoprotein that is considered the scaffolding protein within the γ-secretase complex. One study showed that the ectodomain of Nicastrin binds to APP and Notch and can recruit them into the γ-secretase complex, suggesting that Nicastrin may act as the γ-secretase receptor. APH-1 interacts with Nicastrin to form a stable intermediate in an early assembly stage of the γ-secretase complex. PEN-2 regulates PS endoproteolysis. Each of these four γ-secretase components has been found necessary for the enzymatic activity of the complex with deficiency in any of them dramatically impairing γ-secretase activity. In addition to cleaving APP CTFs, γ-secretase cleaves a series of functionally important transmembrane proteins, including Notch, cadherin and tyrosinase. The cleavage of various substrates appears to be dependent on the subcellular compartment; APP is mainly cleaved in the TGN and early endosomal domains whereas Notch is primarily cleaved at the plasma membrane. Thus a disturbance in the localization of the γ-secretase complex may play some role in abnormal Aβ generation and AD pathogenesis (Zhang et al, 2011).

1.4.4 Caspase processing.

In addition to secretases, caspases (predominantly caspase-3) can directly cleave APP at position Asp664 (based on the APP695 sequence) within the cytoplasmic tail during apoptosis to release a fragment containing the last 31 amino acids of APP (called C31). Additional γ-cleavage further generates the fragment (called Jcasp) containing the region between γ- and caspase-cleavage sites.

During Aβ-induced neurotoxicity, activated caspases cleave APP to generate C31 and Jcasp, which are also neurotoxic, therefore initiating a detrimental cascade. One possible mechanism for C31's toxicity is that C31 complexes with APP to recruit the interacting partners that initiate the signals related to cellular toxicity.

Compared to C31, Jcasp appears to play a minor role in cytotoxicity. Importantly, caspase-cleavage of APP seems to be crucial for Aβ-mediated neurotoxicity, as an APP mutation at position Asp664 to inhibit the caspase-cleavage in transgenic mice negated the synapse, electrophysiology, and behavioral abnormalities, even though Aβ plaques were still abundant in the brain.

1.5 Focus on misfolded protein in AD.

1.5.1 Aβ peptides.

Aβ is a 39- to 42- amino acid peptide located in the membrane-spanning domain of APP (Li et al., 2011). Aβ was originally regarded as an abnormal and toxic species restricted to the brains of aged or demented humans. The discovery of soluble Aβ species in the bodily fluids of various species and in the conditioned medium of cultured cells has refuted this concept and implied a physiological function for Aβ. Soluble Aβ, in fact, can stimulate neurite growth in a short amount of time, which can increase neurons' survival rate (Sun et al., 2012). In addition, low levels of Aβ increase hippocampal long-term potentiation and enhances memory, indicating a novel positive, modulatory role on neurotransmission and memory. Picomolar levels of Aβ can also rescue neuronal cell death induced by inhibition of Aβ generation (by exposure to inhibitors of β- or γ-scretases), possibly through regulating the potassium ion channel expression, hence affecting neuronal excitability (Zhang et al., 2011).

Under normal conditions, brain Aβ is degraded by the peptidases insulin-degrading enzyme, neprilysin, and by endothelin-converting enzyme. Aβ is also cleared from the brain in a process balanced by the efflux, mediated by low-density lipoprotein receptor-related protein, and the influx, mediated by the receptor for advanced glycation end products of Aβ across the blood brain barrier (BBB). There is no evidence for any disturbances in these proteolytic enzymes or transport mechanisms in AD (Blennow et al., 2006).

But Aβ has been the focal point of AD research for over a decade and is generally considered as the upstream causative factor (Ballard et al., 2011). The strongest evidence for this position derives from molecular genetic studies of the three genes (APP, PS1 and PS2) that underlie FAD cases, because they all modulate some aspects of Aβ metabolism, increasing the propensity for Aβ to aggregate (LaFerla and Oddo, 2005). Multiple lines of evidence demonstrate that overproduction of Aβ results in a neurodegenerative cascade leading to synaptic dysfunction and synapses loss, formation of intraneuronal fibrillary tangles, eventually neuron loss and shrinkage of neurites in affected areas of the brain (Zhang et al, 2011). Accumulation of Aβ in the hippocampal region of the brain is thought to induce toxic effects, oxidative stress and inflammatory response that contribute to impairment of cognitive function (Sezgin and Dincer, 2014). However, it remains far from clear how this 4-kD protein fragment compromises neuronal function (Belinova and De Strooper, 2013). It seems that soluble Aβ monomers, due to their amphipathic nature, undergo a conformational

change to high β-sheet content, rendering it prone to spontaneously aggregate into soluble oligomers. Formation of fibril is a multi-step process and further assembly leads to the formation of larger insoluble fibrils that eventually precipitate in the brain (Blennow et al., 2006; Lannfelt et al., 2014). Many studies have confirmed that the neurotoxicity of Aβ is largely dependent on its ability to form β-sheet structures.

In particular, there are two main species, namely $A\beta_{40}$ and $A\beta_{42}$. $A\beta_{42}$ it's only about 10% of the Aβ peptides produced but it is more hydrophobic, more prone to fibril formation and it is considered to be the most neurotoxic form. It is the major component of senile plaques and, probably, the fibrillogenic $A\beta_{42}$ isoform triggers the misfolding of other Aβ species, eliciting inflammatory responses, tau protein aggregation and oxidative stress in AD pathology (Zhang et al., 2011; Sun et al., 2012; Li et al., 2011). Indeed, in a normal brain, the majority of Aβ produced is $A\beta_{40}$, while patients with AD have a high ratio of $A\beta_{42}/A\beta_{40}$.

Aβ neurotoxicity seems to be related to its capacity to disrupt the calcium channels in the cell membranes, enhancing Ca^{2+} influx and leading to a disequilibrium in organisms of this second messenger. As consequence, an intracellular Ca^{2+} overload will impair the ability of mitochondria to buffer or cycle Ca^{2+}, resulting in cell toxicity and eventual cell death. The disruption in calcium homeostasis may in turn cause a variety of secondary effects such as lipid peroxidation and generation of free radicals. Over time, these related actions of Aβ will reduce synaptic integrity (Sun et al., 2012; Heneka et al., 2011).

Additionally, it has been shown that Aβ induces astrogliosis through enhancing phosphorylation of extracellular signal-regulated kinase (ERK) in AD brain. An elevated level of phosphorylated ERK (pERK) was observed in the midfrontal cortex of patients during all stages of AD development in both clinical and neuropathological studies. Moreover, the activation level of pERK in astrocytes was strongly correlated with cognitive performance and the severity of AD neuropathology (Li et al., 2011).

1.5.2 Tau protein.

In its normal state, tau is a soluble protein that promotes microtubule assembly and stabilization which are essential for normal configuration of neuronal extensions, polarization of cells and also for axonal transport (Sezgin and Dincer, 2014). The tau protein is encoded by a single gene (MAPT) located on chromosome 17q21, although it is alternatively spliced to yield six major protein isoforms in the adult human brain. Its phosphorylation is regulated by

the balance between multiple kinases (eg. GSK-3β and CDK5) and phosphates (eg. PP-1 and PP-2A) (Blennow et al., 2006).

Pathological tau protein, by contrast, is the major component of neurofibrillary tangles (NTFs) (Sezgin and Dincer, 2014; Ballard et al., 2011). Almost in parallel with the identification of Aβ in plaques, tangles were shown to be composed of abnormally hyperphosphorylated tau protein (Blennow et al., 2006).

The unusually phosphorylation of tau at certain residues leads to altered solubility properties and tau becomes prone to aggregation into insoluble fibrils in tangles (LaFerla and Oddo, 2005). Tau monomers first bind together to form oligomers, which then aggregate into a β sheet before forming tangles (Ballard et al., 2011). In particular, the amyloid cascade hypothesis proposes that changes in tau and consequent neurofibrillary tangle formation are triggered by toxic concentrations of Aβ. The pathways linking Aβ and tau are not clearly understood, although several hypotheses have been proposed (Ballard et al., 2011). But, it well-documented that tau hyperphosphorylation provokes microtubule destabilization and, as consequence, impairments in axonal transport, compromising neuronal and synaptic function (Blennow et al., 2006). Furthermore, neurofibrillary tangles induce immune response and result in an elevation of proinflammatory markers, thus contribute to the inflammatory stress (Sezgin and Dincer, 2014).

Tau pathology starts early in the disease process in selective neurons in the brains of individuals with AD and from the transentorhinal region, it spreads to the hippocampus and amygdala, and later to the neocortical association areas (Blennow et al., 2006). But NTFs also occur in other neurodegenerative disorders, including frontal temporal dementia with Parkinsonism linked to chromosome 17, Pick's disease, progressive supranuclear palsy and corticobasal degeneration (LaFerla and Oddo, 2005; Oddo et al., 2003).

1.6 The neuropathology of AD.

The neuropathology of AD is very complex and has yet to be completely understood. There are several hypotheses that try to explain the pathological mechanism of the disease (Sun et al., 2012).

1.6.1 Cholinergic dysfunction of the central nervous system.

Cholinergic deficiency was the oldest hypothesis for AD pathology. It was created after the observence of loss of cholinergic activity in the CNS. The brains of AD patients have very low levels acetylcholine (ACh). According to some pathophysiology research, the loss of cholinergic neurons usually occurs in brain areas associated with memory and learning, such as the hippocampus, nucleus basalis of Meynert, and cortex. A reduction in cholinergic activity is thus thought to be associated with cognitive deficits. This reduced activity could affect synaptic transmission and initiates inflammatory processes and thus some reactive oxygen species (ROS) will be produced, with all the consequences above mentioned, resulting in cell death. Furthermore, Aβ can inhibit cholinergic neurons from absorbing choline and halt the action of cholinergic acetyltransferase (chAT), which inhibit the generation of ACh. However, whether the decrease in ACh is the cause of AD needs to be studied further (Sun et al., 2012) since it has not been strongly supported by clinical and experimental investigations and it can not explain byitself all the characteristics of AD (Li et al., 2011).

1.6.2 Amyloid cascade hypothesis.

A major advance in the study of AD came with the sequencing of the main constituent of the senile plaques – the amyloid β peptide (Aβ). This led to the elaboration of a theory of AD known as the amyloid cascade hypothesis. To date, this hypothesis is the most influential model of the pathogenesis of AD. It is widely accepted and it suggests that this peptide is the direct cause of neurodegeneration, synaptic loss and cognitive decline in AD. More precisely, it states that an imbalance between the production and clearance of Aβ in the brain is the trigger for all cases of AD and the initiating event for neurodegeneration, ultimately leading to dementia in AD (Iqbal and Grundke-Iqbal, 2011; LaFerla and Oddo, 2005; Blennow et al., 2006; Steardo Jr. et al., 2015). This hypothesis is supported by different evidences: (a) Aβ is a part of APP, a large type I membrane protein encoded by APP gene on chromose 21; this

gene is mutated in a significant fraction of the FADs cases; (b) individuals with Down's syndrome, who have three copies of APP gene, develop clinical and pathological signs of early onset AD; (c) mutations in the PSEN-1 and PSEN-2 genes can behave as dominant familial AD genes (Herrupt, 2010).

In familial AD (Fig. 7 – top left), mutations in either APP or one of the PSEN genes lead to the brain accumulation of $A\beta_{42}$ that has a high tendency to form β-pleated sheet structures (Blennow et al., 2006; Lannfelt et al., 2014). Amyloid aggregates form – first small oligomers and finally plaques. The amyloid cascade hypothesis proposes that these Aβ aggregates lead in turn to a series of downstream events, ranging from synapse loss to plaque deposition, from inflammation to the triggering of tau hyperphosphorylation and to the death of susceptible neurons.

This is in line with data collected from the transgenic mice 3xTg-AD, the best model right now, which closely mimic the pathology, where, despite equivalent overexpression of human APP and tau, Aβ pathology precedes typical indications of tau pathology (Oddo et al., 2003).

The hypothesis also proposes that sporadic AD develops when the natural history of an individual accelerates a normal age-dependant process of Aβ accumulation (Fig. 7 – top right). At some point, a sufficient amount of Aβ is deposited that the amyloid cascade is engaged. Subsequently, the sporadic disease follows the same pathway to dementia as the familial form (Herrupt, 2010).

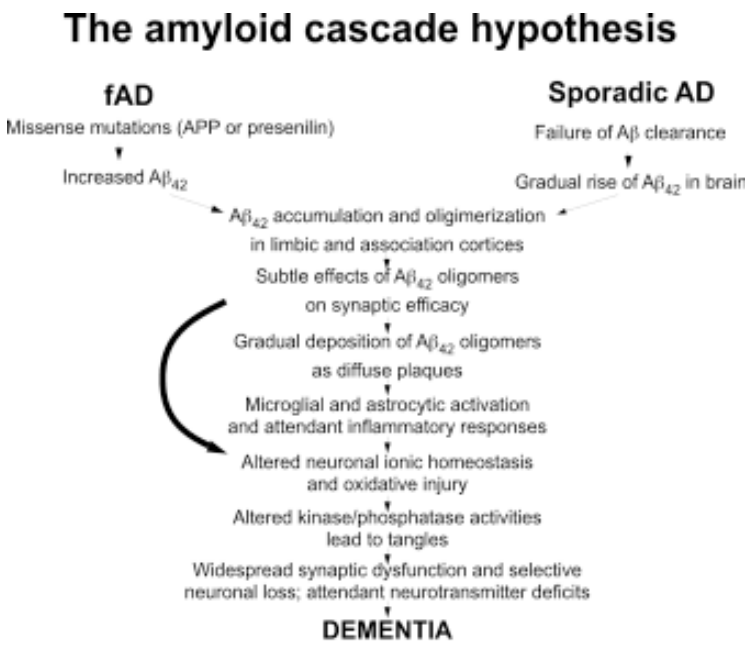


Fig. 7 The amyloid cascade hypothesis of AD. This hypothesis represents the classic theory of the origins of AD. Both familial forms (FAD) as well as later onset forms with no known etiology, (SAD) lead to the production of excess $A\beta_{42}$. Once this toxic event peptide begins to aggregate, a cascade of events is triggered that produces biological and neurological symptoms of AD (Herrupt, 2010).

However, the precise etiology of sporadic AD is not known in detail. In all species, age brings a progressive slowing of brain function. Cognition slows and the ability to form new memories is reduced; motor functions deteriorate and even brain's homeostatic functions become less and less robust. This functional decline is correlated with the loss of structural complexity of brain cells. Neuronal dendrites become less complex, spine and synapse density decrease, the cell bodies of the larger neurons accumulate lipofuscin, astrocytic function declines and the immune system becomes less responsive. In this situation, brain defenses against AD are weakened (Herrupt, 2010). On these bases, a new model was created, which includes as first a precipitating injury that starts the pathogenic process. This injury in turn triggers the second key event, a chronic inflammatory process that adds additional relentless stress to brain cells already weakened by age. Interestingly, also data from animal models and human autopsies revealed that both SP and NTF are co-localized with activated glial cells, suggesting that reactive gliosis might exert a key pathogenesis role in AD (Steardo Jr. et al., 2015). The third event is a major shift in the cellular physiology of the brain cells that marks

the beginning of a cell's degenerative process and leads to a major synaptic dysfunction and neuronal death – the final and most direct cause of Alzheimer's dementia Also a genetic mutation can be such an injury and it triggers a protective response among the cells of the brain, but the age-related failure of normal homeostatic mechanisms means that the response continues, even if after injury itself abates (Herrupt, 2010).

The idea that AD begins with an initiating injury has both theoretical and practical relevance. Theoretically, it means that Alzheimer's is not a part of normal aging, whereas the practical relevance is that, if research can identify the most common sources of injury, we may be able to intervene proactively and delay disease onset.

A cardinal feature of the neuropathology of most AD brains is the evidence for a chronic neuroinflammatory process. There is also a solid epidemiological evidence that inflammation serves as a cause of AD. Long-term use of high doses of certain non-steroidal anti-inflammatory drugs (NSAIDs) lowers the lifetime risk of AD from 30–60%, and there are biochemical evidences of inflammation, as elevated levels of cytokines, such as IL-1, IL-6, TNFα and S100β in human AD brains.

Moreover, it must be considered that neurons are not the only cells of the brain whose cell biology is changed during the progression of AD. Microgliosis as well as astrocytosis are prevalent, and most plaques are surrounded by activated astrocytes and invaded by activated microglia (Heneka and O'Banion, 2007; Rodriguez et al., 2009). The microglial cells are assisted by the responses of astrocytes and brain vascular endothelial cells in maintaining a chronic shift in the inflammation status of the brain. The result of this network-like response is a chronic stress on neurons and their function. Cell cycle proteins are activated, reactive species of oxygen (ROS) are produced, mitochondrial function is reduced, dendritic/axonal transport is impaired. A second tenet of the new model, therefore, is that a chronic immune response, persisting over months and years, creates the unique chemistry and cellular physiology that results in the core symptoms of the AD (Herrupt, 2010). This activated state is accompanied by a shift from an acute pro-inflammatory reaction to a chronic state of activation. Hypothesizing that AD involves a cellular change-of-state from early to late disease, the prediction is that the biology of early AD differs in qualitative ways from the biology that ultimately produces the dementia. This means that the biology of the cells involved has changed and the process in the end is independent of the initiating injury and transformation. It also offers a theoretical explanation for the failure of the prospective human

trials based on NSAIDs: the trials were all begun after manifested AD symptoms. With the disease already in progress, it is likely that many of the neurons in the subjects' brains had gone through their change of state. Their biology no longer required chronic inflammation to sustain their abnormal state.

In conclusion, extracellular Aβ naturally accumulates with age and with time the non-neuronal cells of the brain would be expected to sense its presence and react. The associated release of cytokines then enhance the production of the Aβ peptide. This creates a feed forward reaction. Other factors such as excessive synaptic activity of the type found during excitotoxic injury can also enhance Aβ production. Thus, driven by one or more of these means, Aβ aggregates stimulate the immune response, and the immune response stimulates more Aβ production. In this way a cycle is created – the amyloid deposition cycle (Fig. 8). This pathways is the same also for FAD in which the mutations, by accelerating the deposition of Aβ, enhance inflammation and thus drive the amyloid deposition cycle earlier and harder than normal, strongly favoring the development of a chronic inflammtion (Herrupt, 2010).

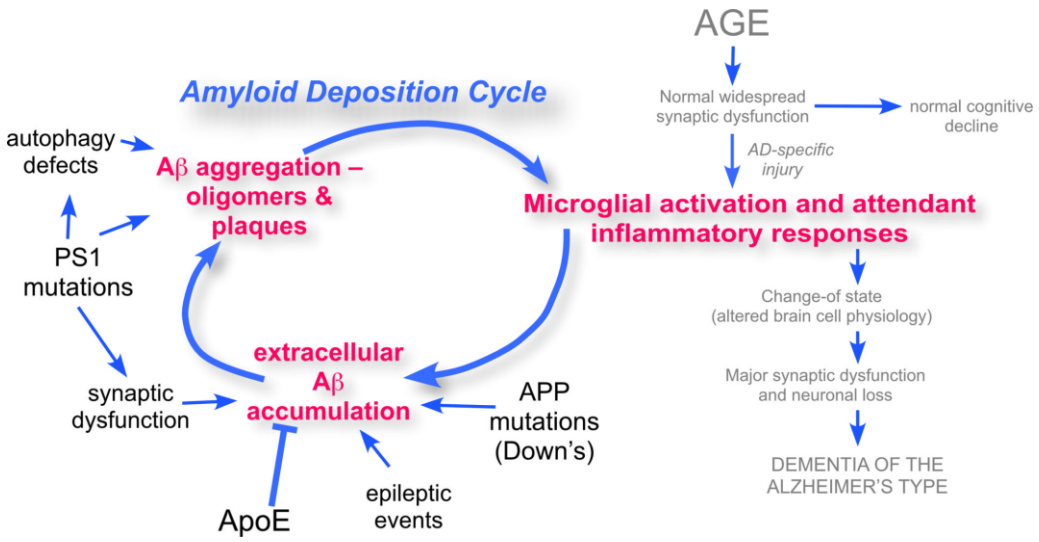


Fig. 8 The amyloid deposition cycle. In this diagram, the amyloid cascade of Fig. 7 is re-envisioned as a feed-forward cycle of amyloid deposition and inflammatory responses.

1.7 Treatments.

Despites all scientific efforts and although we have known about this disease for over a century, to date, no effective, mechanism-based treatment strategy is available to halt the progression or to play a role in the prevention of cognitive decline (Bedse et al., 2015) and medications currently provide only modest and transient benefits to a subset of patients and effective pharmacotherapeutic options are lacking (Lannfelt et al., 2014; Steardo Jr. et al., 2015). One of the primary problem in the treatment of AD is time: neurodegeneration probably occurs throughout the life of an individual and clinically manifests when it crosses a certain threshold (Iqbal and Grundke-Iqbal, 2011). Research advances in the molecular pathogenesis of AD have also led to new drug candidates with disease-modifying potential, which have now come to testing in clinical trials. Epidemiological data have suggested additional drug candidates, some of which have been investigated in randomised trials (Blennow et al., 2006).

1.7.1 Acetylcholinesterase inhibitors.

From the late 60's to the mid of 80's a large body of research placed neurotransmitter dysregulation and inadequate levels, specifically acetylcholine and glutamate, as a contributor to the deterioration of the mental abilities observed in AD (Lannfelt et al, 2014). For these reasons, the aim of the drugs, approved by FDA and used in many countries, is to improve the neurotransmitter levels. In particular, degeneration of cholinergic neurons in the basal forebrain nuclei causes disturbances in presynaptic cholinergic terminals in the hippocampus and neocortex, which is important for memory disturbances and other cognitive symptoms.

One therapeutic approach to enhance cholinergic neurotransmission is to increase the availability of acetylcholine (ACh) by inhibiting acetylcholinesterase (AChE), the enzyme that degrades ACh in the synaptic cleft (Blennow et al., 2006). The acetylcholinesterase inhibitors (AchEIs), namely rivastigmine (Exelon), galantamine (Razadyne, Reminyl) and donepezil (Aricept) have a clinical use in AD. The other one of this group of inhibitors is Tacrine (Cognex), approved in 1993, it is the first drug used in AD treatment but now it is rarely prescribed due to its serious side effects (liver damage) which are not completely understood (Lannfelt et al, 2014; Sun et al., 2012; Bisogno and Di Marzo, 2008).

Donepezil is readily absorbed after oral administration and, compared to tacrine, its effects last longer and it is better tolerated. Galantamine is an extract of the flowers and bulbs of lilies, daffodils, and related plants. Donepezil and galantamine are selective AchEI, whereas rivastigmine inhibits AchE and buturylcholinesterase with similar affinity and galantamine also allosterically modulates presynaptic nicotinic receptors.

Both donepezil and galantamine are metabolised in the liver by the cytochrome P450 enzymes CYP2D6 and CYP3A4, and can thus interact with drugs that inhibit these enzymes, such as fluoxetine and paroxetine, with resulting cholinergic adverse events. Rivastigmine has a non-hepatic metabolism,so it can minimize drug-drug interactions.

The half-life of the drugs also varies, which determines the need for one or two doses per day. Other differences are whether the drug has to be coadministered with food. The efficacy of these drugs has been studied in different randomised double-bind clinical trials. Most of them have improved the cognitive performance as well as activities of daily living but only in patients with mild to moderate forms of AD and only short-term (9-12 months to 5 years) (Karl et al., 2012). Benefits of AChEIs are also seen for functional and behavioural symptoms but there is no evidence that these drugs differ in efficacy.

Donepezil, rivastigmine, and galantamine are efficacious in mild to moderate Alzheimer's disease, and these drugs are also recommended by the Quality Standards Subcommittee of the American Academy of Neurology as first-line pharmacotherapy for symptomatic treatment of the disease. Overall, they are safe drugs, and side-effects are dose-dependent and generally limited to gastrointestinal symptoms, including nausea, vomiting, and diarrhea (Sun et al., 2012). The occurrence of side-effects can usually be reduced by starting treatment with a low dose, which is escalated slowly. Coadministration with food delays absorption of the drug and can also reduce gastrointestinal side-effects.

However, considering the mechanism of action for the AChEIs, they are not expected to change the natural course of AD, but to temporarily mitigate some of the symptoms: they can be effective for up two years and, as emerged from open-label extension studies, some patients can have long-term benefits for up to five years (Blennow et al., 2006). In addition, they all have been shown to modestly slow the progression of cognitive symptoms and reduce problematic behaviors in some people, but at least half of the people who take these drugs do not respond to them (Bedse et al., 2015).

1.7.2 Memantine.

Another symptomatic drug approved by FDA is is memantine (Namenda), a partial non competitive N-metyl-D-aspartate (NMDA) receptor antagonist. Glutamate is the major excitatory neurotransmitter in the brain. Under normal conditions, glutamate and the NMDA receptor have important roles for learning and memory processes. Under abnormal conditions, such as in AD, increased glutamatergic activity can lead to sustained low-level activation of NMDA receptors, which may impair neuronal function. Memantine is believed to protect neurons from glutamate-mediated excitotoxicity without preventing the physiological NMDA-receptor activation needed for cognitive functioning. The drug is well tolerated in general, with few adverse events (Blennow et al., 2006).

Randomised double-blind clinical trials show modest positive effects on cognitive and behavioural symptoms, and improved ability to perform activities of daily living at 6 months in people with moderate to severe AD. Memantine is the only drug that shown to be effective for the later stages of the disease. Additionally, in moderate to severe disease, combination therapy with donepezil and memantine show positive effects on symptoms relative to donepezil alone. Despite the theoretical rationale for neuroprotective properties of memantine, current trials are too short to assess if the drug has any disease-modifying effects (Blennow et al., 2006).

1.7.3 Treatment of behavioral signs.

Behavioural signs, such as aggression, psychomotor agitation, and psychosis (hallucinations and delusions), are very common in patients with Alzheimer's disease, especially in the late stages of the disease. Such symptoms not only affect quality of life for patients and caregivers, but also contribute to care burden and economic cost.

Atypical antipsychotic drugs produce fewer extrapyramidal side-effects (eg, parkinsonism and tardive dyskinesia) than do conventional neuroleptics, and are thus preferred for the management of psychosis or agitation. Several short-term trials show efficacy of risperidone and olanzapine in reducing the rate of aggression, agitation, and psychosis. Alternative treatments include anticonvulsants, such as divalproate and carbamazepine, and short-acting benzodiazepines, such as lorazepam and oxazepam (Blennow et al., 2006).

AD is a nervous system disease that exists in patients for many years. Many efforts are currently underway to explore the pathology of AD and develop appropriate treatments. These strategies focus on slowing disease progression and maintaining patients' quality of life.

Nonetheless, there is no treatment that effectively stops the disease's progression. Therefore, researchers need to recognize early signs of AD and explore new therapies to combat AD (Sun et al., 2012).

