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Differential Effects of Palmitoylethanolamide against Amyloid-β Induced Toxicity in Cortical Neuronal and Astrocytic Primary Cultures from Wild-Type and 3xTg-AD Mice

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Abstract.
Background: Considering the heterogeneity of pathological changes occurring in Alzheimer's disease (AD), a therapeutic approach aimed both to neuroprotection and to neuroinflammation reduction may prove effective. Palmitoylethanolamide (PEA) has attracted attention for its anti-inflammatory/neuroprotective properties observed in AD animal models.
Objective and Methods: We evaluated the protective role of PEA against amyloid-β_{42} ($A\beta_{42}$) toxicity on cell viability and glutamatergic transmission in primary cultures of cerebral cortex neurons and astrocytes from the triple-transgenic murine model of AD (3xTg-AD) and their wild-type littermates (non-Tg) mice.
Results: $A\beta_{42}$ (0.5 μM; 24 h) affects the cell viability in cultured cortical neurons and astrocytes from non-Tg mice, but not in those from 3xTg-AD mice. These effects were counteracted by the pretreatment with PEA (0.1 μM). Basal glutamate levels in cultured neurons and astrocytes from 3xTg-AD mice were lower than those observed in cultured cells from non-Tg mice. $A\beta_{42}$-exposure reduced and increased glutamate levels in non-Tg mouse cortical neurons and astrocytes, respectively. These effects were counteracted by the pretreatment with PEA. By itself, PEA did not affect cell viability and glutamate levels in cultured cortical neuron and astrocytes from non-Tg or 3xTg-AD mice.
Conclusion: The exposure to $A\beta_{42}$ induced toxic effects on cultured cortical neurons and astrocytes from non-Tg mice, but not in those from 3xTg-AD mice. Furthermore, PEA exerts differential effects against $A\beta_{42}$-induced toxicity in primary cultures of cortical neurons and astrocytes from non-Tg and 3xTg-AD mice. In particular, PEA displays protective properties in non-Tg but not in 3xTg-AD mouse neuronal cultured cells overexpressing Aβ.

Keywords: Alzheimer's disease, cell viability, GFAP immunoreactivity, glutamate, MAP2 immunoreactivity

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INTRODUCTION

Alzheimer's disease (AD) is an age-dependent, multifactorial neurodegenerative pathology resulting in the deterioration of selective cognitive performance including memory, and learning impairment [1–3]. The neurodegenerative process in AD is characterized by the presence of two classes of abnormal structures: extracellular amyloid plaques surrounded by activated microglia, reactive astrocytes, dystrophic neurites, and degenerating neurons [4], along with intraneuronal neurofibrillary tangles subsequent to an abnormal tau protein phosphorylation [5–7]. Although the classical so-called "amyloid cascade hypothesis" has been recently revisited, a large body of evidence suggests that the overexpression of the amyloid-β protein precursor (AβPP) and subsequent generation of amyloid-β (Aβ) fragments is central to the neurodegeneration observed in AD patients. Aβ appears to exert some of its neurotoxic effects through numerous secondary pathways, including tau hyperphosphorylation and neurofibrillary tangle formation, oxidation, inflammation, demyelination, and excitotoxicity. For instance, both *in vitro* and *in vivo* findings have demonstrated that Aβ fragments promote a marked neuroinflammatory response, sustained by glial cells, accounting for the synthesis of different cytokines and proinflammatory mediators [8, 9]. As astrocytes outnumber microglia in the brain and their activation seems to last longer, they may have a more important and sustained role over microglia in the enduring neuroinflammation in AD [10]. The secreted proinflammatory factors and alterations in the expression of several proteins that support and accelerate the neurodegenerative events [11], make neurons particularly vulnerable to cytotoxic events, including glutamate excitotoxicity [12]. In fact, it is well demonstrated that a sustained increase in extracellular glutamate levels, associated with overstimulation of N-methyl-D-aspartate (NMDA) receptors, may represent an additional pathogenetic basis of neurodegeneration in AD [13]. In this context, it is worth noting that astrocytes exert a central role in brain homeostasis, in particular via the numerous cooperative metabolic processes that they establish with neurons, such as the supply of energy metabolites and neurotransmitter recycling functions. Thus, it has been reported that impairments in astrocytic function play an important role in neuronal dysfunction, and could contribute to excitotoxic phenomena observed in the neurodegenerative processes [14]. In view of the above, it seems clear that the neuroinflammatory process sustained by excessive and prolonged astrocyte activation might alter neuron/astrocyte cooperation, thus causing deleterious effects on neurons and contributing to the AD neuronal cell loss. A therapeutic approach aimed both to neuroprotection and neuroinflammation reduction may therefore prove effective in slowing the progression of the disease [15].