2. ASTROCYTES.

2.1 Introduction to glial population.

The complexity of the cellular circuitry of human brain is unparalleled by any other living system known so far. The neural cells are exceedingly densely packed within a strictly limited volume of the skull, thus requiring a specific attention to the control of brain homeostasis throughout early development and postnatal functioning. This specific requirement is manifested in the highly developed brain–blood barrier(BBB), which essentially limits the impact of bodily homeostatic systems on the central nervous system (CNS). The brain homeostasis therefore is entrusted to specific population of neural cells known as neuroglia (Heneka et al., 2010).

In the course of late 19th century the cellular nature and morphofunctional heterogeneity of neuroglial cells were firmly established; however, their characterization paralleled the one of neurons and, since astrocytes do not generate action potentials and have ‘‘passive’’ membrane properties, they were just classified as non-excitable cells, and therefore unable to communicate. Hence, they were relegated to submissive roles and, thus, ignored for a few decades (Rossi, 2015; Sofroniew, 2014). However, the neurocentric view of brain function and disease has been challenged by the emerging evidence of the physiopathological potential of neuroglia and it has been introduced the idea that astrocytes have not only trophic and structural role, but rather, they are dynamic components of brain connectivity and function. Thus, their activities and functional relevance cannot be further neglected if one wants to have a comprehensive vision of the CNS performance in health and disease (Colangelo et al., 2014; Verkhratsky et al., 2015; Heneka et al., 2010).

Glial cells are a highly heterogeneous population which differ in ontology, morphology and function, both in different brain regions and at different developmental stages (Steardo Jr. et al., 2015). Nonetheless, each and every neuroglial cell has a conceptual countenance to keep the brain homeostasis, starting from the control of local molecular environment to providing the intrinsic brain defense system. These glial homeostatic functions are many, and their failure are inevitably signals brain pathology.

Astrocytes are the main effectors of the CNS homeostasis and contribute to its defense in pathology, microglial cells are the resident macrophages and they are regarded to be the

innate immune cells in the brain, thus they are the first line of defense against invading pathogens and serve as specialized sensors for brain tissue; oligodendrocytes protect neuronal axons producing the myelin sheath, a fatty insulating layer that facilitates the saltatory conduction of action potentials (Heneka et al., 2010; Verkhrastsky et al., 2015); the NG2 glial cells, known as synantocytes, have a stellate morphology and most likely represent a reservoir of myelinating cells (Steardo Jr. et al., 2015).

2.2 Astrocytes physiology.

Astrocytes, also termed astroglia, are definitely the most abundant and heterogeneous type of glial cells in the CNS. (Steardo Jr. et al., 2015). They are characterized by a star shape morphology with numerous processes that surround neighboring neurons and blood vessels and contain intermediate filaments (glial fibrils). Astrocytes are distributed throughout the nervous system and lack axons and dendrites. These cells have a highly complex and heterogeneous morphology, which differs largely depending on their development stage, subtype, and localization and they are subdivided into **fibrous astrocytes and protoplasmic astrocytes**. The first ones are found in the white matter and have long, thin, unbranched processes (Verkhratsky and Parpura, 2015; Rossi, 2015). Instead, the protoplasmatic ones are found in the gray matter and have many short branching processes, which envelope synapses and the neighbouring blood vessel, where these processes form end-feet plastering the capillary wall and participating in formation of blood-brain barrier (BBB). As a result, astroglial cells integrate neurones and blood vessels into relatively independent neuronal-glial-vascular units. Astroglial endfeet are capable of releasing vasoactive substances that control local blood flow and coordinate the latter with neuronal activity.

Astrocytes typically extend between five and eight major processes and everyone occupies space that is free from processes of any other astrocytes. A single astrocyte in the rodent brain has a volume ≈ 66 000 μm^3, and the membrane of this astrocyte covers around 140.000 synapses lying within the astroglial domain; human astrocytes are considerably larger and more complex and their processes wrap up to 2 millions of synapses. (Fuller et al., 2010; Rodriguez et al., 2009; Heneka et al., 2010).

In addition, in the cerebral cortex, an individual astrocyte was described to enwrap a few neuronal somata and hundreds of dendrites. Thus, it is reasonable to postulate that a given astrocyte can coordinate small groups of neurons, if signals are propagated to neuronal cell

bodies, but it is able to modulate hundreds of neurons when the information is transmitted to dendrites and synapses. Yet, it is true also the reverse situation, as the dendritic tree of a single neuron appears to require numerous astrocytes for complete coverage (Fig. 9). This suggests that the reciprocal signalling between neurons and astrocytes represents a new level of functional integration, which is anatomically different from that of a single synaptic circuit or a single astrocyte territory (Rossi, 2014).

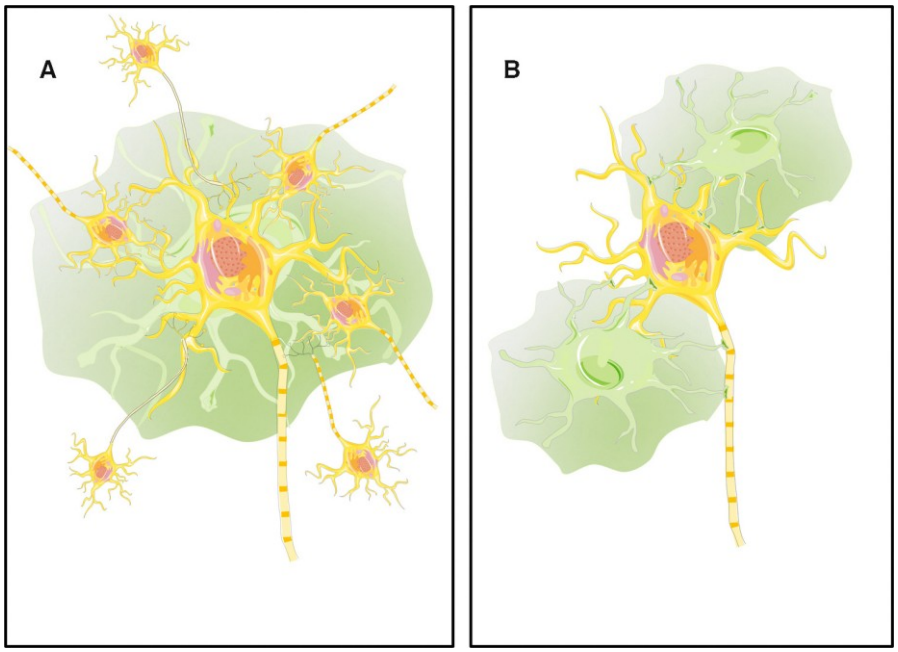


Fig. 9 Schematic representation of the reciprocal interaction between neurons and astrocytes. (A) Diagram showing that a single protoplasmic astrocyte can contact several synaptic structures, neuronal somata and dendrites within its territory. (B) Diagram illustrating that the dendritic tree of a single neuron requires numerous astrocytes for complete coverage.

Furthermore, astrocytes are extensively connected by gap junctions, aqueous channels that connect the cytoplasm of adjacent cells, which are permeable to positively or negatively charged molecules. Gap junctions are evenly distributed along the astrocyte processes, often interconnecting adjacent astrocytic processes derived from the same cell. These gap junctions provide for a glial information-transfer system, as they form pathways for intercellular diffusion, mediating the sharing of molecules such as glucose and other energy substrates (Fuller et al., 2010; Heneka et al., 2010).

It is well-proved that astrocytes control the brain homeostasis and allow neurons to functioning; they are an essential neuro-supportive cell type in brain. Insults to the CNS regardless to their etiology put the strain on the organ homeostasis, with a variety of potential changes in gene expression, cellular structure, and function (Sofroniew, 2014). It is known that astrocytes through dedicated molecular cascades, protect neurons, for example, against glutamate excitotoxicity, extracellular K^+ overload, and ROS damage. Astrocytes also supply stressed neurons with energy substrates (Steardo Jr. et al., 2015; Verkhratsky et al., 2015), and they also clean up neuronal waste, which include not only metabolic bioproducts but also neurotransmitters released during synaptic transmission through active uptake (Li et al., 2011). However, the loss of these critical astroglial functions permits and exacerbates progression of various diseases, including AD.

The astrocytic physiological functions and their alterations with a potential involvement in AD will be outlined in detail in the following sections (Verkhratsky et al., 2015).

2.2.1 Physiological functions of astrocytes.

Numerous functions have been demonstrated for astrocytes and some are essential because they are related to the astroglial cooperation with neurons (Fuller et al., 2010). They are responsible for a massive number of homeostatic tasks in the CNS, highly diverse and regionally specialized. Indeed, astrocytes finely control the environment by regulating pH, ion homeostasis, blood flow, and modulate oxidative stress.

Astrocytes express a wide array of receptors that enable them to respond to virtually all known neuroactive compounds, including neurotransmitters, neurohormones, neuropeptides, growth factors, cytokines, small molecules and toxins. Expression of these receptors allows astrocytes to participate in signaling processes, and additionally, to function as sentinels (Fuller et al., 2010). In addition, by employing a variety of molecular mechanisms (exocytosis, membrane transporters or diffusion through plasmalemmal channels), astrocytes secrete numerous neurotransmitters, neurohormones and trophic factors that regulate synapse formation and maintenance, and dynamically modulate information processing and sinchronization of neuronal networks (Verkhratsky et al., 2015).

2.2.1.1 Metabolic support for neurons.

Astrocytes provide metabolic support to neurons which require a constant input of energy for the neuronal activity (i.e. the generation processing and transmission of impulses) that is metabollically expensive, with the brain accounting for 20% of the body's resting metabolism. Action potentials can be initiated and synapses can transmit signals only if Na^+, K^+ and Ca^{2+} are maintained in electrochemical disequilibrium across the plasma membrane, and approximately 40–50% of total ATP produced in nervous tissue is utilized for this purpose. The synthesis of ATP in the brain is achieved through both mitochondrial oxidative phosphorylation and cytosolic glycolysis, with the main brain fuel being glucose.

Astrocytes are uniquely positioned to provide a nurturing environment for neurons and therefore bidirectional neuron–astrocyte interactions are critical for normal functioning and survival of the CNS. The entire surface of intraparenchymal capillaries is covered by astrocytic end-feet, while other astrocyte processes are wrapped around synaptic contacts, indicating that astrocytes are ideally positioned to sense an increase in synaptic activity and to couple this with an increase in energy metabolism (Fuller et al., 2010).

The metabolic support of neurons is achieved through a glucose-lactate shuttle operative within the astroglial domains. Astrocytes accumulate about 50% of glucose entering the brain tissue, via the GLUT1 transporter, and store it in the form of glycogen (Heneka et al., 2010). In the astrocytes, glucose is used for glycolysis, induced by a Na^+ increased concentration, with the production of lactic acid. This is then transported out of the astrocyte via the H^+-coupled monocarboxylate transporters (MCTs) MCT1 and MCT4. Lactate released is then transported into neurons via MCT2 and converted to pyruvate for use in the citric acid cycle. This happens because neurons can utilize lactate as the major metabolic substrate, sparing glucose for the synthesis of NADPH(H+), ribose-5-phosphate and/or glycerol-borne lipids and because when neurons are stressed, they are unable to upregulate glycolysis because of low 6-phosphofructo-2-kinase/fructose-2. Thus, by actively downregulating glycolysis, neurons rather use glucose to maintain their antioxidant status at the expense of its utilization for bioenergetic purposes, and need astrocytic lactate to cover this metabolic gap. Furthermore, astrocytes are the only neuronal cell type capable of storing glucose as glycogen. Glycogen stores can only be mobilized by a restricted set of neurotransmitters, and breakdown can lead to the release of lactate in the extracellular space. Under hypoglycemic

conditions and periods of increased tissue energy demand, astrocytic glycogen provides the energy substrate for the brain (Fuller et al., 2010).

2.2.1.2 Synaptic function.

At synaptic level, the ability of astrocytes to release chemical transmitters (named gliotransmitters in order to distinguish them from neurotransmitters) is fundamental for their involvement in information processing in neuronal–glial networks. The gliotransmitters include glutamate, ATP, D-serine, GABA, and taurine.

The gliotransmitters can be released from astrocytes through Ca^{2+}-dependent exocytosis, by diffusion though large pore channels or through transporters.

Furthermore, astrocyte are also part of the so called tripartite synapses constituted by the astroglial perisynaptic process, the perisynaptic neuronal terminal and the postsynaptic neuronal membrane (Fig 10). The astrocyte has a dual role in this tripartite synapse. First, by the virtue of neurotransmitter receptors expressed in the astroglial membrane, the astrocyte can sense the transmitter release from the neuronal terminal, and secondly, by releasing gliotransmitters, the astrocyte can modulate efficacy and strength of both excitatory and inhibitory synapses, leading to the activaction of various neuronal receptors such as, for example, NMDA or adenosine receptors. Thus, astrocytes play a crucial role in neurotransmitter homeostasis, and most importantly of glutamate (Heneka et al., 2010; Colangelo et al., 2014).

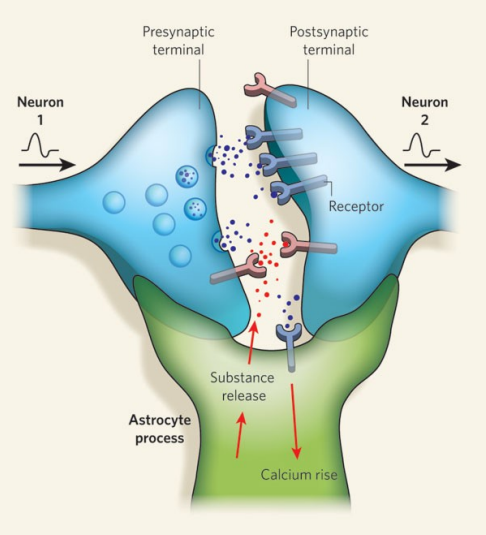


Fig. 10 The tripartite synapse.

As mentioned before, glutamate, despite being the main excitatory transmitter in the CNS, is the most powerful neurotoxin, and every excess of glutamate in the extracellular spaces triggers excitotoxic neuronal death. At glutamatergic synapses,it is released during neuronal activity and astrocytic uptake is the main route for its removal from synaptic cleft. This action is essential not only to terminate its effects as a neurotransmitter, but also to prevent extracellular glutamate levels from reaching excitotoxic levels. From the bulk of glutamate released during synaptic transmission, about 20% is accumulated into postsynaptic neurons and the remaining 80% is taken up by perisynaptic astrocytes (Heneka et al., 2010). The translocation of glutamate is powered by transmembrane ion gradients, and the transport of a single glutamate molecule requires an influx of three Na^+ ions and one H^+ ion coupled with the efflux of one K^+ ion. This intracellular Na^+ elevation puts an extra load on Na^+/K^+–ATPase, causing an increased energy demand in astrocytes, which in turn requires an increase in cellular glucose utilization and glycolysis. The increase in intracellular Na^+ resulting from glutamate has important metabolic consequences as it serves as a signal that couples synaptic activity with glucose consumption (Fuller et al., 2010; Heneka et al., 2010).

The uptake via the excitatory amino acid transporters (EAAT1 and EAAT2 in human brains, in rodents known as glutamate/aspartate transporte (GLAST) and glutamate transporter-1 (GLT-1)) are expressed only in astrocytes where they represent the major mechanism preventing accumulation of glutamate in the synaptic space and thus protects neurons from excessive activation (Colangelo et al., 2014; Heneka et al., 2010). After entering the astroglial cells, glutamate is converted to the glutamine via glutamine synthetase, an enzyme localized primarily in astrocytes. The non-toxic glutamine produced can subsequently be safely transported back to the presynaptic neuronal terminals through the extracellular space, where it is converted back to glutamate, primarily via phosphate-activated glutaminase, thus accomplishing the glutamate-glutamine shuttle (Heneka et al., 2010).

Moreover, astrocytes do not only participate in synaptic transmission, but they act as key elements in synaptogenesis, in synaptic maturation and maintenance. In the *in vitro* condition, the addition of astrocytes triggers very substantial increase (up to seven times) in synapse formation. In fact, these cells produce and secrete cholesterol which is critically important for synapse formation and secrete variety of factors needed for both synaptic maturation and maintenance (Heneka et al., 2010).

2.2.1.3 Synthesis and release of glutathione.

Glutathione (GSH) is a tripeptide, and the predominant low molecular weight thiol in animal cells. Glutathione is synthesized intracellularly, and while its intracellular are usually between 0.5 and 10 mmol/L, the extracellular ones are relatively low (2–20 μmol/L). GSH has many essential roles within the cell. As an antioxidant GSH not only protects cells from the toxic effects of ROS, but also functions in catalysis, metabolism and transport. GSH also participates in reactions involving the synthesis of proteins and nucleic acids. It forms conjugates with a variety of endogenous and exogenous compounds and is a cofactor or co-substrate for various enzymes such as glutathione peroxidase and gluthathione transferase.

When ROS production cannot be balanced by the ROS detoxification processes, oxidative stress occurs. GSH is a major cellular antioxidant, and therefore an important line of defense against this kind of damage, also in neuronal cells. It involves sequential reactions in which glutathione acts as an electron donor and becomes oxidized.

Astrocytes use the cystine/glutamate exchanger (xCT) and other transport mechanisms to take up cystine or cysteine for GSH synthesis. This process is limited by the availability of the amino acid cysteine. GSH is then exported from the astrocyte. Extracellular GSH serves as a substrate for the astroglial ectoenzyme γ-glutamyl transpeptidase (γGT), producing the dipeptide CysGly, which can then be reused for astroglial GSH synthesis or be used as exogenous precursor of neuronal GSH (Fuller et al., 2010).

2.2.1.4 Regulation of extracellular ion concentration.

Neuronal excitability is maintained by relatively large transmembrane fluxes of ions, which are moved by electrochemical gradients. These fluxes affect the extracellular concentrations of ions, which in turn change these gradients (Heneka et al., 2010).

Astrocytes have a critical role in buffering the increase the fluxes., in particular, K^+ concentration resulting from synaptic activity. Excess K^+ ions in the extracellular space can influence neuronal activity, neurotransmitter release, glucose metabolism and cerebral blood flow, and can result in neuronal depolarization, hyperexcitability and seizures. Astrocytes take up excess extracellular K^+ ions via two broad mechanisms: spatial K^+ buffering (passive) and K^+ uptake (active). Spatial K^+ buffering depends on the expression of multiple types of K+ channels, so astrocytes transfer K^+ from areas of a high concentration of K^+ ions to regions

with lower, whereas the active uptake is mediated primarily by the Na^+/K^+–ATPase (Fuller et al., 2010).

2.2.1.5 Formation and maintenance of the blood-brain barrier.

At the level of whole brain, astrocytes form the glia limitants, the blood-brain barrier (BBB): a thin barrier surrounding the brain and spinal cord and containing astrocytic end-feet, that protects the brain from toxic substances in the blood, supplies the CNS with nutrients, and filters excess and toxic molecules from the brain to the blood (Verkhratsky et al., 2015).

Through their perivascular processes forming an endfeet, most protoplasmic astrocytes, contacting capillary endothelial cells, are active components of the BBB and regulate cerebral blood flow and metabolic supply in response to neuronal activity (neurometabolic coupling) (Colangelo et al., 2014; Heneka et al., 2010).

The astrocytic end-feet release signals, which support not only the formation and maintenance of the BBB, but also the expression of transporter molecules in endothelial cells, such as GLUT1.

Astrocytes also have an active role in the short-term modulation of BBB permeability. For example, an increase in neuronal activity within the astroglial domain triggers Ca^{2+} signals, which enter astrocyte endfeet and result in the release of vasoactive substances (Heneka et al., 2010), whereas in pathological conditions, astrocytes may release ATP, cytokines, glutamate or nitric oxide, which can increase BBB permeability. Indeed, the vascular dysfunctions, perivascular amiloidosis and compromised blood–brain barrier are inseparable parts of AD pathology (Fuller et al., 2010).

2.3 Pathological astroglia/ reactive astrogliosis.

Astrocytes, in accordance with their homeostatic function, are deeply involved in neural disease. Acute and chronic brain insults trigger a specific defensive glial reaction, represented by a complex morphofunctional remodeling. Astrocytes, together with microglia, act as crucial effectors of the neuroinflammatory response (Rodriguez et al., 2009; Scuderi et al., 2013; Rossi, 2015).

At the beginning, this reactive state starts as a genuinely survivalistic, defensive brain reaction which is aimed at (i) isolation and removal of the damaged area from the rest of the CNS tissue, (ii) reconstruction of the blood–brain barrier and (iii) facilitation of the

remodelling of brain circuits in areas surrounding the lesioned region (Heneka et al., 2010); however, sometimes, it has deleterious consequences (Fig. 11). In fact, stronger brain insults may push glial homeostatic systems towards a damage exacerbating mode. The severe stress on astroglial energetics with a subsequent loss of ion homeostasis may trigger a massive release of glutamate, a substantial leak of K^+ ions, release of NO and reactive oxygen species—i.e. agents promoting neurotoxicity, given that reactive astrocytes also release cytokines and many other factors that mediate inflammatory responses and remodeling processes (Colangelo et al., 2014). In this situation, reactive gliosis can become a self-perpetuating process which, at the end, exacerbates the injury and, on another hand, it represents a non-physiological state in which astrocytes lose their helpful properties (Steardo Jr. et al., 2015).

In particular, astrogliosis has four key characteristic: (1) astrogliosis is a spectrum of potential molecular, cellular, and functional changes in astrocytes that occur in response to all forms and severities of CNS injury and disease; (2) changes undergone by reactive astrocytes vary with severity of the insult along a graded continuum; (3) changes associated with astrogliosis are regulated in a context-specific manner by many different inter- and intracellular signaling molecules; and (4) changes undergone during astrogliosis have the potential to alter astrocyte activities both through gain and loss of functions. Furthermore, gliosis does not occur in isolation, but it is part of a coordinated multicellular response to CNS insults that incliudes multiple types of glia as well as neurons and different type of non-neuronal cells that are intrinsic to the CNS or that enter from the bloostream, such as leukocytes and platelets (Sofroniew, 2014).

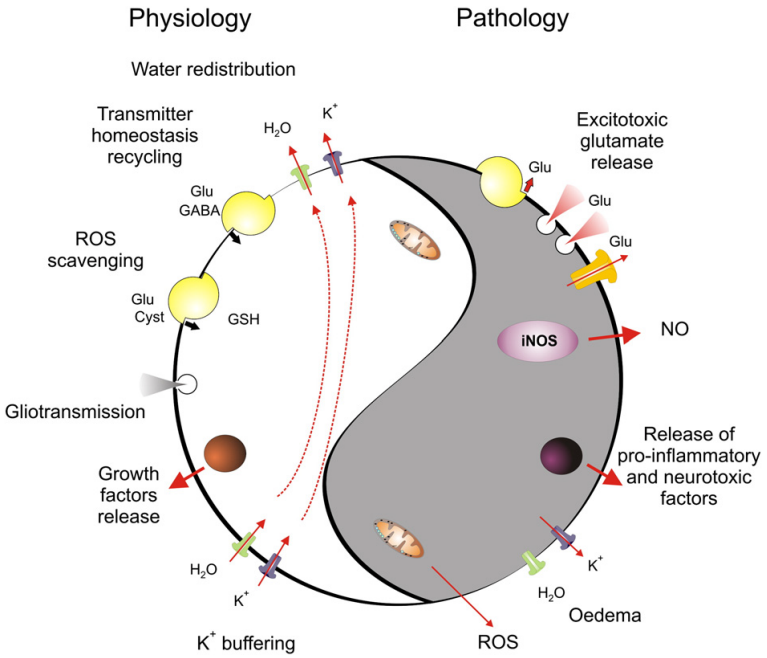


Fig. 11 Dual role of astroglial homeostatic cascades. The homeostatic cascades expressed in astrocytes control extracellular ion homeostasis through K+ buffering, regulate movements and distribution of water, control extracellular concentration of neurotransmitters and provide main reactive-oxygen species scavenging system. In pathological conditions, when astrocytes experience metabolic stress, the same systems may contribute to brain damage (Heneka et al., 2010).

It is important to underline that "activation" or "activated astrocytes" as terms that refer exclusively to astrocyte responses to injury or disease. Astrocytes in healthy tissue continually show physiological activation in the form of transient, ligand-evoked elevations of intracellular calcium that represent a type of astrocyte excitability, involved in mediating many critical dynamic astrocyte functions, including interactions with synapses and regulation of blood (Sofroniew, 2014).

Astrocytes are activated to display reactive astrogliosis around site of injury, a process that leads to excessive scar formation and interfere with the neuronal recovery processes. Reactive astrocytes would become hypertrophic and hyperplastic, concomitant with anomalous functions of astrocytes. One noticeable change in reactive astrocytes include an elevation of expression of glial fibrillary acidic protein (GFAP) which is somewhat proportional to the degree of reactivity. Severe diffuse astrogliosis leads to cellular hyperthophy and some loss of individual astrocytes domains with overlapping of neighboring

astrocyte processes. These changes can extend diffusely over substantive areas, and can occur in various situations, such as chronic neurodegenerative insults, psychiatric disorders, diffuse trauma, diffuse ischemia, or certain types of infection. Because there can be considerable tissue reorganization, the potential for resolution and return to normal structure is reduced and there is a high tendency toward long-lasting tissue reorganization (Sofroniew, 2014; Verkhratsky et al., 2015).

Astrogliosis can be induced, regulated, or modulated by a wide variety of extracellular molecules ranging from small molecules, such as purines, transmitters, and steroid hormones,

to large polypeptide growth factors, cytokines, serum proteins, or neurodegeneration associated molecules like β-amyloid (Sofroniew, 2014). Indeed, this event is particularly patent in AD brain and doctor Alzheimer himself was able to recognize, in autopsied specimens, a marked activation of astroglial cells and described a manifest inflammatory status (Alzheimer, 1907).

In the AD brain, it has been shown that Aβ could trigger astrocytes reactivation in which they are induced to release and/or overproduce many different factors including growth factors, cytokines and chemokines (Li et al., 2011). Data from both humans, treatment of cultured glial cells with aggregated β-amyloid and animal models of AD have demonstrated that reactive astrocytes are the main cellular component found to surround, co-localize and penetrate the senile plaques with their projections and processes replacing the dead or dying neuronal cells, suggesting that the primary aim of this peculiar aim of this localization is probably the creation of a barrier between healthy and injured tissue (Rodriguez et al., 2009; Steardo Jr. et al., 2015). But, in parallel, Aβ challenge provokes alterations of calcium homeostasis, energic modification, and degeneration of co-cultured neurons, along with an increased oxidative and nitrosative stress (Li et al., 2011; Heneka et al., 2010).

2.3.1 Inflammation in the AD brain and role of astroglia.

Biochemical and structural changes of astrocytes during neuroinflammation represent a physiological response to CNS injury to minimize and repair the initial damage. Positive effects of inflammatory processes may occur during the acute phase response (Steardo Jr. et al., 2015), as confirmed in different transgenic animal models where the overall impact of reactive astrogliosis is beneficial and neuroprotective, in particular during early stages of disease.

Nevertheless, sustained inflammatory responses might be driven by positive feedback loops between microglia and astrocytes under conditions of severe and/or prolonged brain insults, thus providing detrimental signals that can compromise astrocytic and neuronal functions and lead to chronic neuroinflammation (Colangelo et al., 2014).

It has been demonstrated that astrocytes, without the influence of other cell types, can be activated by many pathogenic factors to overproduce and respond to a broad array of proinflammatory cytokines and chemokines, such as interleukin-1α, (IL-1α), tumor necrosis factor α (TNFα), interferon γ (IFN-γ), S100β, which have been implicated in contributing to both the development of inflammation within the CNS and progression to disease, as well as resolution of disease (Fuller et al., 2010).

Cytokines and chemokines are secreted proteins with growth, differentiation, and activation functions that regulate and determine the nature of immune responses, and control immune cell trafficking and the cellular arrangement of immune organs. Cytokines are involved in virtually every facet of immunity and inflammation. Functionally, cytokines have been classified as being either pro-inflammatory or anti-inflammatory depending on the final balance of their effects on the immune system. Within the CNS cytokines may exert their effects directly, themselves being present in the brain, or indirectly, with secondary effects that are the result of cytokine action on other targets. Within the CNS, cytokines and their receptors are constitutively expressed in both normal and pathological states, but cytokine overexpression in the brain is an important factor in neurodegeneration.

Chemokines constitute a large family of small cytokines with 4 conserved cysteines linked by disulphide bonds. They are classified structurally into four branches, α-, β-, γ-, and γ-, based on variations in the shared cysteine motif. An impressive number of chemokines have been found in CNS cells, and there is growing evidence that some of them are upregulated in AD (Fuller et al., 2010).

One of the main functions of these chemokines relates to the recruitment of leukocytes to inflammatory sites. It is therefore especially important that the astrocytes in the blood brain barrier (BBB) start to release cytokines and chemokines; as these chemokines would attract leukocytes across the BBB to initiate neuroinflammation in the CNS. This makes astrocytes a very important contributor to the neuroinflammation in the early stage of AD pathogenesis. (Li et al., 2011).

Along with the neuroinflammatory progression, astrocytes remain activated and secret various immunoinflammatory factors. for some of them, studies on their interaction with Aβ and AD development have already been conducted (Li et a., 2011). Evidences have shown that Aβ could elevate the expression of cytokines and chemokines in astrocytes, which could in turn cause reactivation of astrocytes. These reactivated astrocytes could secret more cytokines and chemokines to upregulate the APP mRNA and protein levels in neuron, thereby leading to an enhanced Aβ generation from the APP on the neuronal membrane surface .

Furthermore, under the stimulation of various cytokines, neurons and astrocytes could be involved in the production of Aβ. Neuron is the predominant source of Aβ production in the brain due to the specific localization of APP to the neuronal membrane surface. However, astrocytes could also be induced by proinflammatory stimuli to produce Aβ. It has been demonstrated that IFN-γ in combination with TNFα or IL-1β could induce primary human astrocytes cells to produce Aβ. The astrocyte-secreted proinflammatory factors could promote the expression level of secretases, thereby enhancing the conversion of APP on the membrane of neurons into neurotoxic insoluble fibrillary Aβ. Exogenous Aβ could induce astrocytes to produce more inflammation-like glial responses that could sustain neurodegenerative injuries, including progressive neuronal loss, enhanced astrogliosis, amyloid plaques formation, NFT formation, dystrophic neurite growth, and excessive tau protein phosphorylation. The neurotoxic Aβ could also activate microglia to produce TNFα and ROS, which in turn stimulate astrocytes and microglia to release more inflammatory mediators (Li et al., 2011). These consequences can initiate a chain reaction that leads to further neuronal injury, amyloid deposition and to subsequent amyloid plaque formation. The process of neurodegeneration is therefore closely related to the shift in cytokine balance toward the side of pro-inflammatory cytokines (Fuller et al., 2010).

In particular, for some factors secreted by reactive astrocytes, the interaction with Aβ and AD development have already been documented.

- S100B, for example, is a neurotrophic factor and neuronal survival protein that stimulates neurite outgrowth, modulates long term synaptic plasticity, and promotes neuronal survival and development. Under normal conditions, it is only expressed by NG2 cells but, during injury, S100B could also be upregulated in astrocytes; thus, it could serve as a biomarkers for astrocyte injury in addition to GFAP. S100B secreted by astrocytes has been

shown to promote the synthesis of APP mRNA and APP in neurons, which could serve as a source for additional accumulation of Aβ.

2.3.2 Astroglia as source of ROS and RNS.

As mentioned before, free radicals are potent deleterious agents, causing cell death or other forms of irreversible damage. Astrocytes, as well as microglia, are capable of generating ROS and can significantly increase ROS production in response to injury. Such ROS production may contribute to ROS-induced neuron death, either directly via oxidative damage to neuronal membranes, DNA, or organelles; or alternatively, glial-derived oxidative stress may contribute to neuron death indirectly by increasing astrocyte/ microglia activation and cytokine production. Activated astrocytes release potentially neurotoxic products such as pro-inflammatory cytokines, nitric oxide (NO), as well as the inducible form of nitirc oxide synthetase (iNOS), and ROS. Production of toxic free radicals can lead to the accumulation of reactive oxygen species (ROS) which can result in damage to major components of cells, such as the nucleus, mitochondrial DNA, membranes, and cytoplasmic proteins. (Fuller et al., 2010).

Oxidative damage can activate or inactivate proteins, while oxidative lipid damage produces a progressive loss of membrane fluidity, reduces membrane potential, and increases permeability to ions such as Ca^{2+}. Another consequence of free radical production in the AD brain is that oxidative stress may contribute to aggregation of soluble Aβ into insoluble plaques, leading to amyloid plaque formation. Cross-linking of preformed aggregates of amyloid precursor protein (APP) occurs via free radical attack, leading to formation of the Aβ peptide seen in AD. After generation of a core cross-linked fragment further APP fragments appear to extend the deposition of the amyloid plaque. Also observed as a result of ROS production and oxidative stress is the polymerization of tau, and an increase in advanced glycation end products.

Astroglia have been implicated also in NO-mediated neurotoxicity, and upregulation of iNOS in astroglia in AD has been observed in affected brain regions. In particular, NO-dependent neuronal loss of ATP, leading to neuronal energy depletion, may be a mechanism for NO-mediated neuronal toxicity. Other mechanisms that may be responsible for NO-mediated neurotoxicity include DNA damage and lipid peroxidation, as well as the possibility that NO may liberate iron from ferritin, which favours subsequent hydroxyl radical formation

and oxidative stress. This suggests expression of iNOS, induced by the increase in cytokine expression in the CNS that is seen in AD, by glial cells is neurotoxic and possibly associated with neurodegenerative events. NO can also damage cells by reacting with protein thiol groups and protein-iron complexes. Increased levels of nitrated proteins have been reported in AD brain and cerebrospinal fluid (CSF) demonstrating the potential involvement of RNS in neurodegeneration. The most commonly nitrated amino acid is tyrosine, and numerous proteins are inactivated by nitration of tyrosines, including glutamine synthase. This indicates that the widespread nitration of tyrosine seen in AD brain tissue anr the resulting decreased activity of nitrated proteins may contribute to the neurodegeneration characteristic of AD.

Another mechanism through which NO mediates neurotoxicity is through the activation of inducible cyclooxygenase. NO has been shown to activate both the constitutive and the inducible isoforms of cyclooxygenase, which are upregulated in brain cells under pro-inflammatory conditions. During the catalytic cycle of cyclooxygenase, the release of free radicals and the formation of prostaglandins occur, two events that are closely related to the development of neuroinflammation. Inducible cyclooxygenase is upregulated in the brain of patients affected by Alzheimer's disease, and therefore, the activation of inducible cyclooxygenase can be considered as an indirect way for NO to exert neurotoxicity (Fuller et al., 2010).

2.3.3 Astroglia as source of complements and prostaglandins.

Prostaglandins are long chain, unsaturated fatty acids produced from arachidonic acid by the action of the enzyme cyclooxygenase. They are classified into groups (A, E, F and B) according to their structure. Prostaglandins can cause a variety of effects including increased vascular permeability, neutrophil chemotaxis, smooth muscle contraction and pain. Prostaglandins are also proposed to be released from activated astrocytes, in particular PGE_2. It has been proposed that reactive astrocytes, located around plaques of AD brains, increase secretion of PGE_2 by the elevated expression of PGE synthase, responsible for the isomerisation of PGH_2 into PGE_2, and the evoked activity of COX-2, the enzyme that converts arachidonic acid to PGH_2. This highlights PGE2 as a critical factor in the exacerbation of neurodegeneration in AD.

AD brains show evidence of the deposition of activated complement proteins, indicating that the complement system is involved in the pathogenesis of AD. Senile plaques and NFTs

both show the presence of complement components. As a consequence of complement activation, production of pro-inflammatory peptide fragments and the formation of the cytolytic membrane attack complex occurs. The membrane attack complex is able to insert into and permeabilize the membrane of neuronal precursor cells. Overall, activation of the complement system exacerbates the inflammatory response.

Furthermore, PGE_2 has been shown to upregulate expression of the gene for amyloid precursor protein in neurons. Amyloid precursor protein gives rise to the Aβ peptide, which is directly toxic to neurons and activated astrocytes in the insoluble aggregated form that is found in the senile plaques observed in AD (Fuller et al., 2010).

On the basis of all these considerations on astrocytic dysfunction, it is reasonable to assume that alterations in some of these important neuro-supportive roles can results in injurious consequences for the brain and that astrocyte pathology is important in AD progression. Thus, the hypothesis of an early combination of neuroprotective and anti-inflammatory treatments, aimed at restoring astrocyte functions, may represent might be an appealing way to promote neuroprotection not only in AD but also in a variety of brain disorders (Steardo Jr. et al., 2015; Colangelo et al., 2014).

3. THE ENDOCANNABINOID SYSTEM.

Endocannabinoids (eCBs) are highly lipophilic molecules of the eicosanoid family which are synthesized from lipid membrane precursors and have been shown to be neuromodulators and immunomodulators. These are elements of the eCB system that also includes several enzymes required for their synthesis and metabolism and the cannabinoid (CB) receptors that serve as their molecular targets (Fagan and Campbell, 2014).

Most of the knowledge acquired about cannabinoid receptor pharmacology was made possible by the study of the mechanisms of action of numerous natural, but also synthetic, cannabinoid compounds. Among the natural cannabinoids, the most well-known are Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive compound of the *Cannabis sativa* plant, and cannabidiol (CBD). The characterization of CB_1 and CB_2 receptors permitted the discovered of endocannabinoids or cannabinoids produced and released by nerve cells (Aso and Ferrer, 2014; Pazos et al., 2004; Campbell and Gowran, 2007). The first identified eCB was anandamide (arachidonoylethanolamine; AEA), which is the derivative of ethanolamine and arachidonic acid, the second eCB was 2-arachidonoylglycerol (2-AG), an ester derivative of arachidonic acid and glycerol; then, several others have also been identified, such as 2-arachidonylglyceryl ether (2-AGE), virodhamine, and *N*-arachidonyldopamine.

In addition to the ECs, these classes encompass a number of ECLs (EC-like ligands), which include OEA (Noleoylethanolamide) and PEA (N-palmitoylethanolamide). Since these are substrates for the same catabolic enzymes as the ECs, ECLs are thought to preserve and facilitate the activity of AEA and 2-AG, as well as possessing important biological functions of their own (Maroof et al., 2013).

Endocannabinoids act as neurotransmitters since they are synthesized in the post-synaptic terminals and released by neurons, are able to bind and activate membrane receptors, and are inactivated by reuptake and enzymatic degradation within the cell. However, endocannabinoids have two fundamental characteristics that differentiate them from other neurotransmitters: a) they act as retrograde messengers through the synaptic cleft to activate CB receptors located pre-synaptically; b) they do not accumulate in the interior of synaptic vesicles because they are synthesized and immediately released “on demand”.

They regulate important brain functions including cognition and memory, emotion, motor control, feeding, and pain perception (Wilson and Nicoll, 2002; Fagan and Campbell, 2014).

To date, two subtypes of cannabinoid Gi/o-coupled receptors, CB_1 and CB_2, have been fully characterized and cloned. Their activation through binding of ECs or synthetic agonists, results in the dissociation of the Gα subunit from Gβγ and the consequent stimulation of MAPK pathway and inhibition of adenylate ciclase, attenuating the conversion of ATP to cyclic AMP. CB receptor activation is also tighly linked to ion channel regulation through inhibition of voltage-dependent Ca^{2+} channels and activation of K^+ channels (Maroof et al., 2013; Fagan and Campbell, 2014). However, cannabinoid compounds may also bind to other receptors, such as the G-protein coupled receptor GPR55, the peroxisome proliferator-activated receptors PPARα and PPARγ, and transient receptor potential vannilloid-type 1 (TRPV1) channels. The activation of TRPV1 by the endocannabinoid AEA has been linked to its anti-inflammatory actions (Bedse et al., 2015; Aso and Ferrer, 2014).

CB_1 receptors are the most abundant G protein-coupled receptors in the CNS widely expressed throughout the brain, predominantly in cerebellum, cortex, hippocampus, and basal ganglia, in both neurons and glial cells (Wilson and Nicoll, 2002). They are mostly found on axon terminals of a variety of neuronal populations in the central and peripheral nervous system where act as modulators of excitatory and inhibitory neurotransmission, as well as psychoactivity. As a consequence, ECs are intimately associated with the phenomena of LTP (long-term potentiation) and LTD (long-term depression), two alternative forms of synaptic plasticity underlying learning and memory (Maroof et al., 2013; Fagan and Campbell, 2014).

Moreover, CB_1 receptors are also found in peripheral tissues, playing an important role in energy balance and metabolism (Aso and Ferrer, 2014).

CB_1 activation results in inhibition of adenylate cyclase activity and calcium influx into the axon terminal; thus, CB_1 receptor signaling functions to suppress neurotransmitter release into the synapse.

CB_2 receptors are widely distributed in cells and tissues of immune system and modulate the immune cell migration and the release of cytokines within the CNS and its expression occurs at various stages of inflammation. This expression of CB_2 receptors is mainly located in microglia and relatively low CB_2 receptor expression has also recently been identified in some neurons, in particular, after various insults. Interestingly, neuronal damage can increase the production of endocannabinoids, which may provide a defense mechanism against toxicity (Aso and Ferrer, 2014). The CB_2 exerts its effects through initiation of

phospholipase C (PLC) and inositol 1, 4, 5-triphosphate (IP3) signaling pathways that results in increased levels of intracellular calcium.

AEA is rapidly synthesized by neurons in response to depolarization and consequent Ca^{2+} influx, which leads to the cleavage of its membrane precursor N-arachidonyl phosphatidylethanolamine, by phospholipase D (Fagan and Campbell, 2014).

AEA acts as a partial agonist for CB_1 receptors; 2-AG synthesis, instead, derives from the hydrolysis of phosphatidylinositol- 4,5-bisphosphate (PIP_2) and is mediated by the generation of diacylglycerol (DAG), via the actions of either phospholipase C (PLC) or phospholipase D (PLD). DAG is subsequently converted to 2-AG by DAG lipase. 2.AG behaves as a full agonist for CB_1 and CB_2 receptors.

eCBs are produced by a variety of cell types including endothelial cells, adipocytes, glial cells, and macrophages. 2-AG is more abundant than AEA in the brain and its concentration is about 200 times that of AEA.

After their actions are rapidly eliminated by cellular re-uptake and enzymatic hydrolysis: AEA is metabolized by the fatty acid amide hydrolase (FAAH) expressed mostly by postsynaptic neurons. FAAH metabolizes also other N-acyl ethanolamines, like palmitoylethanolamide (PEA) and oleoylethanolamide, whereas, 2-AG is mainly metabolized by monoacylglycerol lipase (MAGL) in presynaptic neurons. At lesser extent 2-AG is also metabolized by FAAH, serine hydrolase α/β hydrolase 6 (ABDH6), serine hydrolase α/β hydrolase 12 (ABDH12), and cyclooxygenase-2 (COX-2) (Bedse et al., 2015; Pazos et al., 2004).

The understanding of the eCB system is constantly evolving as new discoveries are progressing. The last two decades of research have brought a conspicuous improvement in knowledge of the endocannabinoid system components and functions under physiological and pathological conditions. Among these, pain modulation, motor alterations, neuroinflammation and neuroprotection are some of the most promising lines of research (Pazos et al., 2004).

Therefore, the cannabinoid system appear to be a promising therapeutic target as it has the ability to modulate a range of aspects of AD pathology (Karl et al., 2012).

3.1 The endocannabinoid system and AD.

Multiple data are available showing that the eCB system is implicated in AD progression. Endocannabinoid signaling has been demonstrated to modulate the main

pathological processes occurring during the silent period, including protein misfolding, neuroinflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress (Aso and Ferrer, 2014). Firstly, cortex and hippocampus, key structures for learning and memory functions, are the two brain regions that are affected by AD pathology and they express high levels of CB_1 receptots as well as other components of the eCBs system. Evidence suggests that microglia and astrocytes also express the enzymes involved in the synthesis and degradation of the eCBs and that the activation of cannabinoid receptors expressed by activated microglia controls immune-related function. Moreover, eCBs are known to exert anti-inflammatory, antioxidant and neuroprotective effects.

The modifications described for CB_1 receptors in AD are ambiguous. Whereas some authors have reported a significant reduction in the CB_1 levels in cortical are and in neurons distant from senile plaques, others have described no changes in the expression, distribution, or availability of CB_1 receptors in cortex and hippocampus in AD or have failed to dissociate CB_1 receptor expression changes from normal aging. No correlation between CB_1 levels and any AD molecular marker, Aβ deposition or cognitive status has been found. Furthermore, CB_1 receptor selective radioligand study revealed that CB_1 receptor density increases in early AD and decreases during later stages. In line with these results a decrease in CB_1 protein expression in transgenic mice models of AD have been reported. The increased CB_1 receptor activity during the initial stages of AD might indicate neuroprotective action medicated by eCBs in response to initial neuronal damage. This reduced expression in mice was also associated with astroglial proliferation and elevated expression of cytokines, iNOS and TNFα.

In contrast, there is no controversy regarding the significant increase of CB_2 levels in AD brains, mainly corresponding to receptors expressed on microglia surrounding senile plaques. Also FAAH, the endocannabinoid metabolizing enzyme, is increased in neuritic plaques.

Interestingly, expression levels of CB_2 receptors and hydrolyytic activity of FAAH correlate with $A\beta_{42}$ levels and plaque deposition, although not with cognitive status. Increased FAAH activity may contribute to inflammatory processes by increasing arachidonic acid, through increased AEA metabolism in astrocytes cells surrounding plaques. This pathway may be involved in increasing the production of prostaglandins and related pro-inflammatory

molecules and reactive gliosis that are pertinent to the the inflammatory process of AD (Bedse et al., 2015; Aso and Ferrer, 2014).

Recently, Aβ has been demonstrated to induce hippocampal degeneration, gliosis and cognitive decline, with a concomitant increase in the production of the endocannabinoid, 2-arachidonoyl glycerol, and this may reflect an attempt of the endocannabinoid system to provide neuroprotection from Aβ-induced damage. So robust and early pharmacological enhancement of brain endocannabinoid levels may protect against the deleterious consequences of Aβ. However, the timing of endocannabinoid upregulation by pharmacological intervention in relation to the time-course of development of the disease pathology is crucial, since an increase in eCB levels later in the pathological cascade actually worsens memory retention in rodents and activation of hippocampal CB_1 is negatively associated with the performance of rodents in memory tasks, possibly via a reduction in hippocampal Ach levels (Campbell and Gowran, 2007).

3.2 Beneficial effects of cannabinoids in treatment of AD.

Increasing evidence suggests that the eCBs system could be a potential target for the treatment of AD. Cannabinoids could exert neuroprotective, antioxidant, anti-apoptosis and anti-inflammatory effects. These play a neuroprotective role, through the CB-receptor activation, by preventing excitotoxicity, calcium efflux and inflammation as well as by modulating other signaling pathways (Aso and Ferrer, 2014).

Most of the initial reports on the effects of cannabinoids in AD were investigated in *in-vitro* models of Aβ-induced neuronal toxicity. Later, these investigations were extended to animal models of Aβ-induced toxicity and to transgenic murine models. It has emerged that cells lacking CB_1 receptors are more vulnerable to damage and that neural cannabinoid tone influences neuronal survival, suggesting that augmentation of the endocannabinoid system may offer protection against the deleterious consequences of pathogenic molecules such as Aβ (Campbell and Gowran, 2007).

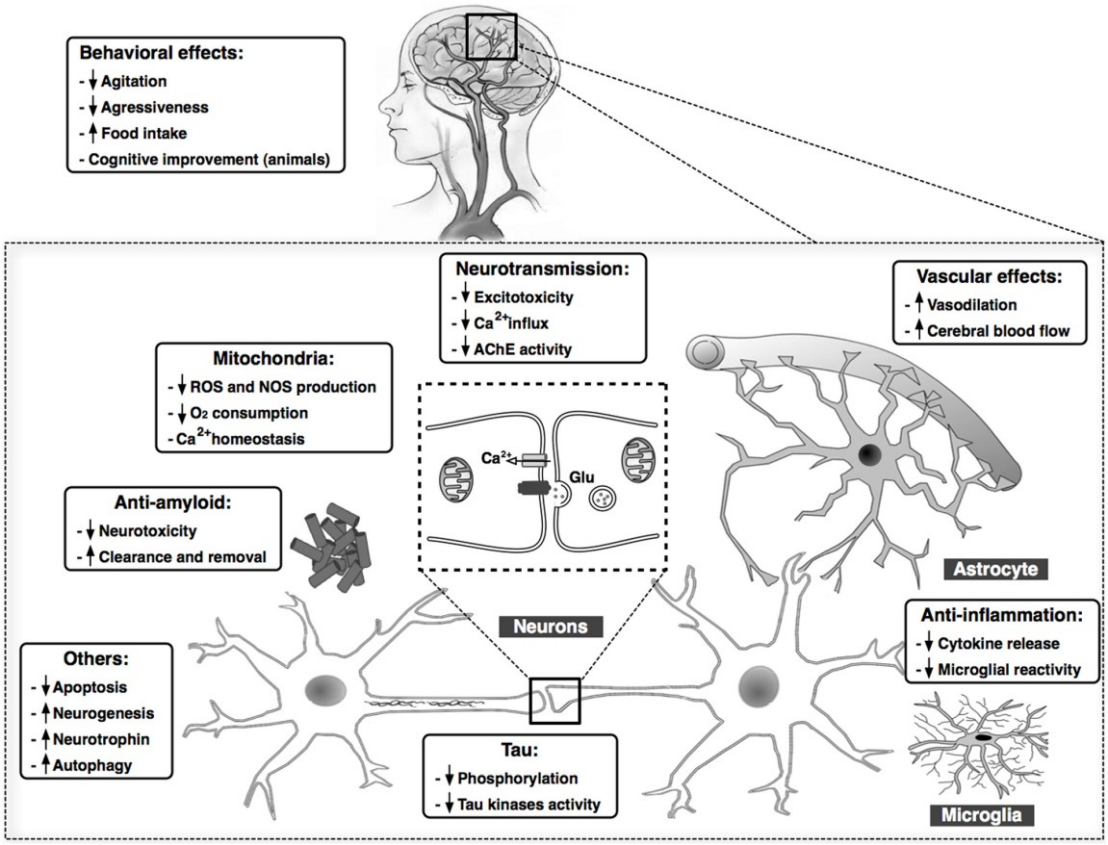


Fig. 12 Summary of the main findings demonstrating beneficial effects of cannabinoid compounds in AD models (Aso and Ferrer, 2014).

3.2.1 Aβ generation and clearance.

Microglia plays an important role in phagocytosis of Aβ, and there is an inverse relationship between cytokine production and Aβ clearance. CB_2 activation is known to reduce microglia activity and inflammatory cytokines productions. So it can be hypothesized that CB_2 agonist could lower Aβ plaques load by increasing Aβ clearance. In line with this hypothesis, it has been shown that *in vitro* activation of CB_2 receptor facilitates the removal of native Aβ from human frozen tissue sections and its agonist was able to induce a prompt Aβ clearance in Aβ-induced animal model of AD. CB_2 mediated decrease in Aβ plaque load is not clear but it might be link to a lower production of inflammatory cytokines and increase of Aβ phagocytosis that may decrease Aβ plaque load. The exact role of CB_1 receptor is not yet clear in same contest but a beneficial role has been proposed. Firstly, Δ^9-THC significantly reduced fibril and aggregate formation, secondly, CB receptor agonist or pharmacological

elevation of eCBs significantly enhanced Aβ clearance from the brain, by increasing the expression of Aβ transport protein, lipoprotein receptor protein 1 (LRP1) (Bedse et al., 2015).

3.2.2 Tau hyperphosphorylation.

Abnormal hyperphosphorylation of tau prompts an accumulation of NFTs in axons of neurons, can impair normal axonal transport, disrupt synaptic plasticity, and finally induce cell loss. But the link connecting Aβ plaques and tau pathologies has remained elusive (Aso and Ferrer, 2014).

Evidence suggests that abnormal activation of kinases like glycogen synthase kinase-3β (GSK-3β), MAPK family members as well as caspases may be responsible for hyperphosphorylation of tau, and Aβ might be involved in the activation of these enzymes. Along with various kinases, NO secreted from astrocytes induces tau hyperphosphorylation in neurons. It has been shown that arachidonoyl-2´-chloroethylamide (ACEA), a selective CB_1 agonist, down regulates iNOS protein expression and NO production in astrocytes, and that leads to a significant inhibition of NO-dependent tau hyperphosphorylation in neurons. It has been also demonstrated that cannabidiol inhibits hyperphosphorylation of tau protein in Aβ-stimulated neuronal cells (Bedse et al., 2015).

3.2.3 Neuroinflammation.

Besides plaques and NFTs, neuroinflammation plays a major role in neurodegeneration and activation of various apoptosis pathways.

Aβ is a proinflammatory molecule, which can induce its own production by increasing the expression of its synthesizing enzymes, such as β-secretase, and through various inflammatory pathways. In particular, it has been recognized that Aβ is able to initiate an inflammatory response, which in turn activates microglia and recruits astrocytes, and therefore the release of inflammatory mediators (IL-1β, TNF-α, and IL-6), reactive oxygen species, and neurotoxic products that have been involved in neuronal and synaptic damage. Neuroprotective effects of eCBs against brain injury and inflammation is associated with reduction of cytokines, ROS, and prostaglandins. eCB modulators can reduce

neuroinflammation in AD by inhibiting glial cell activation and generation of proinflammatory precursor molecules (Bedse et al., 2015; Bisogno and Di Marzo, 2008).

3.2.4 Regulation of glial cell activity.

As mentioned above, CB_2 and FAAH are upregulated in microglia and astrocytes, respectively, in surrounding areas of neuritic plaques and in AD brains (Aso and Ferrer, 2014; Benito et al., 2003). This notion suggests that both microglia and astrocytes play an important role in eCB signaling in AD pathology. CB_2 receptor upregulation in AD seems to be a defensive mechanism at various levels. In particular, CB_2 activation could a) suppress activation of microglia, b) reduce production of inflammation mediators, c) enhance microglial proliferation and its phagocytic activity.