Cannabinoids (CBs) or the modulation of the endocannabinoid signals have been proposed as possible therapeutic approaches to AD [16–18]. The endocannabinoid system is extensively involved in the neuroinflammatory process, exerting an inhibitory and neuroprotective role both at the peripheral level, by modulating plasma and tissue immune cells, and on central glial cells [19, 20]. Anti-inflammatory and neuroprotective functions have been particularly attributed to the endocannabinoids belonging to the acylethanolamide family, like anandamide as well as to anandamide congeners oleoylethanolamide and palmitoylethanolamide (PEA), since their production is greatly increased in the sites of neuronal damage [21]. In the central nervous system, PEA, produced by neurons, microglia, and astrocytes, exerts a local anti-injury function through a down-modulation of mast cells and by protecting neurons from excitotoxicity [22–24]. However, its exact biological roles remain elusive. Recently, PEA has been defined as a cannabinoid receptor-inactive endocannabinoid-related molecule [23], with different mechanisms of action such as the activation of a cell surface receptor [CB2-like or the G-protein coupled receptor 55, orphan (GPR55)] [25], the activation of a nuclear receptor of the peroxisome proliferator-activated receptor (PPAR) family [26, 27] and the action as "*entourage*" compound enhancing endocannabinoid activity at their receptors and/or inhibiting endocannabinoid degradation [28]. In addition, *in vitro* and *in vivo* results have suggested anti-inflammatory and neuroprotective properties of PEA against Aβ-induced neurotoxicity [29–32]. On the contrary, 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), which harbors three mutant human genes ($A\beta PP_{Swe}$, $PS1_{M146V}$, tau_{P301L}) closely mimics many aspects of AD in humans. In fact, these animals are characterized by age-dependent build-up of both plaques and tangles in the cerebral cortex, hippocampus, and amygdala regions [33, 34]. Moreover, evidence of progressive deficits in synaptic plasticity and in cognitive functions has been shown in the 3xTg-AD mice [33, 35]. It has also been reported that the

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primary neuronal cultures from 3xTg-AD mice represent the first *in vitro* model of AD characterized by a simultaneous overexpression of AβPP, Aβ, and tau protein useful to evaluate cellular and molecular mechanisms associated with the pathology of AD in order to investigate new pharmacological approaches [34].

Based on the above findings, the main aim of the present study was to assess the protective effects of PEA on $A\beta_{42}$-induced toxicity by studying cell viability and morphology. Moreover the ability of PEA to modulate the function of cortical glutamatergic neurons and astrocytes, by measuring basal endogenous glutamate release, was evaluated. All experiments were carried out in primary cultures of neurons and astrocytes obtained from the cerebral cortex of wild-type (non-Tg) and 3xTg-AD mice. The cerebral cortex was chosen as the most representative area among the brain regions affected by AD.

MATERIALS AND METHODS

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 [36]. The 3xTg-AD mice harboring $A\beta PP_{swe}$, $PS1_{M146V}$, and tau_{P301L} transgenes were genetically engineered by LaFerla and colleagues at the Department of Neurobiology and Behavior, University of California, Irvine [33, 37]. Genotypes were confirmed by polymerase chain reaction (PCR) after tail biopsies [33]. 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. 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.

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 [38]. 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.

Primary cultures of cerebral 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. [30], 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

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fibrillary acidic protein (GFAP) and only cultures with more than 95% GFAP-positive cells were used for the experiments.

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. The concentration of the substances was chosen according to previous results [29, 30]. Cell viability, cell count and glutamate levels were assessed after 24 h of treatment.

Neutral red assay

The neutral red assay was used to assess cell viability [31, 39]. 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 using a microplate absorbance reader (Sunrise, Tecan). The values of treated cells were referred to control non-exposed cultures, and expressed as percentage variation.