The effects of eCBs on inflammation were demonstrated in different ways. As expected, Aβ peptide activated microglial cells with increased mitochondrial activity, TNF-α release, and cellular morphological changes. Cannabinoid treatment prevented the enhancement of TNF-α release and counteracted Aβ-mediated activation of microglia. Cannabidiol dose dependently reduced Aβ-induced neuroinflammation by suppressing microglial activation, IL-1β and iNOS expression. WIN55,212-2 also inhibited production of chemokines and TNF-α. Both selective CB_1 and CB_2 antagonists partially blocked these effects suggesting the involvement of both receptors (Bisogno and Di Marzo, 2008).

Also different N-acylethanolamides (AEA, PEA, and OEA) were able to exert anti-inflammatory effects in Aβ activated murine astrocytes. Previous studies have shown that N-acylethanolamines activate antiinflammatory nuclease receptor PPAR-α that causes formation of a multiprotein complex along with variable set of protein co-activators. With this multiprotein complex, PPAR-α binds to responsive elements on DNA and enhances the transcription of various anti-inflammatory proteins, that suppress the gene expression of proinflammatory components, such as cytokines (TNF-α, IL-1β) including iNOS and COX-2 (Bedse et al., 2015).

3.2.5 Neurodegeneration.

Aβ has been shown induce cell apoptosis in neuronal cells through a variety of mechanisms that include activation of caspase-3, lysosomal cathepsins and lysosomal membrane permeabilization. Canabinoids at physiological concentrations increase lysosomal stability and integrity and they can also stabilize lysosome against Aβ permeabilization and increase cell survival. eCBs prevented upregulation of tumor suppressor protein p53, and reduced its interaction with lysosomal membrane. Moreover, AEA and 2-AG prevented Aβ-induced increase in DNA fragmentation and caspase-3 activation. Acute *in vivo* administration of Aβ increases 2-AG release in the brain suggesting that endogenous 2-AG plays an important role against in protecting neurons against Aβ-induced toxicity. These effects were mediated by CB_1 and MAPK pathway activation as suggested by the finding that CB_1 antagonist and MAPK inhibitor blocked their neuroprotective effects (Bedse et al., 2015).

3.2.6 Oxidative stress and mitochondrial dysfunction.

Enhanced oxidative stress in brain is generally correlated with cognitive decline and with enhanced risk for development of neurodegenerative diseases. Among the different pro-inflammatory proteins produced in response to Aβ-induced oxidative stress, iNOS and its enzymatic product NO are considered the most important neurotoxic effectors during AD.

NFκB, a redox-sensitive transcription factor that is activated by a family of stress activated kinases (SAPK) including p38 MAP kinase, regulates the expression of different genes involved in cell differentiation, proliferation, and apoptosis, as well as in oxidative, inflammatory, and immune response. As it is well known, NFκB activation is of primarily importance to induce iNOS protein transcription both in Aβ-stimulated neuronal cells and in postmortem AD brains. It is well known that phytocannabinoids have anti-oxidant properties. Cannabidiol, for example, significantly decreases glutamate toxicity, Ca^{2+} -toxicity, iNOs expression and NO production (Aso and Ferrer, 2014; Bisogno and Di Marzo, 2008).

CB_1 receptors are also expressed on mitochondria and regulate its activity. Their activation can decrease oxidative metabolism and, oxygen consumption, ROS production, oxidative phosphorylation. In oxidative stress conditions, cannabinoids have shown protective

actions against mitochondrial damage and have decreased Ca^{2+}-induced cytochrome c release from mitochondria (Bedse et al., 2015).

3.2.7 Addictional effects of endocannabinoids.

Apart from aforementioned mechanisms, few cannabinoids exert their therapeutic effects in similar way of AchE- inhibitors, currently US-FDA approved drugs for AD treatment.

Active component of marijuana, Δ^9-THC, has been demonstrated to competitively inhibit AChE and to thus increase Ach levels. Moreover, Δ^9-THC prevented AchE-induced aggregation of Aβ which can reduce plaques formation. In addition to Δ^9-THC, other CB agonists also showed to have AChE and butyrylcholinesterase inhibition properties. Alternative strategies based on multiple targets such as CB receptors and cholinesterase with single compound is gaining acceptance for treatment of AD.

Besides AChE inhibitors, as above mentioned, current AD treatment includes memantine, a NMDA receptor antagonist,which reduces excitotoxicity by inhibiting Ca^{2+} influx.

In similar way, HU-211 (synthetic cannabinoid devoid of CB_1 and CB_2 agonist activity) protects neurons from excitotoxicity by antagonizing NMDA receptors.

Furthermore, cannabinoids could provide beneficial effects by modulating cerebral blood flow functions. AD is characterized by a decreased regional cerebral blood flow that could result in decrease brain supply of oxygen, glucose, and nutrients. Cannabinoids can improve blood flow to the brain as CB_1 receptor activation can elicit vasodilatation.

CB_2 receptor activation has been shown to improve blood-brain barrier integrity by decreasing adhesion of leukocytes to endothelial cells under inflammatory conditions, which may reduce further exaggeration of inflammation.

However, besides beneficial effects, cannabinoids (especially at high doses) may exert unwanted cannabimimetic and psychiatric side effects such as hypolocomotion, hypothermia, aversion, and anxietyrelated behaviors. Moreover, CB_1 receptor activation may precipitate episodes of psychosis and panic while its inhibition may lead to depression and anxiety-related disorders (Aso and Ferrer. 2014).

4. PEA.

4.1 Birth of PEA.

The story of Palmitoylethanolamide or palmidrol (PEA) started in 1957 with the discovery that the anti-allergic and anti-inflammatory activities exerted by dietary supplementation with soybean, egg yolk, peanut oil and soybean lecithin was due to a specific lipid fraction corresponding to PEA (Fig. 13) (LoVerme et al., 2005(b)).

In 1965, PEA was identified in brain, liver and skeletal muscle from rat and guinea pig (Hansen, 2013). Since then, the presence of PEA has been found in the mouse brain and spinal cord, canine heart extracts, degenerating tissue, testis, paw skin and in peritoneal macrophages (Lambert et al., 2002). During the 1970s in Eastern Europe, PEA was marketed for some time under the brand name of Impulsin^{TM} by the Czech pharmaceutical company SPOFA. for the prevention of virus infection of respiratory tract in school children and soldiers (LoVerme et al., 2005(b)).

More recently, PEA has been emerging as an important analgesic, anti-inflammatory and neuroprotective, antinociceptive mediator, acting at several molecular targets in both central and sensory nervous system as well as immune cells. (Petrosino et al., 2010; LoVerme et al., 2005; Costa et al., 2002). Today PEA is not only a food component but also a nutraceutical use in clinics in Italy and Spain since 2007 under the brand name Normast, a dietary component for special medical purposes, advised for pathological conditions that are sustained by mast cells-mediated tissue hyperactivity. PEA is currently used to restore skin reactivity in animals in a veterinary composition, marketed under the trade name RedonylR by the Italian company, Innovet (LoVerme et al., 2005(b)). Finally, PEA is also an industrial compound widely used as an additive in cosmetics, shampoos, and detergent liquids, as well as a fabric softener, and corrosion inhibitor (Hesselink, 2013).

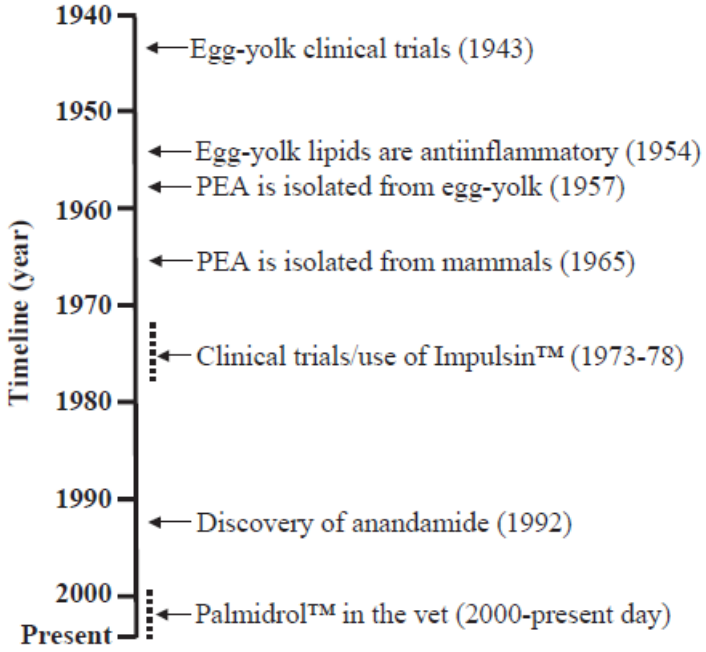


Fig. 13 PEA: a historical perspective.

4.2 PEA "life cycle".

Palmitoylethanolamide (PEA) is an endogenous lipid compound belonging to the class of fatty acids amides (Balvers et al., 2013). Fatty acid amides in general are conjugates of fatty acids with ethanolamine, amino acids, or mono-amine neurotransmitters and are widely abundant in nature. The N-acylethanolamines (NAEs) (or acylethanolamides, AEs), subclass, to which PEA belongs, also includes the first endocannabinoid to be discovered, anandamide (AEA, N-arachidonyl-ethanolamine) and the anorectic mediator N-oleoyl-ethanolamine (OEA) (Balvers et al., 2013; Mattace Raso).

Chemically, PEA (N-(2-hydroxyethyl)hexadecanamide) is an amide between palmitic acid and ethanolamine (Fig. 14).

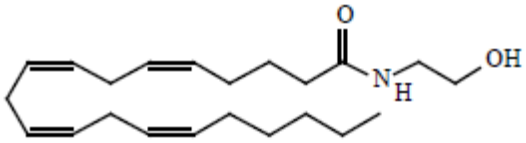


Fig. 14 Chemical stucture of PEA.

PEA is synthesized through complex metabolic pathways which ultimately use palmitic acid (PA; C16:0) as substrate, that is one of the most abundant satured fatty acids in our body and present in the human diet in considerable amounts (Balvers et al., 2013; Lambert et al., 2002).

Synthesis and degradation of PEA occur in various cell types, including those relevant for chronic pain and inflammation signaling, such as immune cells, neurons and microglia.

PEA is not stored in vescicles, but it is produced "on demand" between the lipid bilayer, mainly from its precursors, N-acyl-phosphatidiyl-ethanolamines (NAPEs), by the biosynthetic enzyme NAPE-selective phospholipase D (NAPE-PLD). However, other possible pathways are reported for the conversion of NAPEs into the corresponding NAEs, including: 1) a secretory phospholipase 2 (sPLA2) that hydrolyzes NAPEs into N-acyl-lyso-phosphatidyl-ethanolamines (lyso-NAPE),which are then hydrolyzed to NAEs by a lysophospholipase D; 2) an alpha/beta-hydrolase 4 (Abh4) that acts as a lysophospholipase/phospholipase B for the formation of glycerol-phospho-NAEs, which are then converted to NAEs by the glycerophosphodiester phosphodiesterase GDE1; and 3) a PLC-dependent pathway for NAPE conversion to phospho-NAEs, followed by formation of NAEs via the protein tyrosine phosphatase N22 (PTPN22) (Esposito and Cuzzocrea, 2013; Petrosino et al., 2010; Hansen, 2013).

In vivo, the action of PEA and anandamide are relatively short-lived, due to their rapid metabolism. PEA degradation is catalized by the degradative fatty acid amide hydrolase (FAAH), or in case of inflammatory status by N-acylethanolamine-hydrolyzing acid amidase (NAAA) that is highly expressed in macrophages and the lungs, as well as in various rat tissues including the brain (Lambert et al., 2002; Petrosino et al., 2010). FAAH has been molecularly cloned and extensively characterized and selective inhibitors that block its activity in vivo have been developed. This serine hydrolase is present in all mammalian tissues, but is particularly abundant in brain and liver (LoVerme et al., 2005(b)). FAAH is known to be located in the endoplasmic reticulum, while NAAA is located inside the lysosomes. Furthermore, the intracellular integral membrane protein of 597 amino acids FAAH is responsible for the hydrolysis of both PEA and AEA but the uptake processes for the two fatty acid amides are different. Anandamide is taken up into cells predominantly by an energy-independent mechanism of facilitated transport whereas at least 50% of cellular

PEA uptake is brought about by passive diffusion, because with its high lipophilicity, PEA can easily flip between the phospholipid bilayer, and the other part is taken up by cells through a facilitated transport system that is apparently inhibited by AEA and 2-AG (Esposito and Cuzzocrea, 2013; Hansen et al., 2013).

4.3 Endogenous levels of PEA.

PEA exists in all cells, tissue and body fluids, with a widespread occurrence both in plants and animals. The levels of endogenous PEA in normal muscle and adipose tissues (0.04 - 6.00 pmol/mg tissue) are similar to those observed in the central nervous system (CNS) and somewhat higher compared to the gastrointestinal tract (0.05 - 1.50 pmol/mg lipids), skin (0.3 - 0.69 pmol/mg of lipid extract) and eye (0.10 – 0.64 pmol/mg). PEA is abundant in the CNS and it is conspicuously produced by glial cells. In blood, the physiological levels of PEA, which is likely bound to albumin, range from 4 to 45 pmol/lipid mg (Esposito and Cuzzocrea, 2013; Esposito and Cuzzocrea, 2013(b); Hansen et al., 2013).

The physiological stimuli that regulate PEA levels in mammalian tissues are largely unknown; however, multiple studies indicate that this lipid accumulates during cellular stress, particularly following tissue injury (LoVerme et al., 2005(b)). For example, changes in PEA levels have been demonstrated in osteoarthritis, atopic dermatitis, gut inflammation, eye degenerative diseases, and in response to ultra-violet-B irradiation in mouse epidermal cells (Esposito and Cuzzocrea, 2013).

When inflammation and degeneration was induced by the injection of cadmium chloride, the total amount of NAEs in rat testis increased up to 25-fold with a preponderance of PEA. Cell death, as in post-mortem tissues, or, more simply, cell damage, as during ischemic conditions or in glutamate-induced neurotoxicity, also caused a several-fold increase of PEA and NAE levels (Lambert et al., 2002). The levels of PEA in the cerebral cortex was also dramatically increased in a model of focal cerebral ischemia, while levels of AEA and 2-AG had only minor increase or remain unchanged , respectively. Interestingly, PEA levels behaved the same in rats with acute stroke and in a human patient with left-side hemispheric infarction. All these findings greatly substantiate the hypothesis that PEA formation may serve a cytoprotective role in relation to neuronal injury, maybe by facilitating the production

of apoptosis in injured cells or neighboring cells, thus inhibiting the spread of a necrotic process (Esposito and Cuzzocrea, 2013).

4.4 Mechanims of action.

Despite its potential clinical significance, the cellular mechanism responsible for the actions of PEA has long remained unidentified, and a great deal of controversy has surrounded its identity. Evidence indicates that PEA is an important anti-inflammatory, analgesic, and neuroprotective mediator acting at several molecular targets in both central and peripheral nervous systems.

The presence of acylethanolamine and their cognate precursors in various tissues and their pharmacological properties suggested that these molecules are not only autocrine and paracrine regulators for the reduction of inflammation, but play also a role as regulators of peripheral functions, therefore AEs were initially called Autacoid Local Injury Antagonism Amides or ALIAmides. Indeed, PEA accumulates in tissues following injury and exerts a local, autacoid, anti-injury function via down-modulating mast cells and protecting neurons agaist excitotoxicity, thus reducing tissue inflammation, decreasing hyperalgesia, neuronal damage and exerting a neuroprotective function (Esposito and Cuzzocrea, 2013; Mattace Raso et al., 2014). In fact, several studies demonstrate that, like with the endocannabinoids, AEA and 2-AG, also the tissue concentrations of PEA are altered during different pathological conditions (Petrosino et al., 2010). This first mechanism does not exclude the other two.

The second one is the “entourage effect”, which is discussed in detail in the next section, and the “receptor mechanism” is based on the capability of PEA to directly stimulate the nuclear peroxisome proliferator-activated receptor-α (PPAR-α), which clearly mediates many of the anti-inflammatory effects of this compound.

Furthermore, it has recently demonstrated that PEA induced allopregnanolone synthesis in astrocytes in a PPAR-α dependent manner, opened a new field to better understand PEA effects in the CNS. In particular, an increase in allopregnanolone levels was found in the brainsteam of PEA-treated mice, leading to a reinforcement of the hypnotic effect of pentobarbital, due to a positive modulation of GABA(A) receptor by the hormone. Among the neuroactive steroids, allopregnanolone seems to share many of the effects showed by PEA,

including analgesic, anticonvulsivant, and antiallodynic activities. This hormone has been recognized to be a positive activator of the GABA(A) receptor, increasing the Cl^- currents that lead to neuronal hyperpolarization, also achieved by PEA through the opening of the IKCa and BKCa channels. In summary, the main direct and indirect mechanisms of action of PEA are illustrated in Fig. 15 (Mattace Raso et al., 2014; Esposito and Cuzzocrea, 2013; LoVerme et al., 2005(b); Petrosino et al., 2010).

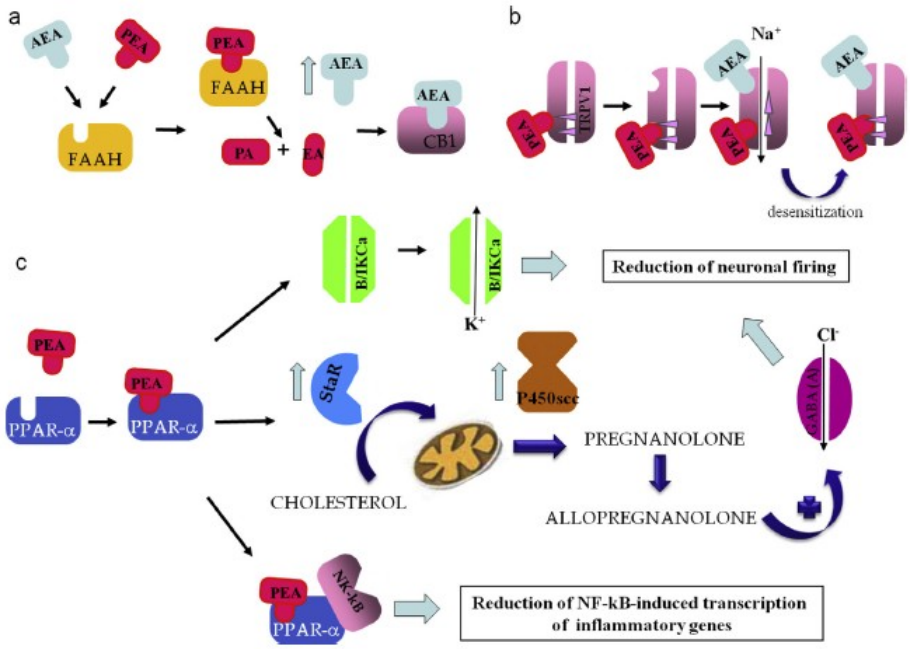


Fig. 15 Direct and indirect mechanisms of action of PEA. The indirect mechanism would involve PEA potentiation of AEA effects through (a) a competitive inhibition of AEA metabolism by FAAH, leading to an increase in AEA levels and its binding to CB1 (b); an allosteric activity on TRPV1, increasing AEA affinity to this receptor, and inducinglater TRPV1 desensitization. (c) Through a PPAR-α, PEA increases the gating properties of IKCa and BKCa channels, resulting in a fast reduction of neuronal firing, increases the expression of StaR and P450scc, involved in cholesterol transfer into the mitochondria and its metabolism in pregnanolone, respectively. The resulting increase in allopregnanolone levels leads to a positive allosteric activation of GABA(A) receptors, an increase in Cl− currents and a reinforcing effect on the reduction of neuronal firing. PEA anti-inflammatory effect appears to be related to a cytoplasmatic complex, that reduces NF-kB transcription activity, dampening the transcription of pro-inflammatory gene.

4.5 The PEA receptors.

Currently, several receptors have been proposed so far to explain the anti-inflammatory and anti-hyperalgesic effects of PEA.

At the beginning, the structural and functional similarities between AEA and PEA, PEA was considered to be an endocannabinoid and it was first suggested that these two lipid mediators might share the same receptor (LoVerme et al., 2005; Lambert et al., 2002; Mattace Raso et al., 2014).

In general, there is a consensus that PEA does not interact with CB_1 receptors at physiologically relevant concentrations, whereas the interactions of PEA with CB_2 receptors was controversial for a while. In support of this idea, PEA was initially reported to displace the binding of the high-affinity cannabinoid agonist [^{3}H]WIN55,212-2 in RBL-2H3 cell membranes which are known to express CB_2 mRNA. However, these results have not been subsequently replicated and it is now accepted that PEA does not bind to CB_2 receptors (LoVerme et al., 2005(b)). But, blockade of CB_2 receptors with the selective antagonist SR144528 was found to prevent PEA-induced antinociception but not the prolonged anti-inflammatory effects of this fatty acid amide (Costa et al., 2002). More recent studies, however, have failed to demonstrate an interaction between PEA and CB_2 receptors unless very high (100 μM) concentrations are used. This inactivity of PEA on CB receptors has been confirmed also by binding studies and it can thus be concluded with reasonable confidence that the physiological effects of PEA are not the result of direct actions at CB receptors (Lambert et al., 2002). To reconcile these contradictory observations, it was proposed that PEA may act by inhibiting anandamide hydrolysis ("entourage effect"), which is catalyzed by FAAH. According to this hypothesis, PEA may compete with AEA for degradation, causing an increase in tissue AEA levels, an enhanced activation of CB_2 receptors and consequently an anti-inflammatory and anti-nociceptive AEA-mediated effect. There could also be an allosteric activation of transient receptor potential vanilloid receptor type 1 (TRPV1) channels, modulating anti-nociception (LoVerme et al., 2005; Mattace Raso et al., 2014; Esposito and Cuzzocrea, 2013(b)).

PEA is also reported to have affinity for orphan receptors such the orphan receptor G-protein coupling receptor, GPR55, since PEA was shown to mediate GTP gamma S formation in cells transfected with the human cDNA for this receptor GPR55 activation has been

suggested to account for some of the non-CB1, non-CB2 effects reported for certain cannabinoid ligands (Esposito and Cuzzocrea, 2013; Esposito and Cuzzocrea, 2013(b); Petrosino et al., 2010; Mattace Raso et al., 2014).

However, the effects of PEA are not only restricted to an enhanced endocannabinoid signal. After the observation that OEA, a lipid amide structurally related to PEA, elicits satiety and stimulates lipolysis in rodents by activating the nuclear receptor PPAR-α, along with the fact that activation of PPAR-α by synthetic agonists causes profound anti-inflammatory effects prompted to question whether PEA might also interact with this receptor to inhibit inflammation. Actually, it is well-documented that PEA activates the nuclear receptor PPAR-α, whereas it does not engage two related PPAR isoforms, PPAR-β/δ and PPAR-δ (LoVerme et al., 2005; LoVerme et al., 2005(b); Mattace Raso et al., 2014). The activation of these intracellular receptor seems to be responsible for the anti-inflammatory activities of PEA.

This can also explain why the CB_2 antagonist SR144528 inhibits the antinociceptive but not the antiinflammatory effects of PEA (LoVerme et al., 2005(b)). Conti and colleagues in fact demonstrated that PEA-induced antiinflammation was not influenced by the presence of SR144528 (Conti et al., 2002; LoVerme et al., 2005).

4.5.1 The PPARs.

The PPARs belong to the family of nuclear hormone receptors that comprise 48 human ligand-inducible transcription factors, whose activity is regulated by steroids and lipid metabolites. Functionally PPARs are involved in adipocyte differentiation, lipid storage and glucose homeostasis of the adipose tissue, brain, placenta and skin (Sodhi et al., 2011; Rakhshandehroo et al., 2010).. The natural ligands of these receptors include long chain fatty acids, eicosanoids, oxidised lipoproteins and lipids.

Till date, three PPAR isoforms (α, β and γ), each encoded by different genes but with similar protein structure, have been characterized in mammals.

PPAR-α and PPAR-γ have been identified as key regulators of cellular differentiation processes and the anti-inflammatory regulation, whereas PPARβ/δ has been associated with modulation of immune response (Sodhi et al., 2011).

4.5.1.1 PPAR-α.

PPAR-α were first discovered in the early 1990s and since then has been identified as the master regulator of hepatic lipid metabolism. In addiction, PPAR-α has been shown to govern glucose metabolism, lipoprotein metabolism, liver inflammation, amino acid metabolism and hepatocyte proliferation. Synthetic agonists of PPAR-α lower plasma triglycerides and raise plasma high-density lipoprotein (HDL) levels and are thus used clinically in the treatment of dyslipidemia (Rakhshandehroo et al., 2010).

However, more recently, it has been recognized that also the main PEA pharmacological effects are mediated by activation of peroxisome proliferator-activated receptor PPAR-α (LoVerme et al., 2005; D'Agostino et al., 2012; Scuderi et al., 2011). Also its monounsatured analog OEA, which shows anorexic effects, activates the intestinal PPAR-α. As demonstrated by LoVerme and colleagues, PEA selectively activates PPAR-α, because the compound failed to engage PPAR-β/δ or PPAR-γ in HeLa cells expressing these receptor isoforms. Then, in an inflammatory response in mouse abdominal skin, elicited by applying the phorbol ester TPA, topical PEA treatment (150 nmol * cm^{-2}, 45 min and 4h after TPA) caused a marked elevation in skin of PPAR-α mRNA levels which was associated with a parallel reduction in TPA-induced edema. In transgenic PPAR-$\alpha^{-/-}$ mice, the reduction of edema was absent. The same results were obtained when the edema was induced by carrageean: it disappeared in PEA-treated wild-type mice but not in PPAR-$\alpha^{-/-}$ mice (LoVerme et al., 2005). Furthermore, PEA treatment did not enhance AEA levels in skin but rather decreased them, indicating that PEA activity is not only correlated to prevent AEA hydrolysis (LoVerme et al., 2005). Interestingly, the same anti-inflammatory effects were obtained with the PPAR-α agonist Wy-14643 (30 mg/kg ip) (LoVerme et al., 2005). Instead, systemic administration of a maximal dose of SR144528 (2 mg/kg i.p.) failed to inhibit the anti-inflammatory effects of either PEA or GW7647 in the TPA model, which is suggestive of a lack of involvement of CB_2 receptors in this response and a further confirmation that PEA modulates inflammation by engaging PPAR-α (LoVerme et al., 2005; Costa et al., 2002).

Another evidence comes from the observation that mice lacking the gene encoding for PPAR-α display prolonged inflammatory responses and synthetic agonists of this receptor cause profound anti-inflammatory actions. These effects are accompanied by reduced

expression of inducible nitric oxide synthase (iNOS), cyclooxigenase-2 (COX-2) and various inflammatory cytokines, including IL-1β, PGE_2 and TNF-α (Mattace Raso wt al., 2014).

In addition, PPAR-α activators have been shown to inhibit monocyte differentiation and neutrophil function, including endothelial extravasation (LoVerme et al., 2005(b); Esposito and Cuzzocrea, 2013).

PEA is predominantly expressed in tissues that are characterized by a high rate of fatty acid metabolism, such as liver, kidney, the intestines, heart, skeletal muscles, adrenal gland and pancreas. The stimulation of these receptors upregulates the expression of several catabolic enzymes that are involved in mitochondrial and microsomal ω-oxidation. Then, this subset of receptors regulates the energy homeostasis by their innate ability to stimulate the breakdown of fatty acids and cholesterol, promoting gluconeogenesis and reduction in serum lipid level. Moreover, the injection of a PPAR-α agonist into the hypothalamus was shown to induce expression of PPAR-α target genes and reduce food intake. The majority of the tissues expressing PPAR-α plays a role in whole-body metabolic homeostasis and is incline to inflammation when metabolism is impaired, a condition which may or not promote pathological conditions, such as obesity, non alcoholic steatohepatitis, type 2 diabetes or cardiovascular diseases. The anti-inflammatory effect of several PPAR-α agonists suggested the potentiality to target this receptor in metabolic disorders often associated to the activation of inflammatory pathways. Therefore, it is conceivable that PPAR-α may have a key role in all those diseases where inflammation is a major determinant of complications including overweight and obesity, underlining the relationship between nutrition, metabolic organs, and vascular tissues (Mattace Raso et al., 2014).

4.5.1.2 PPAR-β/δ.

PPAR-β/δ is expressed ubiquitously in vascular smooth muscle cells as well in the endothelial cells, in addition to liver, kidney, abdominal adipose tissue and earlier during the fetal development.

PPARβ/δ agonists also inhibit lipopolysaccharide-inducible genes, such as iNOS and COX-2 in murine macrophages. Additionally, they have been implicated in the maintenance of lipid homeostasis, keratinocyte proliferation and hyperplasic development of adipose tissue

in animals under a high-fat diet. The modulators of PPARβ/δ include prostacyclin PGI2, and synthetic ligands GW 501516, GW 0742, GW- 2433, GW-9578, L-165041 and L-783483 (Sodhi et al., 2011).

4.5.1.3 PPAR-γ

There are two isoforms of PPAR-γ. PPAR-γ1 isoform is abundantly expressed in white adipose tissue, pancreatic beta cells, spleen, intestines and heart. PPAR-γ2 isoform is expressed in white and brown fat and plays a pivotal role in fat cell differentiation and lipid storage. The predominant function of PPAR-γ include lipid and carbohydrate metabolism. The activation of these receptors by their ligands, the thiazolidinediones, have emerged as a powerful class of antidiabetic drugs. The exogenous ligands for PPAR-γ include Rosiglitazone, Pioglitazone and Troglitazone (Sodhi et al., 2011).

4.5.1.4 Activation mechanism of PPARs.

All PPARs share the same molecular mode of action. Under the inactivated conditions, these heterodimers reside in the nucleus and are associated with corepressors, like nuclear receptor corepressor-1 (NCoR), silencing mediator of retinoid and thyroid-responsive trancription (SMRT) and histone deacetylase (HDAC) complexes, which inhibit the gene expression.

Upon ligand binding to PPARs, there is a conformational change in the complex that facilitates a co-repressor for co-activator complex exchange and transcriptional activation of target genes. indeed, in the presence of its ligand, the PPARs form permissive heterodimers with the nuclear receptor RXR, and in this way it can bind to specific DNA-response elements in target genes, known as peroxisome proliferator response elements (PPREs) and regulate gene expression (Fig. 16). PPREs are characterized by a common core sequence consisting of a direct repeat of the consensus sequence AGGTCA interspaced by a single nucleotide.

Activaction of transcription by PPARs is dependent on a number of different steps including ligand binding to PPAR, binding of PPAR to the target gene, removal of corepressors and recruitment of coactivators, remodeling of the chromatin structure and finally facilitation of gene transcription. (Rakhshandehroo et al., 2010; Sodhi et al., 2011).

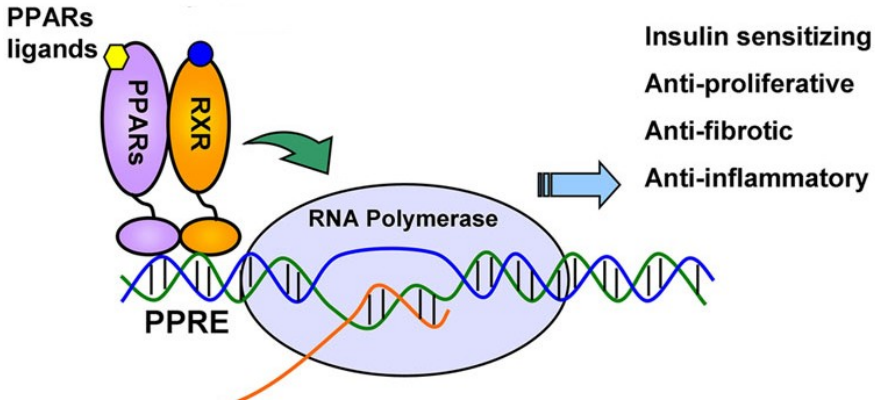


Fig. 16 A basic mechanism of PPAR signaling. Following ligand binding, PPAR forms a heterodimer with RXR, which binds to the PPRE of target genes and regulates the transcription of genes.

4.6 Pharmacological properties of PEA.

In the last century, the properties of PEA have been explored with growing interest. In addition to its known anti-inflammatory activity, PEA also produces analgesia, anti-epilepsy, and neuroprotection. PEA also inhibit food intake, reduces gastrointestin al motility and cancer cell proliferation and protect the vascular endothelium in the ischemic heart.

PEA is abundant in the CNS: its levels have been reported to range from around 0.13 to 6.84 pmol/mg. circadian variations occurs in the CNS concentration of PEA, levels being higher in the cerebrospinal fluid, hippocampus and hypothalamus during dark period compared to the light period. Its concentration shows significant changes during pathological conditions, as shown in glutamate-treated neocortical neurons *ex-vivo,* in cortex after CNS injury and in microdyalisis fluid from a patient with stroke (Esposito and Cuzzocrea, 2013; D'Agostino et al., 2012).

Although pain perception is thought to be controlled mainly by neurotransmitter systems that operate within the CNS, antinociceptive mechanisms also occur in peripheral tissues. It is known that PEA elicited analgesia in acute and inflammatory pain. In fact, it has been reported that exogenous administration of PEA exerted antinociceptive effects in various models of inflammatory and neuropathic pain in the mouse, such as carrageenan-induced

hyperalgesia and chronic constriction injury (Mattace Raso et al., 2014). The analgesic activity of PEA may be ascribed to a more direct action on mast cells, *via* an autacoid local injury antagonism mechanism, combining a dual activity both on neurons of nociceptive pathway and on non neuronal cells, such as mast cells in the periphery and glia in the spinal cord. These actions are accompanied by changes in nitric oxide production, neutrophil influx, and expression of proinflammatory proteins such as inducible nitric oxide synthase and cyclooxygenase-2 (Petrosino et al., 2010; LoVerme et al., 2005; Costa et al., 2002). For example, in one study, animals treated with PEA for 3 days (10 mg kg^{-1}, orally, once daily) following a carrageenan-induced inflammatory insult displayed significantly lower levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) (Costa et al., 2002) and decreased edema induced by the local injection of the polysaccharide (LoVerme et al., 2005). Conti and colleagues showed additionally that the reduction of edema induced by carrageenan was already evident after 1h after also when PEA was given orally (10 mg/kg) (Conti et al., 2002). Furthermore, broad-spectrum analgesia by PEA has been documented in a variety of pain models**.** PEA reduces pain behaviors elicited by formalin, magnesium sulfate, carrageenan, nerve growth factor and dextran (Costa et al., 2002; Conti et al., 2002).

Moreover, PEA was found to inhibit hyperalgesia both after sciatic nerve ligation and in carrageenan-induced injury (Conti et al., 2002; Esposito and Cuzzocrea, 2013). In a model of traumatic spinal cord injury (SCI) in rats an acute overproduction of PEA was observed in lesioned animal 1 day after the contusion. This damage provoked also activaction of astrocytes and microglia, which partecipate in release of glutamate, pro-inflammatory cytokines, ATP, and ROS. PEA modulated tissue injury events associated with spinal cord trauma in mice in secondary damage induced by experimental SCI in mice. Repeated PEA administration (10 mg/kg ip, 30 min before and 1 and 6 hours after SCI) significantly reduced the degree of spinal cord inflammation and tissue injury, neutrophil infiltration, pro-inflammatory cytokine expression, nuclear transcription factor activation-kappaB activation (Mattace Raso et al., 2014), and apoptosis. Also significantly less mast cell density and degranulation were observed in samples collected from PEA-treated mice. Moreover, PEA treatment significantly ameliorated the recovery of motor function (Esposito and Cuzzocrea, 2013).

Other studies have demonstrated that PEA reduces inflammation (Conti et al., 2002; LoVerme et al., 2005).

The increased PPAR-α levels and the reduced expression of cyclooxygenase (COX)-2 and inducible NOS (iNOS) were evidenced in the spinal cord, which represents the main relay station of the neural firing between the inflamed area and the CNS. PEA is also able to attenuate the degree of peripheral inflammation in another animal model of peripheral nerve injury, the chronic constriction injury, which is associated to a profound local inflammatory response that involves T cells and macrophages.

The anti-inflammatory actions of PEA, leading to a reduction of peripheral and central sensitization, are mediated by both neuronal and non-neuronal cells. The latter comprise glia (in particular, astrocytes and microglia) as well as peripheral and central mast cells. In particular, mast cells in the CNS have been shown to play a pivotal role in inflammatory and neurodegenerative diseases. Emerging evidence suggests that the cross-talk between mast cells and glia has an important role in neuroinflammation, exacerbating the acute inflammatory response, accelerating neurodegenerative disease progression and promoting pain perception. In this context, PEA can function in maintaining cellular homeostasis, not only by inhibiting mast cell activation in the CNS and regulating microglial cell activity, but also by blocking peripheral mast cell activation and hence signaling pathways from the periphery to the brain (Mattace Raso et al., 2014).

In addition to the effects on inflammation and pain, there is also literature suggesting that PEA inhibits food intake and cancer cell proliferation (Esposito and Cuzzocrea, 2013(b)).

Increasing evidence points to an antiepileptic and neuroprotective action of PEA. For example, in one study, intraperitoneal administration of the compound was shown to inhibit electroshock-induced and chemically induced seizures. Other neuroprotective actions have also been reported; for example, in a separate study, PEA dose-dependently protected cultured mouse cerebellar granule cells from glutamate toxicity. All these properties are clearly resumed in Fig. 17.

Finally, it must be mentioned that morpho-functional changes of astrocytes and microglial cells after traumatic or toxic insults to the CNS (namely, reactive gliosis) disrupt the complex neuro-glial networks underlying homeostasis and connectivity within brain circuits.

The observation that PEA may be protective in a delayed post-glutamate paradigm of excitotoxic death opened the way to several *in vivo* and *in vitro* studies on the neuroprotective properties of PEA. The growth of knowledge about the molecular mechanisms underlying AD has highlighted the role of neuroinflammation in the pathophysiology of this disorder. AD is classically characterized by the deposit of misfolded proteins: the extracellular accumulation of beta amyloid peptide (Aβ), and the formation of intracellular neurofibrillary tangles (Mattace Raso et al., 2014). Scuderi et al. demonstrated that PEA exhibits anti-inflammatory properties able to counteract Aβ-induced astrogliosis (Scuderi et al., 2011). PEA (10^{-7}M) blunted the expression of proinflammatory molecules in rat primary astrocytes activated by soluble $A\beta_{42}$ (1μg/ml). This effect was reduced by a PPAR-α antagonist. These results indicate that PEA is able to counteract Aβ-induced astrogliosis, and suggest a novel treatment for neuroinflammatory/neurodegenerative processes.

Further in vitro study evaluated the neuroprotective effect of PEA on astrocyte activation and neuronal loss in models of Aβ neurotoxicity in mixed neuroglial cultures and organotypic hippocampal slices via PPAR-α (Scuderi et al., 2012). The anti-inflammatory properties of PEA show that the reduction of reactive gliosis subsequently induces a marked rebound of neuroprotective effect on neurons. More recently, these protective effects of PEA in rat neuronal cultures and organotypic hippocampal slices challenged with Aβ were investigated. PEA reduced Aβ-induced astrocyte activation showing a protective effect on neurons, reverted by a selective PPAR-α antagonist (Scuderi et al., 2013).

Subsequently, a first *in vivo* study by D'Agostino and collaborators described the neuroprotective activities of PEA in mice injected intracerebroventricularly with Aβ(25-35) peptide. Spatial and non-spatial memory tasks to evaluate learning and memory dysfunctions showed that PEA, administered for 2 weeks once a day (10mg/kg, subcutaneously) starting 3hours after Aβ, reduced or prevented behavioral impairments induced by Aβ injection. Acute treatment with PEA was ineffective. According to the neuroprotective profile of PEA observed during behavioral studies, also lipid peroxidation, protein nitrosylation, inducible nitric oxide synthase expression, and caspase-3 activation, were reduced by PEA treatment. The finding that GW7647, a synthetic PPAR-α agonist, mimics the procognitive effect of PEA add further support to the notion that this nuclear receptor is the primary molecular target of PEA, confirming the physiopathological role of PEA/PPAR-α signaling in the CNS

(D'Agostino et al., 2012). These data disclose an unknown therapeutic possibility to treat memory deficits.

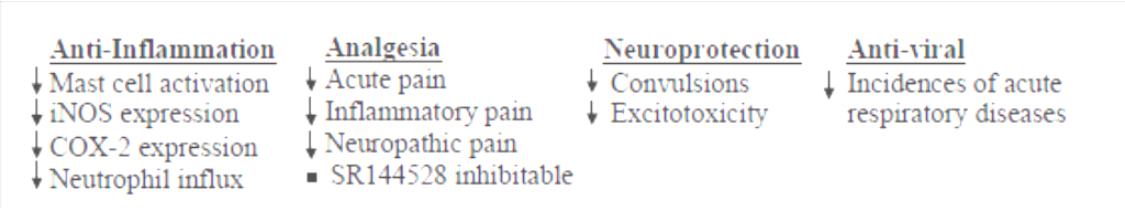


Fig. 17 Pharmacological consequences of PEA administration. (LoVerme et al., 2005 (b)).

Additionally, PEA has been researched also in several different cell types *in vitro,* without exhibiting any toxic effect. In the several preclinical studies the biopharmacologic effects of PEA were not associated with any changes in behavior in rodents, indicating that all the tested doses were well-tolerated. Then, the body weight gain of palmitoylethanolamide-treated rats was similar to that of control animals Interestingly, the anti-inflammatory effect of PEA is not accompanied by tolerance following repeated administration of high doses, which is very important for the potential therapeutic utility of the compound. Actually, the lack of any side effect has been repeatedly reported in published clinical studies, both in animals and humans. No serious adverse events were reported and the tolerance was very good. Indeed, PEA has been explored in a variety of indications such as sciatic pain, diabetic pain neuropathic pain, pain due to arthritis and pain in multiple sclerosis in the period 1992-2010 and around 20 clinical trials have documented its safety and efficacy in these chronic pain states.

PEA is therefore most probably the best-documented nutraceutical around, with a pharmacological profile described in more than 350 scientific papers (Hellink et al., 2013; Esposito and Cuzzocrea, 2013; Costa et al., 2002).

5. ANIMAL MODELS OF AD.

Alzheimer's disease, as every other dementia, is a sole prerogative of humans; no animal suffers from AD. Hence, substantial efforts were invested in producing relevant animal models of AD (Rodriguez et al., 2009)

Animal models of AD play an important role in all areas of AD studies. Proper AD models are needed to study the mechanisms underlying AD pathogenesis, the genetic interactions of the genes of interest, and environmental risk factors that cause sporadic AD, as well as to test the therapeutic effects of AD drug-candidates on neuropathology and cognitive function. First, animal models of AD need to show histopathology such as plaques, NFTs, neuronal loss, and behavioral pathology including cognitive deficits (LaFerla and Oddo, 2005). Second, animal models of AD need to fulfill a substantial similarity between mechanisms underlying changes in behavioral, pathophysiological, and neuronal components in the model and those in AD. Third, animal models of AD need to provide predictive value of AD pathogenesis, allowing extrapolation of the effects of an experimental manipulation. Ideal animal models of AD may have homology to the neurobehavioral pathology and underlying mechanisms of human AD.

Initial models of AD were simply normal aged animals, which showed cholinergic involution associated (in monkeys) with β-amyloid deposition. After the discovery of loss of cholinergic neurons, several cholinergic models of the disease were created. Among these, the most relevant were the rodent models with lesions in the nucleus basalis magnocellularis that is the equivalent of Meynert in humans. These models offered the possibility to investigate the differences in structure, function and behavior of the cholinergic systems in young and aged animals. The majority of these models, however, were created by using non-selective excitotoxic toxins such as NMDA, ibotenic acid, quisqualic acid and certain alkaloid substances. None of these models, however, mimicked the histopathology (plaques and tangles) and progression of AD (Rodriguez et al., 2009).

Therefore several dozens of experimental models have been developed to mimic the genetic cause and the neuropathological features of human AD by generating transgenic mice that overexpress mutant forms of human APP, presenilins, and/or tau protein in the brain.

Each model has some peculiarities and they have been classified as APP models, CTF models, presenilin models, APP+PS1 double transgenic models, tau models, and a triple

(APP+PS1+tau) transgenic model, with presenting a summary of key features relevant to AD pathology (Lee and Han, 2013). However, no transgenic model recapitulates its complete neuropathological spectrum. For example, the overexpression of mutant isoforms of human APP in transgenic mice leads to amyloid deposition in the murine brain, but is insufficient for triggering the full spectrum of AD neuropathology. Numerous models have successfully replicated amyloid plaque deposition, generally by deriving mice with relatively high levels of APP overexpression, and inclusion of a mutant PS1 allele can accelerate the deposition rate as well as exacerbate the pathological severity. The discovery of tau gene mutations in frontotemporal dementia with parkinsonism linked to chromosome 17 kindreds facilitated the development of tauopathies. One surprising aspect of the overwhelming majority of extant AD models has been that the successful development of one hallmarks pathological lesion has been insufficient to trigger the development of other signature lesions. Consequently, the concomitant manifestation of both plaques and tangles in a mouse requires aggressive biotechnical strategies to introduce multiple transgenes into the same mouse which has generally been achieved by crossing several independent transgenic lines, or alternatively, by microinjecting pathological protein into the brains of single-transgenic mice (Oddo et al., 2003; Oddo et al., 2003 (b)).

Below, it will be described briefly some models, and more attention will be placed on the transgenic mice with a triple mutation (3xTg-AD) that were used to conduct the experiments of this thesis.

5.1 APP models.

APP models are transgenic mice that express mutant forms of human APP, which are associated with early-onset of disease. Mutations in APP around the cleavage sites by β-secretase or γ-secretase increase $A\beta_{42}$ secretion in the brain. Indeed, overexpression of mutant forms of human APP in the brains of mice results in $A\beta_{42}$ accumulation, plaque deposition, Aβ-triggered pathology, and cognitive impairment, which have some similarities to those in human AD. For example, Tg2576 mice express the human APP695 with the Swedish double mutations (K670N/M671L) at the β-secretase cleavage site. They show abundant accumulation of $A\beta_{40}$ and $A\beta_{42}$ at 6-9 months and plaque deposition begins at 9 months of age and cognitive impairment at 6 months (Rodriguez et al., 2009). Tg2576 mice are widely used

partly due to the initial generosity of Dr. K Hsiao to users and later commercial availability from Taconic Inc.

Similarly, TgAPP23 mice express the human APP751 containing the Swedish double mutations (K670N/M671L) at the β-secretase cleavage site. They show typical plaques at 6 months and neuritic and synaptic degeneration as well as tau hyperphosphorylation in aged brains; development of cognitive decline is at 10 months. Furthermore, they have 14% loss of hippocampal CA1 pyramidal neurons (Lee and Han, 2013).

5.2 CTF models.

In this group there are TgCTF104 and TgβCTF99 mice. The first express the carboxy-terminal fragment (CTF) of APP (CTF104 of APP591-695) and they develop plaque deposition at 8-10 months, severe cognitive deficits at 8 months, and neuronal loss at 18-22 months.

TgβCTF99 express the β-secretase-cut carboxyl terminal fragment (βCTF99) with the V717F mutation. They display no plaque deposition but develop cognitive deficits at 11-14 months and neuronal loss in the hippocampus and cerebral cortex at 16-18 months (Lee and Han, 2013).

5.3 PS1 and PS2 models.

PS1 and PS2 transgenic lines carry FAD mutant forms of PS1 and PS2 (M146L or M146V), respectively. Mice show enhanced levels of $A\beta_{42}$, supporting the hypothesis that the presenilin mutations cause AD pathogenesis through a gain of deleterious function. However, this kind of transgenic mice do not develop plaques, unless they are crossed with plaque-forming APP lines (eg., Tg2576). In this case, presenilin FAD mutations produce elevated levels of $A\beta_{42}$ and cause earlier and more extensive plaque deposition (Lee and Han, 2013).

5.4 APP + PS models.

APP and PS1, carrying the transgene for both mutant APP and mutant PS1 show increased $A\beta_{42}$ production and more extensive plaque deposition. Tg-APPswe/PS1dE9 mice

express the human APP with the Swedish mutations (K670N/M671L) at the β-secretase cleavage site and PS1 (PS1dE9), which result in plaque deposition in the brain starting at 6 months of age and behavioral deficits at 6-8 months of age. Tg-APPswe/PS1dE9 mice do not develop NFTs or neuronal loss.

5XFAD mice express the human APP with the Swedish mutations (K670N/M671L) at the β-secretase cleavage site and two FAD associated mutations (I716V/V717I) at the γ-secretase cleavage site, and human PS1 with the M146V and L286V. these mice develop plaque deposition at 2 months and cognitive deficits at 4-6 months (Lee and Han, 2013).

5.5 Tau models.

Transgenic mice overexpressing mutant tau produce tau pathology, with somatodendritic localisation of hyperphosphorylation of tau, but they do not develop Aβ deposition (Rodriguez et al., 2009). These mutations do not lead to AD, but rather to another form of dementia called frontotemporal dementia with parkinsonism-17, which is marked by neurofibrillary pathology similar to that in AD, although without any amyloid deposition (Oddo et al., 2003). **JNPL3 mice** express the human tau with the most common mutation (P301L), which causes the fronto-temporal dementia and parkinsonism-linked to chromosome 17 (FTDP-17 mutation) in human. JNPL3 mice develop NFTs at 6.5 months and progressive motor disturbance in hemizygous animals. When they are crossed with Tg2576, the resulting double mutant (tau/APP) progeny and the Tg2576 parental mice develop Aβ deposits at the same age; however, the tau/APP double mutants exhibit enhanced NFT pathology in the limbic system and olfactory cortex compared to that of the JNPL3 parent strain (Lee and Han, 2013).

5.6 Triple (APP + PS + Tau) models.

5.6.1 Generation of 3xTg-AD mice.

The triple transgenic mice (3xTg-AD) harbor three mutant transgenes: $PS1_{M146V}$, APP_{Swe}, and tau_{P301L}. The 3xTg-AD mice develop an age-dependent and progress neuropathology that includes plaque and tangle pathology. So this is the first transgenic model

to develop both plaques and tangle pathology in AD-relevant brain regions and it represents the most advanced animal model of AD (Rodriguez et al., 2009).

This transgenic model was created by Oddo and LaFerla in 2003 and instead of crossing three independent lines, they used the direct comicroinjections of two independent transgenes, encoding human amyloid precursor protein APP_{Swe} and human tau_{P301L}, both under the control of the mouse Thy1.2 regulatory element, into a single-cell embryos harvested from homozygous mutant $PS1_{M146V}$ knocking (PS1-KI) mice (Fig 18). It has been shown with Southern blotting that both transgenese co-integrated at the same locus and the analysis of transmission frequency among the offspring confirmed that both transgenes were co-inherited. Because the APP and tau transgenes cointegrated at the same site and thus are unlikely to independently assort, and because the M146V mutation was "knocked in" to the endogenous mouse PS1 locus, these 3xTg-AD mice essentially breed as readily as a "single" transgenic line and exist in both a hemizygous and homozygous genotype. This is important not only for the establishment and maintenance of the mouse colony, but also because 3xTg-AD mice are of the same genetic background, thereby reducing a confounding biological variable that is unavoidable when crossing independent transgenic lines (Oddo et al., 2003 b; LaFerla and Oddo 2005).

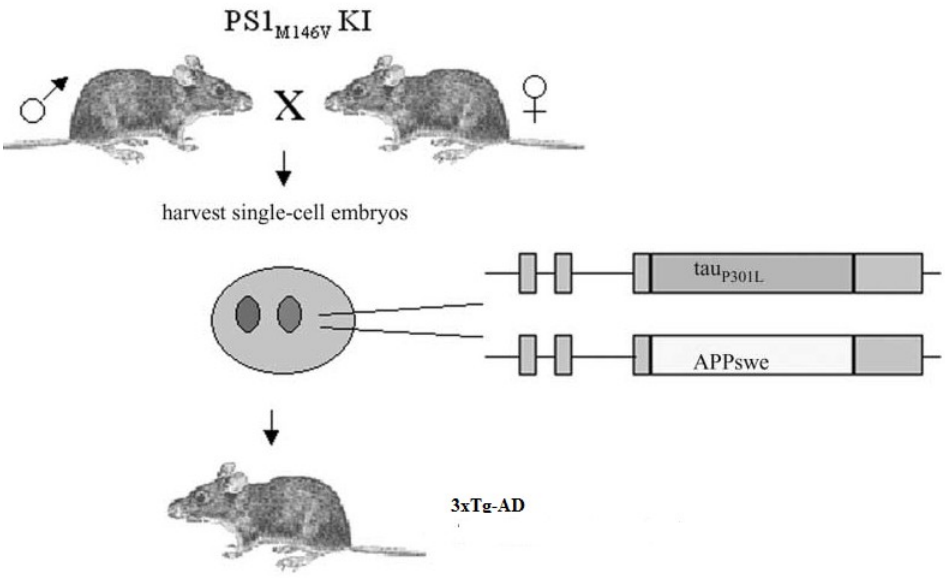


Fig. 18 Strategy used to develop 3xTg-AD Mouse Model.

5.6.2 Aβ pathology and Tau pathology.

Protein extracts from multiple tissues from 3xTg-AD mice, analyzed by Western blot using human-specific antibodies targeted to APP or tau, confirmed that their expression was predominantly restricted to the CNS. In particular, AD-relevant regions, including the hippocampus and cerebral cortex were among the regions containing the highest levels of both the transgene-derived human APP and tau proteins. In particular, these APP proteins are processed to liberate Aβ. In fact from hemizygous and homozygous brain homogenates, it emerged that Aβ is twice as abundant in the homozygous versus hemizygous brains but it is undetectable in NonTg brain. In particular, there is a progressive increase in Aβ formation as a function of age in 3xTg-AD brains and a particularly pronounced effect on $A\beta_{42}$ levels (Fig 19).