Immunocytochemistry

Cells were rinsed in 0.1 M PBS and then fixed with 4% paraformaldehyde in Sorensen's buffer 0.1 M, 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 (MAP2) (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. Nuclei were stained with Hoechst 33258 (1 μg/ml; Sigma Aldrich, St. Louis, MO, USA) added to the secondary antibody solution. 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-MAP2 antibody, anti-GFAP antibody, rhodamine-conjugated anti-rabbit antibody, and fluorescein isothiocyanate-conjugated anti-mouse antibody were purchased from Chemicon, Temecula, CA.

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% tetrahydrofuran, 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 [40].

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

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Newman-Keuls test for multiple comparisons. $p < 0.05$ was the accepted level of significance.

RESULTS

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

Effects of Aβ42 exposure in the presence and in the absence of PEA on cellular viability

The exposure to Aβ42 (0.5 μM; 24 h) induced a significant decrease in the cell viability in cultured cortical neurons obtained from non-Tg mice (Fig. 1A), but not in cultured cortical neurons obtained from 3xTg-AD mice (Fig. 1B). Pretreatment with PEA (0.1 μM) fully counteracted Aβ42-induced decrease of cell viability in cultured cortical neurons obtained from non-Tg mice (Fig. 1A). By itself, PEA did not affect cell viability in cultured cortical neurons obtained from non-Tg mice (Fig. 1A) or 3xTg-AD mice (Fig. 1B).

Effects of Aβ42 exposure in the presence and in the absence of PEA on endogenous extracellular glutamate levels

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, respectively). Aβ42 (0.5 μM; 24 h) exposure reduced extracellular glutamate levels in cultured cortical neurons obtained from non-Tg mice (Fig. 2A), but not in cultured cortical neurons obtained from 3xTg-AD mice (Fig. 2B). Pretreatment with PEA (0.1 μM) counteracted Aβ42-induced decrease of extracellular glutamate levels in cultured cortical neurons obtained from non-Tg mice (Fig. 2A). 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. 2B).

Effects of Aβ42 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 Aβ42-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 MAP2, which can be considered an index of the integrity of the cytoskeleton

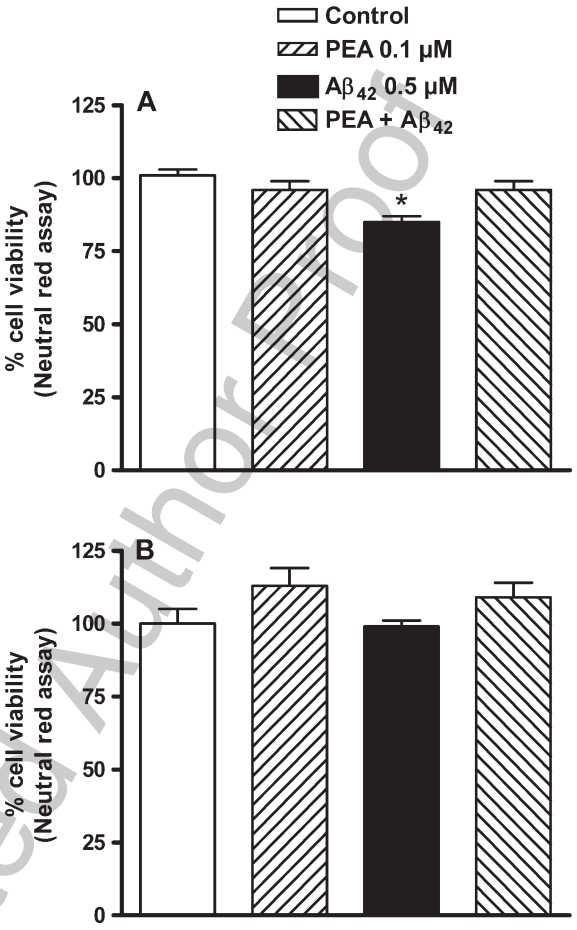

Fig. 1. Effects of Aβ42 exposure (0.5 μM; 24 h), alone or in combination with PEA (0.1 μM), on cell viability in primary cultures of cerebral cortical neurons from non-Tg (A) and 3xTg-AD (B) mice. PEA was added 1 h before Aβ42 and maintained in contact with the cells during Aβ42 exposure. Cell viability 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 + Aβ42 groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

in AD [41]. Control cultured cortical neurons presented a high number of healthy neurons, which developed a complex neuronal network characterized by highly arborized dendritic trees and MAP2 immunoreactivity homogeneously distributed in the cell bodies and dendrites (Fig. 3). On the contrary, in cultured cortical neurons exposed to Aβ42 (0.5 μM; 24 h) the neuronal network appeared fragmented (Fig. 3). In particular, a dishomogeneous distribution of MAP2 immunoreactivity along the neurites was observed, and the dendrites often appeared truncated. To quantify the effect of Aβ42, the number of vital MAP-2 immunostained cultured neurons was determined. As shown in