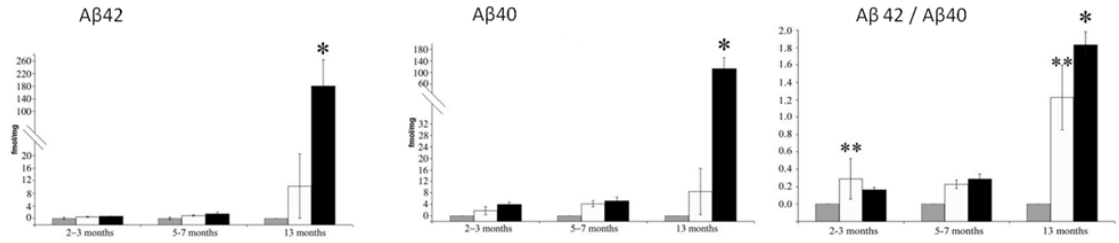


Fig. 19 $A\beta_{40}$ and $A\beta_{42}$ levels measured by ELISA from different-aged mice (n= 3/group). Non-Tg, hemizygous, and homozygous mice are depicted as gray, white, and black bars, respectively. Statistical difference between hemizygous and NonTg mice is denoted by "**," whereas "*" indicates that homozygous mice are statistically different from hemizygous and NonTg mice.

Intraneuronal Aβ immunoreactivity is one of the earliest neuropathological manifestations, first detectable in neocortical regions and subsequently in CA1 subfield pyramidal neurons. It is apparent between 3 and 4 months of age in the neocortex and by six months of age in CA1 subfield of the hippocampus, becoming readily evident by 12 months, both in hemizygous and homozygous brains. Later, Aβ deposits become detectable in other cortical regions, suggesting that there is an age-related, regional dependence to Aβ deposition in the 3xTg-AD mice. Furthermore, these extracellular deposits are recognized by the $A\beta_{42}$-specific antibody and some of them are co-localized with reactive astrocytes.

The 3xTg-AD mice develop a progressive and age-dependent Aβ- and tau- pathology (Rodriguez et al., 2009), but Aβ deposits are evident prior to tangle formation, in spite of equivalent overexpression of the human βAPP and human tau transgenes. This is in line with the Aβ amyloid cascade hypothesis which predicts that Aβ is the trigger for all cases of AD and that the tau pathology and other degenerative changes are a downstream consequence of Aβ pathology.

In fact, when Aβ deposits in cortex are apparent by six months in 3xTg-AD mice, no tau alterations are detectable at this age. Extensive human tau immunoreactivity is first evident when the 3xTg-AD mice reach about 12 months of age, firstly in CA1 subfield, particularly within pyramidal neurons, and then later progresses to involve cortical structures. In this way, 3xTg-AD profile closely mimics the distribution pattern that occurs in human AD brains. Additionally, tau is normally phosphorylated at different amino acids residues; however, in pathological conditions, the amount of phosphorylation is greatly increased at specific sites, including Ser202 and Thr205. As in human AD brain, in 3xTg-AD mice there is an age-related progression in alterations of the tau proteins, detectable using conformational-specific and phospho-tau antibodies. None of these antibodies bind to normal tau, nor are any of these immunoreactive structures detected in Non-Tg brains. Thus, tau is conformationally altered and hyperphosphorylated at multiple residues in the brains of the 3xTg-AD mice in an age-related and regional-dependent manner, becoming evident between 12 to 15 months of age.

Moreover, as mentioned above, it has been reported that Aβ pathology could affects the development of tau pathology, even if they initiate in different brain regions in the 3xTg-AD mice (i.e. cortex for Aβ and hippocampus for tau). The finding that tau and Aβ immunoreactivity colocalize to the same neurons, as revealed by double-labeling immunohistochemistry, further supports the concept that Aβ is poised to influence tau.

Consequently, it is likely that soluble intracellular Aβ (or an intracellular Aβ-containing APP derivative), which is the first detectable pathological manifestation, affects the development of the tau pathology. $A\beta_{42}$-specific epitopes are also found to be associated with intracellular neurofibrillary tangles in tha AD brain (Oddo et al., 2003 b; LaFerla and Oddo, 2005).

5.6.3 Synaptic dysfunction and cognitive deficits.

Modeling both plaques and tangles in AD-relevant brain regions enables one to establish the relationship of these proteinaceous structures to crucial neurologic processes, such as learning and memory, synaptic plasticity and brain inflammation.

The finding that intraneuronal Aβ triggers the onset of cognitive deficits is consistent with the finding that it also induces synaptic dysfunction. As with the onset of cognitive decline, the synaptic plasticity deficits manifest prior to extracellular Aβ deposition and tangles and best correlate with intraneuronal Aβ.

The synaptic plasticity deficits emerge in an age-dependet manner. At six months, the 3xTg-AD mice showed a profound functional and cognitive impairment, including LTP, spatial memory and long-term memory deficits. Most importantly, functional deficits precede the appearance of histological markers (Rodriguez et al., 2009).

Notably, the memory and cognitive decline observed in AD patients correlates better with the synaptic pathology than either plaques or tangles, and thus, synaptic dysfunction is likely the most significant factor contributing to the initial stages of memory loss (Oddo et al., 2003 b; LaFerla and Oddo, 2005).

However, all animal models of AD developed so far have some limitations, the most serious one being the absence of significant neuronal loss. This may reflect some intrinsic differences between human and animal brain, shorter lifespan of experimental animals or influence of other yet unknow factors (Rodriguez et al., 2009).

5.6.4 Glial atrophy and astrogliosis.

In recent studies performed on different regions of the brains of 3xTg-AD mice, both astrogliosis and astroglial atrophy were found.

In a plaque infested brain the reactive astrocytes were concentrated around the Aβ plaques, whereas astroglial cells distant to the plaques had atrophic features (Heneka et al., 2010).

The decrease in complexity of astrocytes from several brain regions, which indicated their atrophy, began to be observed before the formation and consolidation of neuritic plaques. In very young animals (3 months old), which did not have any signs of AD

pathology, astroglial morphology was already somewhat altered, particularly in the enthorinal cortex. There was a slight reduction in astrocyte complexity that was directly associated with a decrease in the number of processes and overall decrease in both surface and volume of GFAP-labelled structures. Similar reduction in GFAP labelled profiles was observed at all ages, although this decrease becomes significant only at 12 months of age. In 12-month-old animals the complexity was reduced, whereas GFAP-positive surface decreased as well as the volume. This decrease in complexity is reflected by reduced number of processes and their arborization. Incidentally, the same decrease in astrocytes complexity was found in the postmortem brain of dementia patients. In particular, it is the specific population of astrocytes surrounding amyloid plaques that displays the typical reactive characteristics, showing thick processes and enlarged cell bodies. Atrophic astrocytes lose the ability to perform their essential homeostatic functions, hence instigating a profound and irreversible chain of pathological changes in the brain (Rodriguez et al., 2009; Verkhratsky et al., 2015). For example, the 3xTg-AD animals show other changes that could be associated with glial malfunction. It has been observed a transient increase in the number of asymmetric excitatory synapses in AD mice at 2 months of age, which subsequently dropped to control levels at 6–9 months. Nonetheless, this increase in excitatory synaptic contacts re-emerged again at 12 months of age when the brain parenchyma was infested with plaques, and neurons showed tangles (Rodriguez et al., 2009). Additionally, the decrease in GFAP content was accompanied with reduced immunoreactive for glutamine synthetase in the prefrontal cortex. Therefore, deficient homeostatic support of neuronal networks may result in a decrease of synaptic activity and synaptic los, which represent an early event in AD progression (Verkhratsky et al., 2015).

6. AIM OF THE STUDY.

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by impairment of cognitive functions and memory loss. Its core neuropathological hallmarks are extracellular senile plaques and intraneuronal neurofibrillary tangles (Ballard et al., 2011). Recently, it has been recognized that also neuroinflammation is deeply involved in AD, accounting for the synthesis of different cytokines and pro-inflammatory mediators released by astrocytes and microglia (Steardo Jr. et al., 2015). In this context, the role of microglia has been extensively described, whereas the astrocytes involvement remains to be better elucidated (Scuderi et al., 2011). Astrocytes in AD brains, changing from a basal to a reactive state, fail to provide their neuro-supportive functions, as consequence, neurons are more vulnerable to toxic molecules (Fuller et al., 2010; Verkhratsky et al., 2015), including glutamate and its excitotoxicity which represents an additional pathogenic basis of neurodegeneration in AD (Sastre et al., 2008; Fernàndez-Ruiz et al., 2010).

At present, no therapies are able to effectively impact or to prevent the disease course (Bedse et al., 2015). Therefore, the new therapeutic approach should aim both to neuroprotection and neuroinflammation reduction, modulating astrocytes activation (Vesce et al., 2007).

Cannabinoids (CBs) or the modulation of their signals have been proposed as possible therapeutic approaches to AD (Bisogno and Di Marzo, 2008; Bedse et al., 2015). The endocannabinoid system is extensively involved in the neuroinflammatory process, exerting an inhibitory and neuroprotective role both at the peripheral level and on central glial cells, particularly, those compounds belonging to the acylethanolamide family (Campillo and Paez, 2009).

Among these molecules, palmitoylethanolamide (PEA) has attracted a lot of attention for its numerous pharmacological properties and very low toxicity (Hesselink, 2013). PEA has been studied extensively for its anti-inflammatory and neuroprotective effects, mainly in models of peripheral neuropathies (Conti et al., 2002; Petrosino et al., 2010). However, its physiological role and pharmacological properties in the CNS remain, for the most part, unclear (Scuderi et al., 2011). Recently, *in vitro* and *in vivo* results have suggested anti-inflammatory and neuroprotective properties of PEA against Aβ-induced neurotoxicity

(Scuderi et al., 2011; 2014; D'Agostino et al., 2012). But, there are not exhaustive data on the possible neuroprotective effects of PEA in genetically modified mouse model of AD.

The triple-transgenic murine model of AD (3xTg-AD) closely mimics many aspects of AD in humans and primary cultures from 3xTg-AD mice represent the first *in vitro* model of AD and provide a valuable approach to study the cellular and molecular basis of the disease and to investigate new pharmacological approaches (Vale et al., 2010).

Based on the above findings, the main aim of the first part of the study investigation of the toxic effects of $A\beta_{42}$ (0.5 μM, 24 hours) in primary cultures from both non-Tg and 3xTg-AD mice.

The cerebral cortex was chosen as the most representative among the brain regions primarily affected by AD.

In the second part of the study, it was investigated the possible neuroprotective effects of PEA (0.1 μM), given in pre-treatment, on $A\beta_{42}$-induced neurotoxicity in primary cultures of neurons and astrocytes prepared wild-type mice and of 3xTg mice.

The effects of Aβ and PEA were proved by the evaluation of three different parameters: neurochemical, morphological and biochemical.

a) Neurochemical: assessment of cortical glutamatergic function.

The effects of $A\beta_{42}$ and PEA were investigated in terms of changes in basal endogenous glutamate levels. Glutamate was determined by HPLC technique coupled with fluorimetric detection.

b) Morphological: evaluation of the morphology and the number of neuronal and glial cells.

The effects of Aβ42 and PEA were investigated with immunocytochemical methods (MAP-2 immunoreactivity in neurons and GFAP immunoreactivity in glial cells). Examination was made using a Nikon Microphot FXA microscope.

c) Biochemical: assessment of the cell viability.

The effects of $A\beta_{42}$ and PEA were investigated in terms of ability of viable cell to incorporate and bind the supervital Neutral red. The absorbance was read at 540 nm using a microplate absorbance reader.

The analysis of the results obtained with the different preparations, i.e. neuronal cultures and glial cultures from both non-Tg and 3xTg-AD mice, may contribute to better elucidate the role played by glial cells, exposed to beta-amyloid, in the pathogenic mechanisms of AD, the phenomenon of neurotoxicity and its involvement in neurodegeneration, along with the contribution of glial cells, the morphological changes, in astrocytes and in neurons, induced by Aβ and PEA treatments and their possible correlation with the functional changes and finally the toxic effects of Aβ and the neuroprotective effects of PEA in an animal model of Alzheimer's disease.

7. MATERIALS AND METHODS.

7.1 Animals.

Colonies of 3xTg-AD mice and wild type littermates (non-Tg) were established at the animal facilities of the Puglia and Basilicata Experimental Zooprophylactic Institute (Foggia, Italy), according to the procedures previously described (Cassano et al., 2012). The 3xTg-AD mice harboring AβPPswe, PS1M146V, and tauP301L transgenes were genetically engineered by LaFerla and colleagues at the Department of Neurobiology and Behavior, University of California, Irvine (Oddo et al., 2003; Oddo et al., 2003(b)). Genotypes were confirmed by polymerase chain reaction (PCR) after tail biopsies (Oddo et al., 2003(b)). The housing conditions were controlled (temperature 22° C, light from 07:00–19:00, humidity 50%–60%), and fresh food and water were freely available.

Experiments were carried out in strict accordance with the European Communities Council Directive (86/609/EEC) and the Guidelines released by the Italian Ministry of Health (D.L. 116/92) and (D.L.172 111/94-B). A formal approval to conduct the experiments described was obtained by the local Ethics Committee (University of Ferrara, Italy). Efforts were made to minimize the number of animals used and to reduce their discomfort.

7.2 Primary cultures of cerebral cortical neurons.

Primary cultures of cortical neurons were prepared from embryonic day 18 (E18) non-Tg and 3xTg-AD mouse embryos and cultured as previously described by Antonelli et al., 2008. Briefly, cortices free of meninges were dissociated in 0.025% (w/v) trypsin at 37°C followed by mechanical repeated gentle pipetting through wide- and narrow-bore fire-polished Pasteur pipettes in culture medium [Neurobasal medium (Gibco, Grand Island, NY, USA) supplemented with 0.1 mM glutamine (Sigma Chemical Co., St. Louis, MO, USA), 10 µg/ml gentamicin (Sigma Chemical Co.) and 2% B-27® Supplement (50X), serum free (Gibco®)].

Cells were counted and then plated on poly-L-lysine (5 µg/ml)-coated multiwells (24 wells; Nunc A/S, Roskilde, Denmark) at a density of 200,000 cells per well and on 96-well at a density of 50,000 cells per well. For immunocytochemistry, the cells were plated on glass

coverslips at a concentration of 200,000 cells per well. Cultures were grown at 37°C in a humidified atmosphere, 5% CO_2/95% air. Cytosine arabinoside (10 µM; Sigma Chemical Co.) was added within 24 h of plating to prevent glial cell proliferation. After 8 days of in vitro incubation (days in vitro: DIV), cultures were used for experiments.

7.3 Primary cultures of cortical astrocytes.

Primary cultures of cerebral cortical astrocytes were obtained from newborn non-Tg and 3xTg-AD mice (1 or 2 days old) and cultured as described by Scuderi et al. (Scuderi et al., 2012), with slight modifications. Cerebral cortices were removed and dissociated by mild trypsinization at 37°C, followed by mechanical trituration to obtain single cells. Cells were suspended in the culture medium [DMEM, 5% inactivated fetal bovine serum (Gibco), 100 IU/ml penicillin, and 100 µg/ml streptomycin (all from Sigma-Aldrich, Milan, Italy)] and then seeded in 75-cm^2 flasks at a density of 3×10^6 cells/flask. The cells were incubated at 37°C in a humidified atmosphere, 5% CO_2/95% air. The culture medium was replaced after 24 h and again twice weekly until astrocytes were grown to form a monolayer firmly attached to the bottom of the flask (12 or 14 days after dissection). At cell confluence, flasks were vigorously shaken to separate astrocytes (which remained adherent in the bottom of the flasks) from microglia and oligodendrocytes (which floated on the supernatant). Collected astrocytes were counted and then plated on poly-L-lysine (5 µg/ml)-coated multiwells (24 wells) at a density of 200,000 cells per well and on 96-multiwell plates at a density of 50,000 cells per well. For immunocytochemistry, the cultured astrocytes were plated on glass coverslips at a concentration of 200,000 cells per well. The purity of the cells in culture was tested with monoclonal anti-glial fibrillary acidic protein (GFAP) and only cultures with more than 95% GFAP-positive cells were used for the experiments.

7.4 Neuronal and astroglial culture pharmacological treatments.

Both neuronal and astroglial cultures were treated with $A\beta_{42}$ (0.5 µM; Tocris Bioscience, Bristol, UK) for 24 h with or without PEA (0.1 µM; Tocris Bioscience, Bristol, UK), added 1 h before $A\beta_{42}$ and maintained in contact with the cells during the peptide exposure (Fig. 20). The concentration of the substances was chosen according to previous

results (Scuderi et al., 2011; Scuderi et al., 2012). Cell viability, cell count and glutamate levels were assessed after 24 h of treatment.

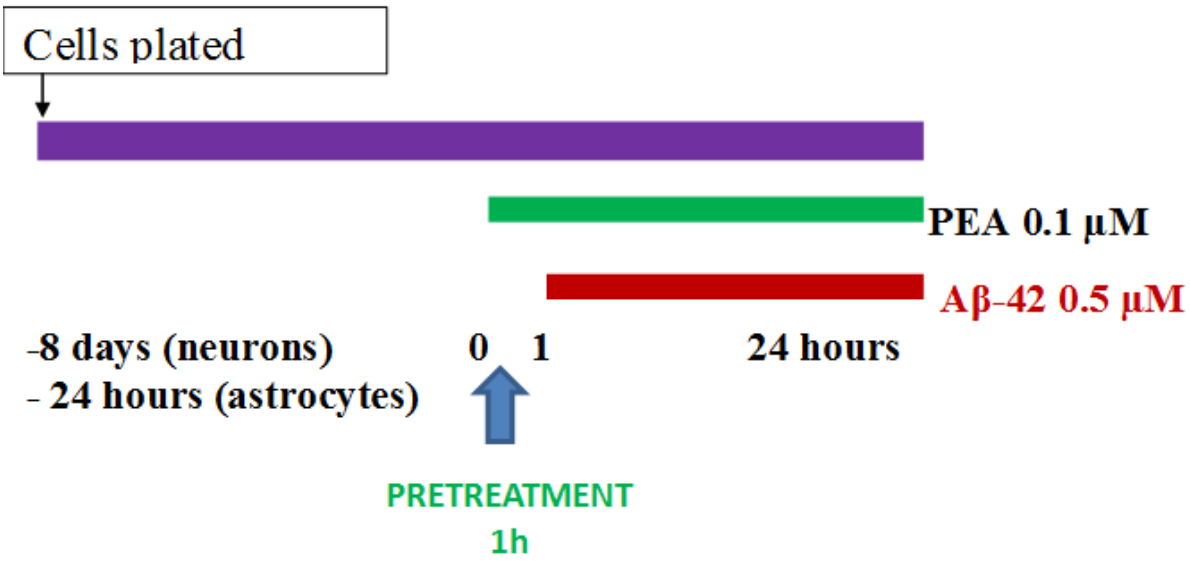


Fig. 20 Experimental scheme for treatments in the primary cultures of cerebral cortical neurons and astrocytes from wild-type and 3xTg-AD mice. PEA 0.1 µM, was added 1h before $A\beta_{42}$ 0.5 µM and maintained in contact with the cells during the peptide exposure.

7.5 Neutral red assay.

The Neutral red assay was used to assess cell viability (Scuderi and Steardo, 2013; Repetto et al., 2008). Cells were cultured in 96-multiwell plates and treated as described above. 24 h after pharmacological treatments, the plates were incubated for 3 h at 37° C with a neutral red working solution (50 µg ml-1 in PBS 1X without calcium and magnesium, Sigma-Aldrich, St. Louis, MO, USA). The cells were washed and the dye removed from each well through a destain solution (ethanol:deionized water: glacial acetic acid, 50:49:1, v/v). The absorbance was read at 540 nm sing a microplate absorbance reader (Sunrise, Tecan). The values of treated cells were referred to control non-exposed cultures, and expressed as percentage variation.

7.6 Immunocytochemistry.

Cells were rinsed in 0.1M PBS and then fixed with 4% paraformaldehyde in Sorensen's buffer 0.1M, pH 7.4, for 20 min. After rinsing in PBS (three times for 5 min each), the cells were incubated overnight at 4°C in 0.3% Triton X-100/PBS solution (v/v) containing the following primary antibodies: anti-microtubule-associated protein 2 (MAP-2) (1:1000 dilution, Chemicon, Temecula, CA) and anti-GFAP (1:200 dilution Chemicon, Temecula, CA). The cells were then washed three times with PBS and incubated for 60 min at room temperature with the proper secondary antibodies: rhodamine-conjugated anti-rabbit antibody (1:100 dilution Chemicon, Temecula, CA) and fluorescein isothiocyanate-conjugated anti-mouse antibody (1:100 dilution Chemicon, Temecula, CA), respectively.

After 3 washes in PBS, the cells were mounted in glycerol and PBS (3:1, v/v) containing 0.1% 1,4-phenylenediamine and examined using a Nikon Microphot FXA microscope. For cell counts, five separate non-overlapping fields were randomly chosen in each coverslip and the images were taken using the x20 objective.

Anti-MAP-2 antibody, anti-GFAP antibody, rhodamine-conjugated anti-rabbit antibody, and fluorescein isothiocyanate-conjugated anti-mouse antibody were purchased from Chemicon, Temecula, CA.

7.7 Endogenous extracellular glutamate levels.

On the day of the experiment, cells were rinsed twice (1 min/rinse) by replacing the culture medium with a warmed (37°C) Krebs Ringer-bicarbonate buffer (mM: NaCl 118.5, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11, pH 7.4). Thereafter, 400 µl of this solution were added to each plates and, after 50 min, 100 µl of the solution were collected. After rinsing, the procedure was repeated to collect a second 50 min fraction. Then, cells were treated by adding 400 µl of Krebs Ringer-bicarbonate buffer containing $A\beta_{42}$ (0.5 µM) and/or PEA (0.1 µM) and a third fraction was collected 24 h later. Control cell cultures were treated with Krebs Ringer-bicarbonate buffer. The first two fractions were used to assess basal endogenous glutamate levels. The effects of the treatments on endogenous extracellular glutamate levels during the third fraction were reported and expressed as percentage changes of basal values, as calculated by the means of the two fractions collected prior to treatment.

Endogenous glutamate levels were quantified using a high-performance liquid chromatography/fluorimetric detection system, including a precolumn derivatization o-phthaldialdehyde reagent and a Chromsep 5 (C18) column (3 mm internal diameter; 10 cm length). The mobile phase (flow: 0.75 ml/min) consisted of 0.1 M sodium acetate, 10% methanol, and 2.5% tetrahydro-furan, pH 6.5. For fluorimetric detection, excitation and emission wavelengths were set at 370 and 450 nm, respectively. The limit of detection for glutamate was 30 fmol per sample (Ferraro et al., 2000).

7.8 Statistical analysis.

Results are expressed as means±standard error of mean. The statistical analysis was carried out by analysis of variance (ANOVA) followed by the Newman-Keuls test formultiple comparisons. $p < 0.05$ was the accepted level of significance.

8. RESULTS.

8.1 Primary cultures of cerebral cortical neurons from 3xTg-AD and non-Tg mice.

8.1.1 Effects of $A\beta_{42}$ exposure in the presence and in the absence of PEA on cellular viability in cultured cortical neurons from non-Tg mice and 3xTg-AD mice.

The exposure to $A\beta_{42}$ (0.5 μM; 24 h) induced a significant decrease in the cell viability in cultured cortical neurons obtained from non-Tg mice (Fig. 21A), but not in cultured cortical neurons obtained from 3xTg-AD mice (Fig. 21B). Pretreatment with PEA (0.1 μM) fully counteracted $A\beta_{42}$-induced decrease of cell viability in cultured cortical neurons obtained from non-Tg mice (Fig. 21A). By itself, PEA did not affect cell viability in cultured cortical neurons obtained from non-Tg mice (Fig. 21A) or 3xTg-AD mice (Fig. 21B).

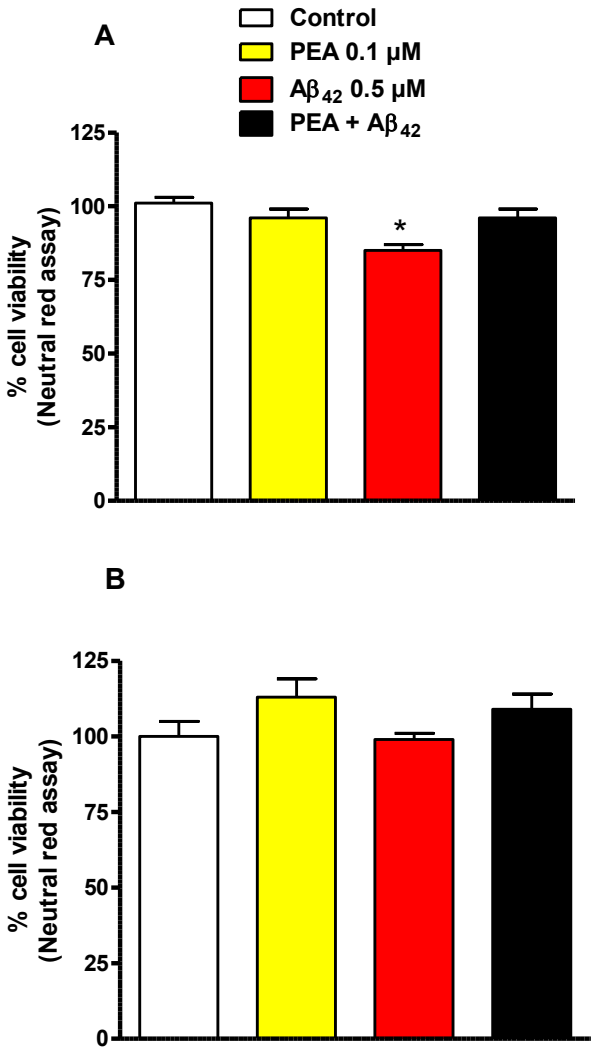


Fig. 21 Effects of $A\beta_{42}$ exposure (0.5 µM; 24 h), alone or in combination with PEA (0.1 µM), on cell viability in primary cultures of cerebral cortical neurons from non-Tg (A) and 3xTg-AD (B) mice. PEA was added 1 h before $A\beta_{42}$ and maintained in contact with the cells during $A\beta_{42}$ exposure. Cell viability was assessed by Neutral red assay and expressed as percentage of control values.

Each histogram represents the mean±S.E.M. (n = 30–40).

$*p < 0.05$ significantly different from control, PEA and PEA + $A\beta_{42}$ groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

8.1.2 Effects of $A\beta_{42}$ exposure in the presence and in the absence of PEA on endogenous extracellular glutamate levels in cultured cortical neurons from non-Tg mice and 3xTg-AD mice.

Basal extracellular glutamate levels in cultured cortical neurons obtained from non-Tg mice were significantly higher than those observed in cultured cortical neurons obtained from 3xTg-AD mice (0.328±0.029 μM and 0.063±0.005 μM, respetively) (Fig. 22). $A\beta_{42}$ (0.5 μM; 24 h) exposure reduced extracellular glutamate levels in cultured cortical neurons obtained from non-Tg mice (Fig. 23A), but not in cultured cortical neurons obtained from 3xTg- AD mice (Fig. 23B). Pretreatment with PEA (0.1 μM) counteracted $A\beta_{42}$-induced decrease of extracellular glutamate levels in cultured cortical neurons obtained from non-Tg mice (Fig. 23A). By itself, PEA did not affect extracellular glutamate levels in cultured cortical neurons obtained from non-Tg mice (Fig. 2A) or 3xTg-AD mice (Fig. 23B).

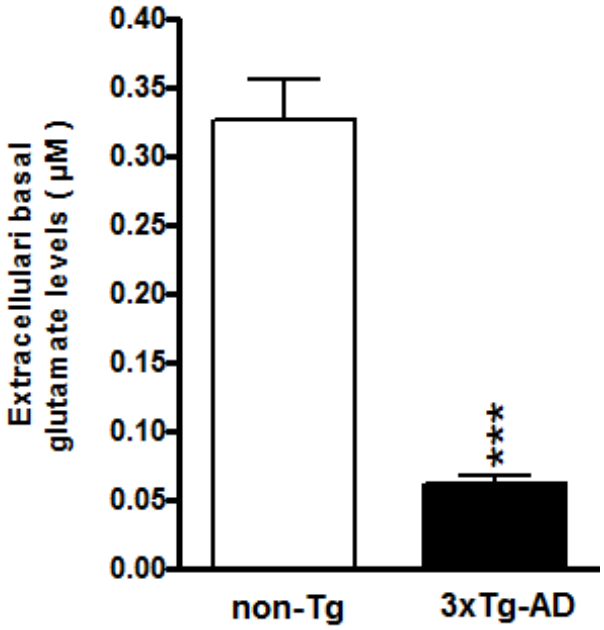


Fig. 22 Extracellular basal glutamate levels in primary cultures of cerebral cortical neurons from non-Tg and 3xTg-AD mice. The basal endogenous extracellular glutamate levels are the mean of the two first fraction collected before PEA pretreatment and $A\beta_{42}$ treatment.
Each histogram represents the mean±S.E.M. (n = 30–40).
$^{***}p < 0.001$ significantly different from non-Tg group, according to Student's *t*-test.

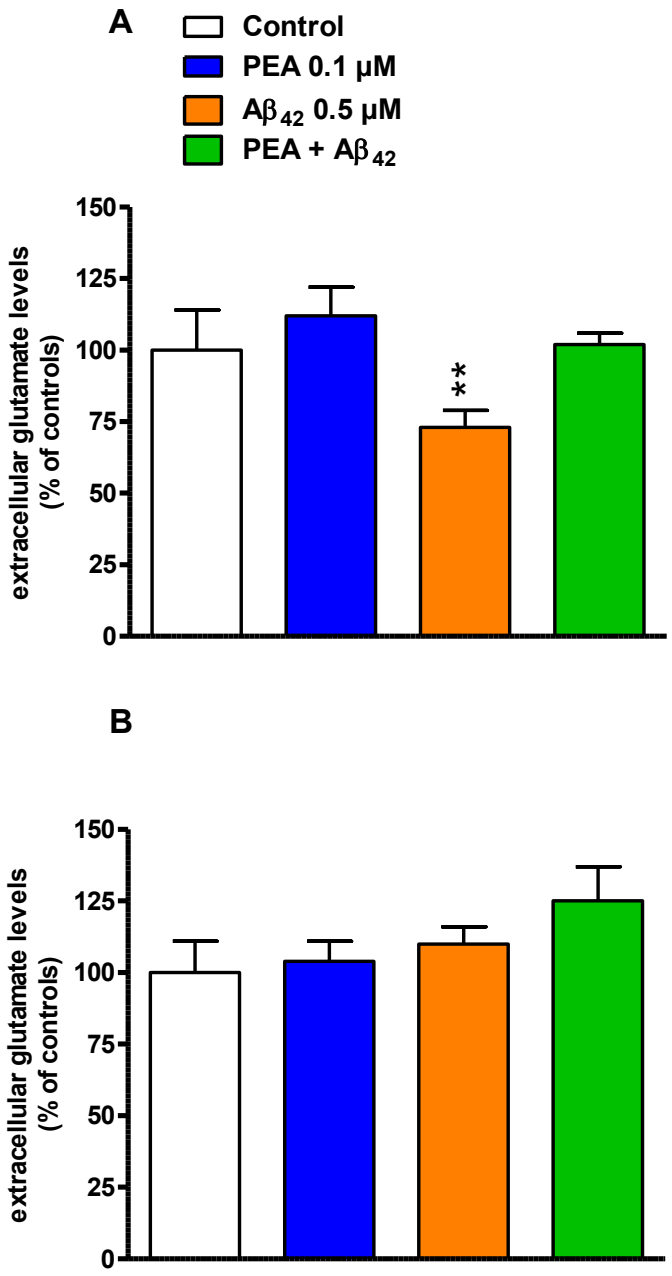


Fig. 23 Effects of $A\beta_{42}$ exposure (0.5 µM; 24 h), alone or in combination with PEA (0.1 µM), on extracellular glutamate levels in primary cultures of cerebral cortical neurons from non-Tg (A) and 3xTg-AD (B) mice. PEA was added 1 h before $A\beta_{42}$ and maintained in contact with the cells during $A\beta_{42}$ (24 h).

Each histograms represents the mean±S.E.M. (n = 32–42).

$^{**}p < 0.01$ significantly different from control, PEA and PEA + $A\beta_{42}$ groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

8.1.3 Effects of $A\beta_{42}$ exposure in the presence and in the absence of PEA on MAP-2 immunoreactivity in cultured cortical neurons from non-Tg mice and 3xTg-AD mice.

In view of the above results, the possibility that $A\beta_{42}$-exposure could affect morphological development and proliferation of cultured cortical neurons obtained from non-Tg mice, was explored. To this purpose, cultured cortical neurons were stained with an antibody for the neuronal marker MAP-2, which can be considered an index of the integrity of the cytoskeletonin AD (Takahashi et al., 2013). Control cultured cortical neurons presented a high number of healthy neurons, which developed a complex neuronal network characterized by highly arborized dendritic trees and MAP-2 immunoreactivity homogeneously distributed in the cell bodies and dendrites (Fig. 24, left panel). On the contrary, in cultured cortical neurons exposed to $A\beta_{42}$ (0.5 μM; 24 h) the neuronal network appeared fragmented (Fig. 24, right panel). In particular, a dishomogeneous distribution of MAP2 immunoreactivity along the neurites was observed, and the dendrites often appeared truncated.

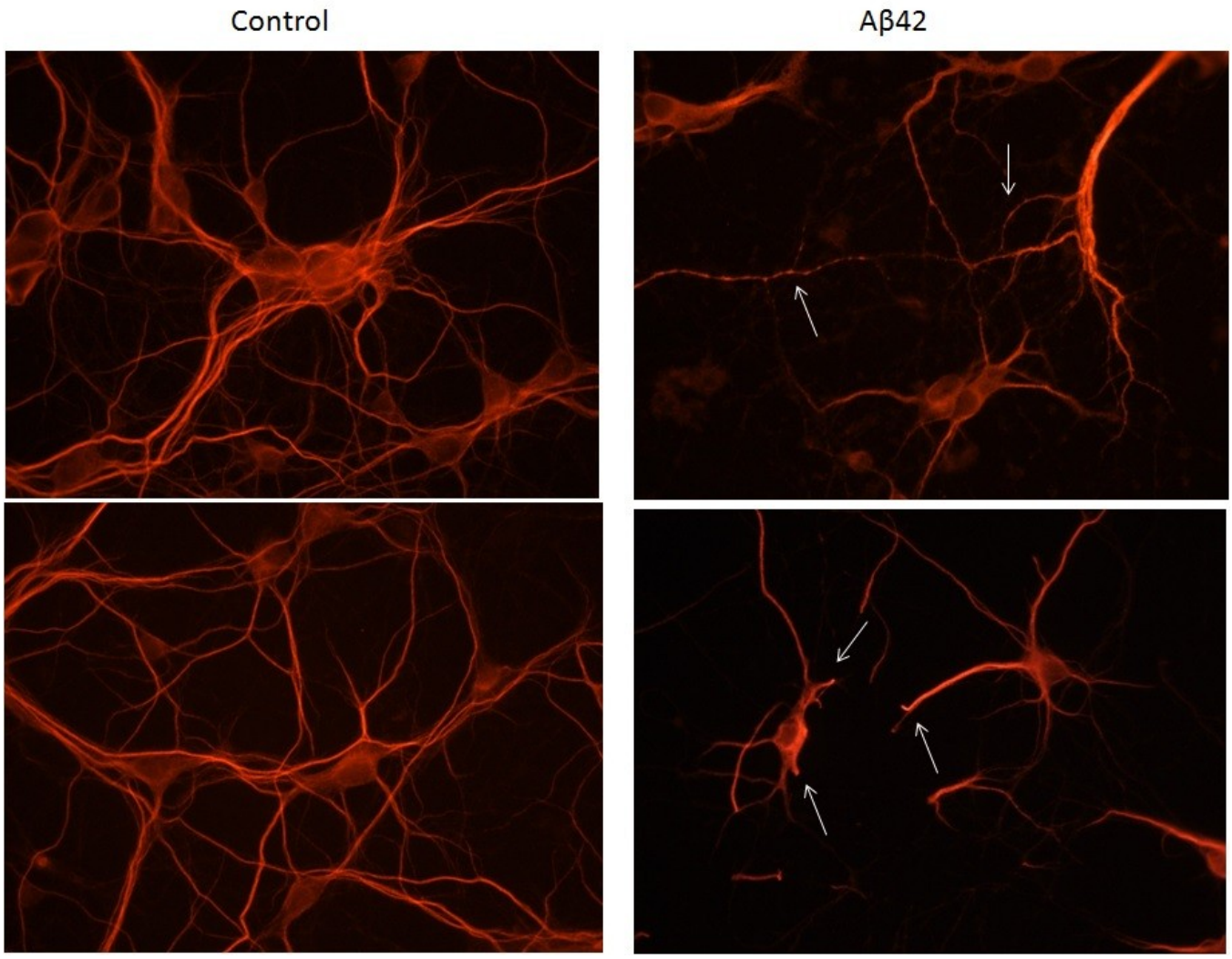


Fig. 24 Representative fluorescence photomicrographs of MAP-2 immunoreactivity in primary cultures of cerebral cortical neurons from non-Tg mice not exposed (left panel) and exposed to $A\beta_{42}$ (0.5 μM; 24 h; right panels). Local neuronal network fragmentation was indicated by the white arrows (right panels). Neurons were stained with anti-MAP-2 antibody and observed in sample fields under fluorescent microscope (magnification x40).

To quantify the effect of $A\beta_{42}$, the number of vital MAP-2 immunostained cultured neurons was determined. As shown in Fig. 25A, the number of neurons was significantly lower in cell cultures exposed to $A\beta_{42}$ than in control cultures from non-Tg mice, but not in cultured cortical neurons obtained from 3xTg- AD mice (Fig. 25B).

Pretreatment with PEA (0.1 μM), by itself ineffective, counteracted $A\beta_{42}$-induced decrease of neuron number in cultured cortical neurons obtained from non-Tg mice (Fig. 25B). By itself, PEA did not affect the number of neurons in cultured cortical neurons obtained from non-Tg mice (Fig. 25A) or 3xTg-AD mice (Fig. 25B).

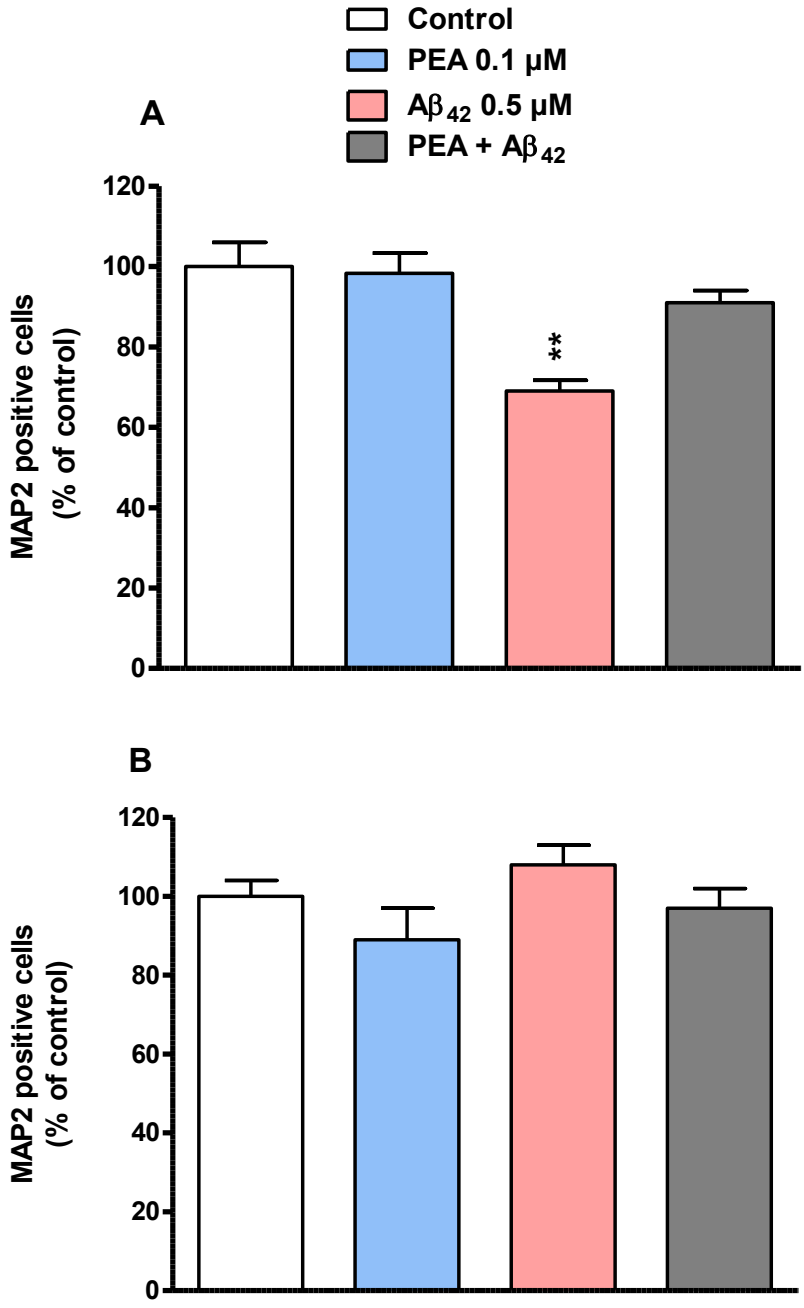


Fig. 25 Effects of $A\beta_{42}$ exposure (0.5 µM; 24 h), alone or in combination with PEA (0.1 µM), on the number of MAP-2 positive cells in primary cultures of cerebral cortical neurons from non-Tg (A) mice and 3xTg-AB (B) mice. PEA was added1 h before $A\beta_{42}$ and maintained in contact with the cell during $A\beta_{42}$ (24 h).

Neurons were stained with anti-MAP-2 antibody and observed under fluorescent microscope. Each histograms represents the mean±S.E.M. (n = 8/12).

**$p < 0.01$ significantly different from control, PEA and PEA + $A\beta_{42}$ groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

Finally, control cultured cortical neurons obtained from 3xTg-AD mice displayed morphological alterations similar to those observed in $A\beta_{42}$-exposed cultured cortical neurons obtained from non-Tg mice (Fig. 26, left panel). The exposure to $A\beta_{42}$ (0.5 μM) (Fig 26, right panel) or PEA (0.1 μM) (data not shown.) did not modify these alterations in cultured cortical neurons obtained from 3xTg-AD mice.

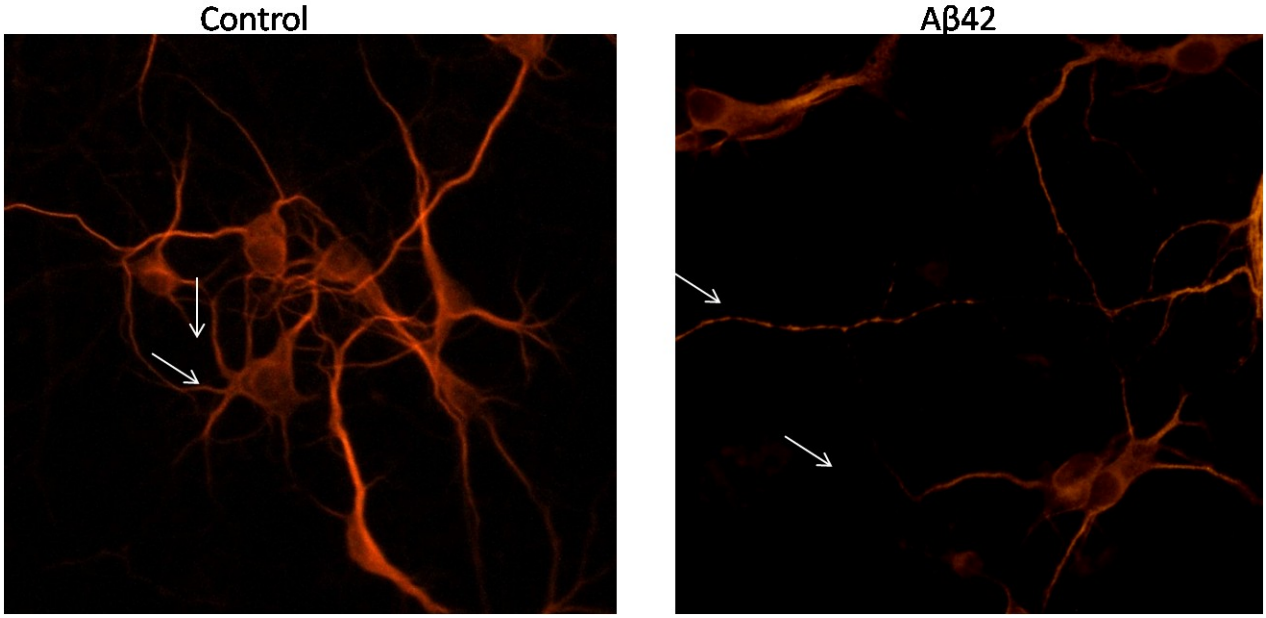


Fig. 26 Representative fluorescence photomicrographs of MAP-2 immunoreactivity in primary cultures of cerebral cortical neurons from 3xTg-AD mice not exposed (left panel) and exposed to $A\beta_{42}$ (0.5 μM; 24 h; right panels). Local neuronal network fragmentation was indicated by the white arrows (right panels). Neurons were stained with anti-MAP-2 antibody and observed in sample fields under fluorescent microscope (magnification x40).

8.2 Primary cultures of cerebral cortical astrocytes non-Tg and from 3xTg-AD mice.

8.2.1 Effects of $A\beta_{42}$ exposure in the presence and in the absence of PEA on cellular viability in cultured cortical astrocytes from non-Tg mice and 3xTg-AD mice.

$A\beta_{42}$ (0.5 μM; 24 h) exposure significantly increased the astroglial proliferation, as indicated by the enhanced cell viability measured in cultured cortical astrocytes obtained from non-Tg mice (Fig. 27A).

On the contrary, $A\beta_{42}$ exposure failed to modify the cell viability value in cultured cortical astrocytes obtained from 3xTg-AD mice (Fig. 27B). PEA pretreatment (0.1 μM) counteracted $A\beta_{42}$-induced astroglial proliferation in cultured cortical astrocytes obtained from non-Tg mice (Fig. 27A). By itself, PEA did not affect cell viability in cultured cortical astrocytes obtained from non-Tg mice (Fig 27A) or 3xTg-AD mice (Fig. 27B).

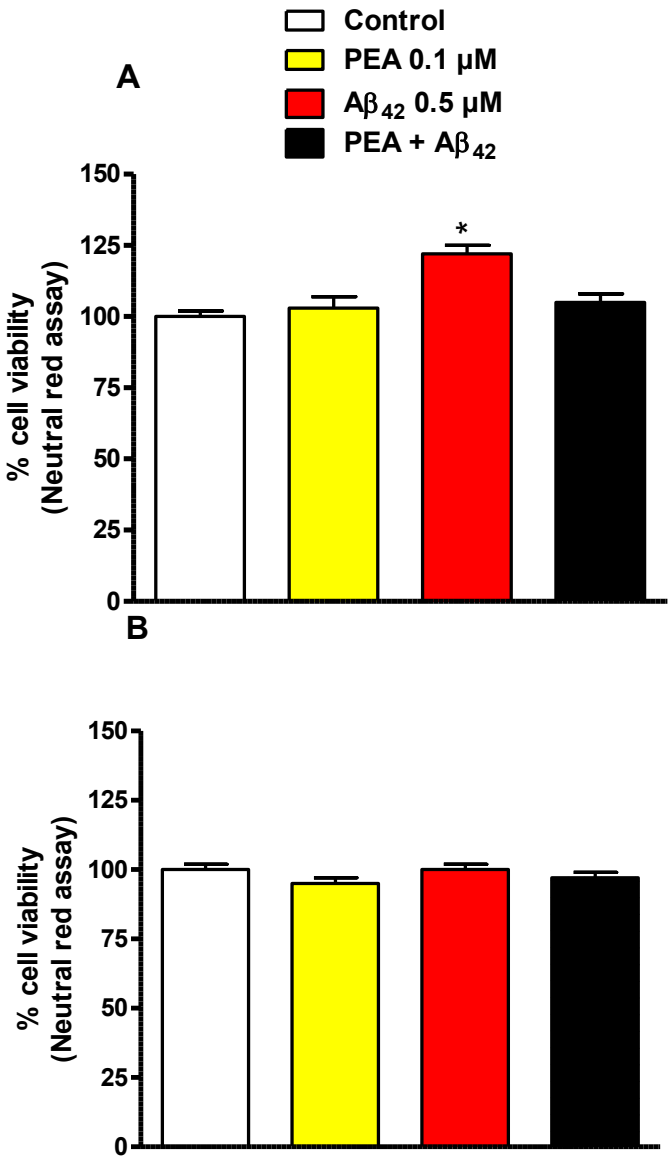


Fig. 27 Effects of $A\beta_{42}$ exposure (0.5 µM; 24 h), alone or in combination with PEA (0.1 µM), on cell viability in primary cultures of cerebral cortical astrocytes from non-Tg (A) and 3xTg-AD (B) mice. PEA was added 1 h before$A\beta_{42}$ and maintained in contact with the cells during $A\beta_{42}$ exposure (24 h). Cell viability was assessed by Neutral red assay and expressed as percentage of control values.

Each histogram represents the mean±S.E.M. (n = 30–40).

*p < 0.05 significantly different from control, PEA and PEA + $A\beta_{42}$ groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

8.2.2 Effects of $A\beta_{42}$ exposure in the presence and in the absence of PEA on endogenous extracellular glutamate levels in cultured cortical astrocytes from non-Tg mice and 3xTg-AD mice.

Basal extracellular glutamate levels in cultured cortical astrocytes obtained from non-Tg mice were significantly higher than those observed in cultured cortical astrocytes obtained from 3xTg-AD mice (1.994±0.122 μM and 0.087±0.007 μM, respectively). $A\beta_{42}$ (0.5 μM; 24 h) exposure increased extracellular glutamate levels in cultured cortical astrocytes obtained from non-Tg mice (Fig. 28A) but not in cultured cortical astrocytes obtained from 3xTg- AD mice (Fig. 28B). Pretreatment with PEA (0.1 μM) counteracted $A\beta_{42}$-induced increase of extracellular glutamate levels in cultured cortical astrocytes obtained from non-Tg mice (Fig. 28A). By itself, PEA did not affect extracellular glutamate levels in cultured cortical astrocytes obtained from non-Tg (Fig. 28A) or 3xTg-AD mice (Fig. 28B).

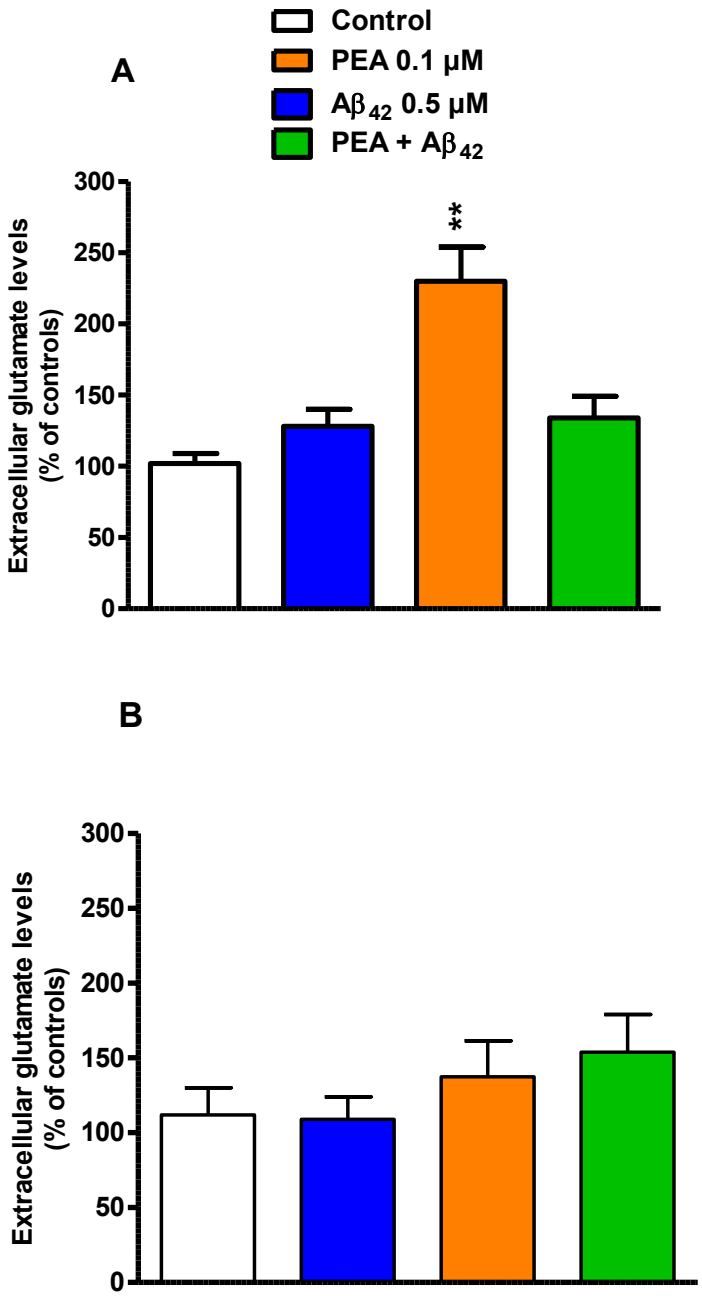


Fig. 28 Effects of $A\beta_{42}$ exposure (0.5 µM; 24 h), alone or in combination with PEA (0.1 µM), on extracellular glutamate levels in primary cultures of cerebral cortical astrocytes from non-Tg (A) and 3xTg-AD (B) mice. PEA was added 1 h before $A\beta_{42}$ and maintained in contact with the cells during $A\beta_{42}$ exposure (24 h).

Each histograms represents the mean±S.E.M. (n = 32–42).

∗∗p <0.01 significantly different from control, PEA and PEA + $A\beta_{42}$ groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

8.2.3 Effects of $A\beta_{42}$ exposure in the presence and in the absence of PEA on GFAP immunoreactivity in cultured cortical astrocytes from non-Tg mice and 3xTg-AD mice.

Morphological changes induced by $A\beta_{42}$ exposure were also evaluated in GFAP immunostained cultured cortical astrocytes obtained from non-Tg and 3xTg-AD mice. In control cultured cortical astrocytes from non-Tg mice, GFAP positive cells showed numerous branched processes, extending outward from the somata in multiple directions (stellate shape), that are typical of healthy astrocytes (Fig. 29, left panel), whereas the control cortical astrocytes from 3xTg-AD mice showed already morphological alterations, similar to those in $A\beta_{42}$-treated astrocytes from non-Tg cortical cultures (Fig. 29, right panel).

Following the exposure to $A\beta_{42}$ (0.5 μM; 24 h), the morphology of cultured astrocytes from non-Tg mice resulted changed since convolutions and swellings in the terminal part of the processes were clearly evident (Fig. 30).On the contrary, the exposure to $A\beta_{42}$ (0.5 μM; 24 h) failed to affect the morphology of cultured astrocytes from 3xTg-AD mice (data not shown). To quantify the effect of $A\beta_{42}$ exposure, the number of vital GFAP immunostained cultured astrocytes from non-Tg mice was determined. As shown in Fig. 31, the number of astrocytes was significantly higher in cell cultures exposed to $A\beta_{42}$ than in control cultures. Pretreatment with PEA (0.1 μM), by itself ineffective, counteracted the $A\beta_{42}$-induced decrease of neuron number (Fig. 31).

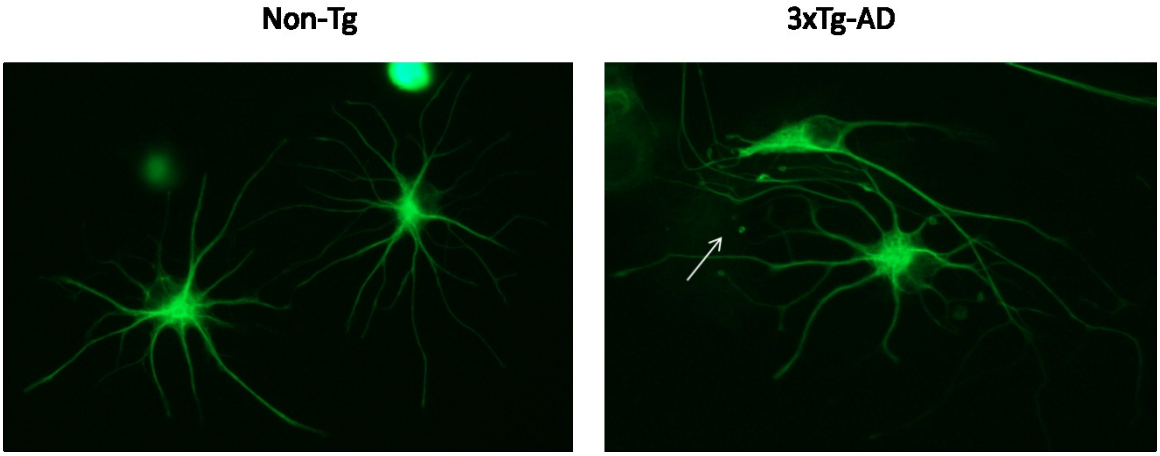
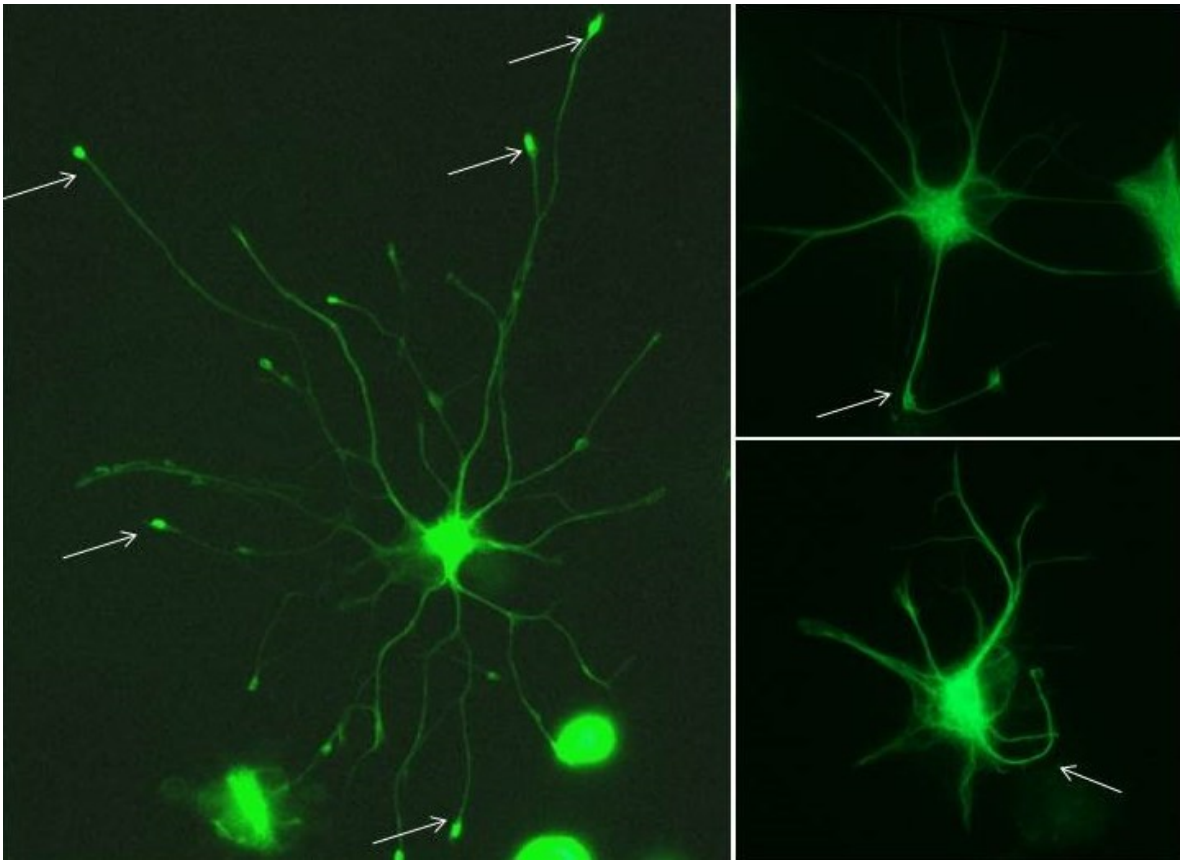


Fig. 29 Representative fluorescence photomicrographs of GFAP immunoreactivity in primary cultures of cerebral cortical astrocytes from non-Tg (left panel) and 3xTg-AD mice (right panel) after 24 h of $A\beta_{42}$ (0.5 μM) exposure. Astrocytes were stained with anti-GFAP antibody and observed in sample field under fluorescent microscope (magnification ×40). Alterations of the morphology are indicated by the white arrows.



Fig, 30 Representative fluorescence photomicrographs of GFAP immunoreactivity in primary cultures of cerebral cortical astrocytes from non-Tg mice after 24 h of $A\beta_{42}$ (0.5 μM) exposure. Alterations of the morphology are indicated by the white arrows. Astrocytes were stained with anti-GFAP antibody and observed in sample fields under fluorescent microscope (magnification ×40).

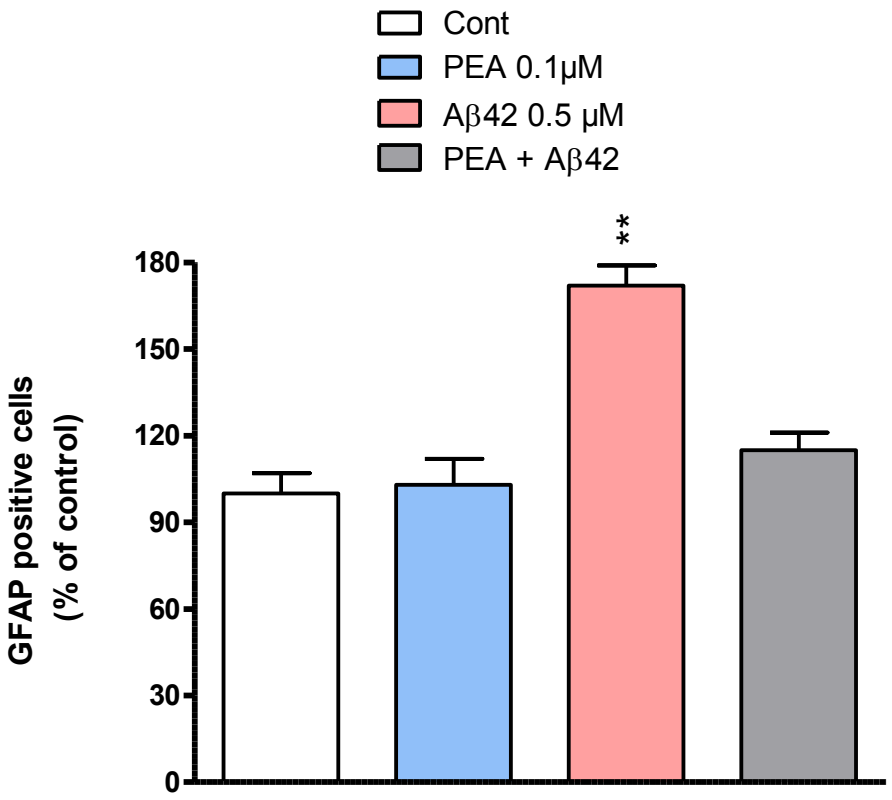


Fig. 31 Effects of $A\beta_{42}$ exposure (0.5 μM; 24 h), alone or in combination with PEA (0.1 μM), on the number of GFAP positive cells (expressed as percentage of control value) in primary cultures of cerebral cortical astrocytes from non-Tg mice. Astrocytes were stained with anti-GFAP antibody and observed under fluorescent microscope.

Each histograms represents the mean±S.E.M. (n = 8/12).

**$p<0.01$ significantly different from control, PEA and PEA + $A\beta_{42}$ groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

9. DISCUSSION.

In the present study we compared, for the first time, the effects of $A\beta_{42}$ exposure on cell viability and glutamatergic transmission in primary cultures of cerebral cortex neurons and primary cultures of cerebral cortex astrocytes from 3xTg-AD and non-Tg (i.e., wild-type) mice. Moreover, the possible protective role of PEA against $A\beta_{42}$ toxicity was also evaluated in 3xTg-AD and non-Tg mouse cell cultures.

Primary cultures of cerebral cortex neurons

As expected, treatment with $A\beta_{42}$ caused degeneration in cortical neurons obtained from non-Tg mice, as demonstrated by the biochemical and morphological approaches. In particular, after 24 h of $A\beta_{42}$ exposure, a very small, but significant, decrease in cell viability was observed, and this effect was also associated with a reduction of vital MAP-2 immunostained cultured neuron number. This latter parameter has been used as an index of neurodegeneration since MAP-2 cytoskeletal protein, predominantly expressed in neurons, plays important roles in the outgrowth of neuronal processes, synaptic plasticity, and neuronal cell death. Staining of the neurites with MAP-2 antibody in non-Tg cortical cell cultures exposed to $A\beta_{42}$ revealed an abnormal outgrowth of these cell projections, mainly characterized by the fragmentation of neuronal network, reaching in some cases the breakage of the neuronal processes. The impairment induced by $A\beta_{42}$ in the neurite development observed in the present study is in agreement with recent data, reporting that axon degeneration in cultured hippocampal neurons is a key component of neuronal death following $A\beta_{42}$ exposure (Alobuia et al., 2013). Furthermore, a microtubule deregulation after $A\beta_{42}$ treatments has been observed in other studies. In particular, Mota et al. (Mota et al., 2012) documented $A\beta_{42}$-induced decreases in total and polymerized levels of β-III tubulin along with polymerized β-tubulin, and these alterations were correlated with a reduced neurite length. Finally, $A\beta_{42}$-induced microtubule depletion and loss of spines (Zempel et al., 2010) as well as a retraction of synaptic contacts (Rönicke et al., 2011) were also observed.

In cortical cell cultures from non-Tg mice, $A\beta_{42}$ treatment also impaired glutamatergic signaling as demonstrated by the reduction of extracellular glutamate levels. This effect is more evident than the observed $A\beta_{42}$-induced reduction of cultured cell number. Although a

direct correlation is not possible, this observation led to speculate that also other mechanism(s), such as impairment of neurite outgrowth and other nervous terminal injuries, could contribute to $A\beta_{42}$-induced reduction of extracellular glutamate levels. The damage of nervous terminals could, in fact, impair the exocytotic mechanisms, leading to reduced glutamate efflux from cortical neurons. At this regard, several studies showed synaptic dysfunction associated with $A\beta_{42}$ exposure, particularly at presynaptic level (Cuello, 2005; Calkins et al., 2011; Mezler et al., 2012; Quiroz-Baez et al., 2013; Mota et al., 2014). Deleterious effects of $A\beta_{42}$ on multiple steps of synaptic vesicle trafficking, leading to weaken synaptic transmission have also been reported (Park et al., 2013). $A\beta_{42}$-treated neurons also displayed reduced number of synaptic vesicles and a reduction in several presynaptic proteins (Parodi et al., 2010). A reduction in the density of the vesicular glutamate transporter 1 (VGluT1) and a decrease in the number of vGluT1-immunopositive hippocampal nerve terminals were observed in a mouse model of AD (Canas et al., 2013), suggesting a particular susceptibility of glutamatergic nerve terminals to $A\beta_{42}$-induced toxicity. In fact, intracellular accumulation of $A\beta_{42}$ dramatically affects glutamatergic synaptic function at both presynaptic and postsynaptic levels (Ripoli et al., 2014). Finally, there is strong evidence for $A\beta_{42}$-induced impairments in mitochondrial transport, dynamics and function that contribute to synaptic degeneration (Calkins et al., 2011; Ferreira et al., 2010; Reddy et al., 2010).

Interestingly, the exogenous $A\beta_{42}$-induced reductions of cell viability and extracellular glutamate levels were not observable in cortical cell cultures from 3xTg-AD mice. The discrepancies between the results observed in cultured cells from the two genotypes could be due to the expression of endogenous intra- and extra-neuronal $A\beta_{42}$ peptides in 3xTg-AD mouse-derived cell cultures. In fact, an early in vitro $A\beta_{42}$ overexpression associated with increased $A\beta_{42}$ levels was evident in cultured cortical neurons of 6 DIV obtained from 3xTg-AD mice (Vale et al., 2010). Furthermore, an altered calcium homeostasis and decreased glutamatergic response were also observed in cultured cortical neurons from 3xTg-AD mice (Vale et al., 2010; Stutzmann et al., 2004; Stutzmann et al., 2007; Smith et al., 2005). In view of these data, it could be suggested that in the present study the exogenous $A\beta_{42}$ was ineffective in 3xTg-AD mice-derived cortical neurons as this cells at 8 DIV were already exposed to a quite high concentration of endogenous $A\beta_{42}$ fragments. This view is supported by i) the demonstration that control cultured cortical neurons obtained from 3xTg-AD mice displayed morphological alterations similar to those observed in $A\beta_{42}$-exposed cultured

cortical neurons obtained from non-Tg mice; ii) the evidence that basal extracellular glutamate levels in cortical cell cultures from 3xTg-AD mice were significantly lower than those measured in non-Tg mouse cultured neurons. This finding is in line with previous data demonstrating a modification of the plasma membrane electrical excitability, leading to changes on synaptic function and consequently on glutamate transmission (Vale et al., 2010). Furthermore, in vivo microdialysis studies reported a significant decrease of basal glutamate release in the frontal cortex and hippocampus of 18-month-old 3xTg-AD-mice (Cassano et al., 2012) and a reduction of KCl-stimulated glutamate release in the hippocampus of 17-month-old APdE9 mice (Minkeviciene et al., 2008). Interestingly for the possible translational aspects of the present findings, there are data in literature reporting reduced glutamate tissue levels in AD brains (Minkeviciene et al., 2008; Hyman et al., 1987; Lowe et al., 1990).

Primary cultures of cerebral cortex astrocytes

It is known that astrocytes are the principal homeostatic cells of the central nervous system. Although the role of astroglia in AD pathogenesis remains generally unknown, the interest in astroglial remodeling in the course of neurodegeneration has increased substantially during the last decade (Grolla et al., 2013). Thus, in the present study primary cultures of cerebral cortex astrocytes have been chosen as an *in vitro* experimental model to study the contribution of astrocytes to the possible protective effects of PEA against $A\beta_{42}$ toxicity. $A\beta_{42}$ exposure (24 h) induced a proliferation of cultured astrocytes from non-Tg mice. This result suggests that $A\beta_{42}$ causes reactive astrogliosis (Lü et al., 2009) as previously observed in human AD tissues (Lü et al., 2009) and in cultured animal astrocytes (Scuderi et al., 2011; Scuderi et al., 2012; Casal et al., 2004). It is worth noting that, in the present study, $A\beta_{42}$ exposure also induced an alteration in the morphology of cultured astrocytes obtained from non-Tg mice, which represents another important sign of reactive astrogliosis. In particular, $A\beta_{42}$ caused an alteration in the growth and development of astrocytic processes, with the appearance of convoluted processes and terminal swellings, while control cultures presented processes extending radially from the somata to the periphery. These $A\beta_{42}$-induced changes in astrocyte morphology were probably indicative of an activation state, at which the cells released proinflammatory mediators (Garwood et al., 2011). Interestingly, similar

alterations have been observed in 1–12 months 3xTg-AD mouse astrocytic morphology (Olabarria et al., 2010; Yeh et al., 2011), but not in the present study.

These $A\beta_{42}$-induced effects probably indicated a rearrangement of cytoskeleton filaments, which could modify the functionality of astrocytes. It has been shown that in pathological conditions, such as in AD, the activated glial cells produce inflammatory mediators, including TNF-α and prostaglandin E2, which increase intracellular Ca^{2+} levels in astrocytes, leading to the release of gliotransmitters, such as glutamate (Hamilton and Attwell, 2010). In view of these findings, the effects of $A\beta_{42}$ on astrocyte functionality have been assessed by evaluating extracellular glutamate levels. The exposure of non-Tg mouse cultured astrocytes to $A\beta_{42}$ (24 h) significantly increased extracellular glutamate levels. A previous study demonstrated that astrocytes exposed (72 h) to 10 μM $A\beta_{25-35}$ exhibit increased glutamate release (Orellana et al., 2011). This effect could be due at least to two different mechanisms. Firstly, $A\beta_{42}$ could cause the release of pro-inflammatory cytokines by cultured astrocytes (Scuderi et al., 2011; Garwood et al., 2011; Orellana et al., 2011) along with a consequent increase of intracellular Ca^{2+} levels leading to exocytotic glutamate release. Secondly, $A\beta_{42}$ could decrease glutamate uptake (Abe and Misawa, 2003) and compromise the activity of glutamate transporters GLT-1 and GLAST (Matos et al., 2008; Matos et al., 2012), thus reducing glutamate reuptake. Previous studies reported a significant reduction in the activity of glutamate transporters in human AD tissues (Scott et al., 1995; Masliah et al., 1996) and in animal models of AD (Masliah et al., 2000; Scott et al., 2002). Based on these data, we expected to observe higher basal glutamate levels in cultured astrocyte from 3xTg-AD mice than in those from non-Tg mice. Surprisingly, an opposite result was obtained. This finding could be the consequence of increased glutamate reuptake in this specific animal model of AD. In fact, an increase of GLT1 expression has been shown in frontal cortex of 3xTg-AD mice (Cassano et al., 2012). Differently, Kulijewicz-Nawrot and colleagues (Kulijewicz-Nawrotet al., 2013) did not find any changes in the expression of GLT-1 in prefrontal cortex astrocytes from 3xTg-AD mice. However, other mechanisms could underlie the reduction of basal glutamate levels in cultured astrocytes from 3xTg-AD mice and other experiments will be necessary to explain this phenomenon. As observed in primary cultures of cerebral cortex neurons, $A\beta_{42}$-induced effects on extracellular glutamate levels, cell viability, and cell morphology have not been detected in 3xTg-AD mouse cultured astrocytes. The loss of responsiveness to a challenge with $A\beta_{42}$ in astrocytes from 3xTg-AD mice was already

observed in a recent work (Grolla et al., 2013). In particular, these authors observed significant effects of $A\beta_{42}$ exposure on the expression of mGluR5 and inositol 1,4,5-trisphosphate receptor type 1 as well as on parameters of metabotropically stimulated $[Ca^{2+}]_i$ transients in entorhinal cortex and hippocampal astrocytes derived from non-Tg mice. These effects were absent in entorhinal cortex and hippocampal astrocytes derived from 3xTg-AD mice. Furthermore, they demonstrated that senile plaque formation in 3xTg-AD mice triggers astrogliosis in hippocampal but not in entorhinal cortex astrocytes. The authors suggested that the expression of AD-related mutant genes in the transgenic mice could deregulate Ca^{2+} homeostasis and signaling in astroglia (Grolla et al., 2013). Therefore, we may speculate that also a deregulation in some pathways regulating glutamate release/efflux could be responsible of the lack of $A\beta_{42}$ effect in the cultured astrocytes from the animal model of AD.

PEA-induced protection against $A\beta_{42}$ toxicity.

An anti-inflammatory neuroprotective role has been suggested for the endogenous fatty acid amide PEA, member of N-acyl-ethanolamines (Esposito and Cuzzocrea, 2013; Esposito and Cuzzocrea, 2013(b); LoVerme et al., 2005; D'Agostino et al., 2012; Paterniti et al., 2013).

Furthermore, a recent study reported that PEA, by activating PPAR-α, rescues altered molecular pathways as well as behavioral impairments that can mimic some early traits of AD. Based on these findings, in the present study the possible protective role of PEA against $A\beta_{42}$ toxicity has been also investigated in primary cultures of cortical neurons and astrocytes from both the mouse genotypes. PEA pretreatment counteracts the reduction of cell viability induced by $A\beta_{42}$ in cultured cortical neurons from non-Tg mice. These data were in agreement with previous morphological and biochemical studies, showing that PEA pretreatment significantly reduced $A\beta_{42}$-induced neuronal loss in rat organotypic hippocampal slice cultures and rat neuronal cultures (Skaper and Facci, 2012; Scuderi and Steardo, 2013). The present results also suggested protective effects of PEA in non-Tg mouse cultured cortical astrocytes, where the compound was able to prevent the $A\beta_{42}$-induced cell proliferation. A similar result has been recently obtained in rat organotypic hippocampal slice cultures exposed to $A\beta_{42}$ (Scuderi et al., 2012). Furthermore, evidence that PEA reduced the astrocytic production of proinflammatory molecules and cytokine release in an in vitro model of Aβ neurotoxicity, has been also provided (Scuderi et al., 2011). Interestingly, the present

study also described, for the first time, a protective effect of PEA pretreatment on the $A\beta_{42}$-induced alterations of glutamatergic signaling, observed both in cultured neurons and in cultured astrocytes from non-Tg mice. Overall, these results suggest that PEA could be effective in preventing not only the $A\beta_{42}$-induced cell death, but also the loss of functionality of cortical neurons and astrocytes triggered by the exposure to the peptide. Further studies are necessary for elucidating the possible involvement of PPARs, GPR55, CB2, or other receptors in the protective effects of PEA. However, recent studies suggest some possibilities. Thus, the neuroprotective effect of PEA could be dependent on its ability to counteract the inflammatory processes, through the activation of the anti-inflammatory nuclear receptor PPAR-α and the consequent gene expression regulation (Scuderi et al., 2012). Furthermore, PEA by activating PPAR-α could enhance the number of peroxisomes and/or the activity of the peroxisomal matrix protein catalase counteracting the redox perturbation following the $A\beta_{42}$ excess (D'Agostino et al., 2012).

In the present study, we propose that the lack of $A\beta_{42}$-induced toxicity in cultured cells from 3xTg-AD mice could be ascribed to the expression of endogenous intra- and extra-neuronal Aβ peptides in this in vitro animal model of AD (see above). Thus, one would expect that PEA exerted by itself positive effects on cell viability and functionality in primary cultures of cerebral cortex neurons and astrocytes from 3xTg-AD mice. On the contrary, the present results demonstrated that PEA did not display any effects on cell viability and extracellular glutamate levels in cultured cortical neurons from 3xTg-AD mice. A possible explanation of this result is that, in this study, the cultures were used after 8 DIV, while early in vitro Aβ overexpression associated with increased $A\beta_{42}$ levels was already evident in cultured cortical neurons of 6 DIV obtained from 3xTg-AD mice (Vale et al., 2010). Furthermore, previous studies have demonstrated that the expression of fatty acid amide hydrolase (FAAH) enzyme is elevated in astrocytes in AD (Benito et al., 2003) and in Down's syndrome, sometimes referred to as a human model of AD-like $A\beta_{42}$ deposition (Núñez et al., 2008). This could also contribute to the lack of effects of PEA, which is a substrate of FAAH in cultured astrocytes obtained from transgenic mice.

CONCLUSIONS

The present study indicates that PEA exerts differential effects against Aβ-induced toxicity in primary cultures of cortical neurons and astrocytes from non- Tg (wild-type) and 3xTg-AD mice. In particular, PEA displays protective properties in wild-type mouse cell cultures but not in 3xTg-AD mouse neuronal cultured cells overexpressing Aβ. Taken together, these findings suggest that the compound may be effective in the early AD or when Aβ is accumulating and initiating damage in the central nervous system. In this context, it will be relevant to evaluate the effects of PEA on cellular viability and glutamate release in vitro choosing a period of exposure of the 3xTg-AD mouse neurons to the toxic peptide preceding the development of Aβ accumulation and tau hyperphosphorylation (Vale et al., 2012; Alonso et al., 2011).

"EXCESSIVE RESERVATIONS AND PARALYSING DESPONDENCY HAVE NOT HELPED THE SCIENCES TO ADVANCE NOR ARE THEY HELPING THEM TO ADVANCE, BUT A HEALTHY OPTIMISM THAT CHEERFULLY SEARCHES FOR NEW WAYS TO UNDERSTAND, AS IT IS CONVINCED THAT IT WILL BE POSSIBLE TO FIND THEM."

Alzheimer's research motto.

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