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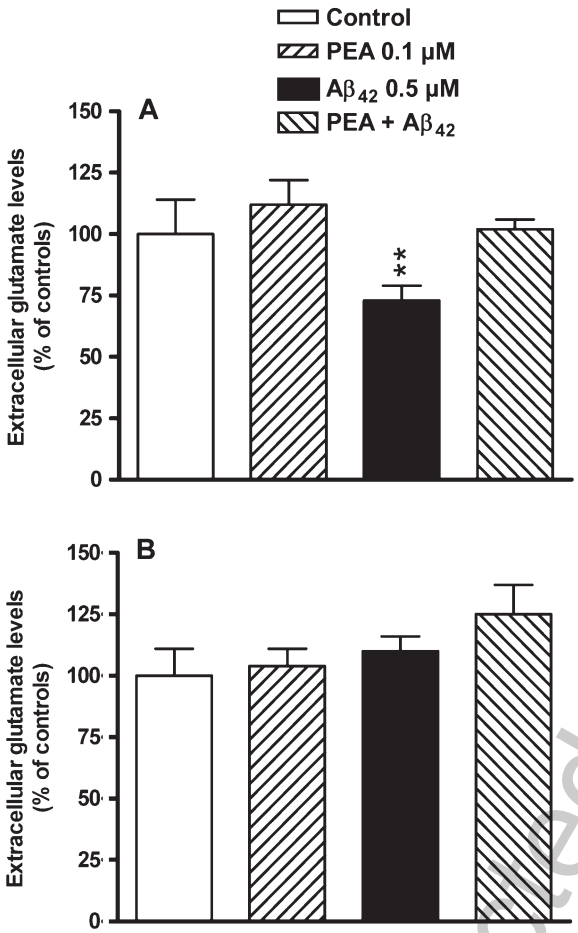

Fig. 2. Effects of $A\beta_{42}$ exposure (0.5 μM; 24 h), alone or in combination with PEA (0.1 μM), on extracellular glutamate levels in primary cultures of cerebral cortical neurons from non-Tg (A) and 3xTg-AD (B) mice. PEA was added 1 h before $A\beta_{42}$ and maintained in contact with the cells during $A\beta_{42}$ 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.

Fig. 4, the number of neurons was significantly lower in cell cultures exposed to $A\beta_{42}$ than in control cultures. Interestingly, pretreatment with PEA (0.1 μM), by itself ineffective, counteracted $A\beta_{42}$-induced decrease of neuron number (Fig. 4).

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. 5). The exposure to $A\beta_{42}$ (0.5 μM) or PEA (0.1 μM) did not modify these alterations in cultured cortical neurons obtained from 3xTg-AD mice (data not shown).

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

Effects of $A\beta_{42}$ exposure in the presence and in the absence of PEA on cellular viability

$A\beta_{42}$ (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. 6A). On the contrary, $A\beta_{42}$ exposure failed to modify the cell viability value in cultured cortical astrocytes obtained from 3xTg-AD mice (Fig. 6B). PEA pretreatment (0.1 μM) counteracted $A\beta_{42}$-induced astroglial proliferation in cultured cortical astrocytes obtained from non-Tg mice (Fig. 6A). By itself, PEA did not affect cell viability in cultured cortical astrocytes obtained from non-Tg mice (Fig 6A) or 3xTg-AD mice (Fig. 6B).

Effects of $A\beta_{42}$ exposure in the presence and in the absence of PEA on endogenous extracellular glutamate levels

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. 7A) but not in cultured cortical astrocytes obtained from 3xTg-AD mice (Fig. 7B). 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. 7A). By itself, PEA did not affect extracellular glutamate levels in cultured cortical astrocytes obtained from non-Tg (Fig. 7A) or 3xTg-AD mice (Fig. 7B).

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, GFAP positive cells showed numerous branched processes, extending outward from the somata in multiple directions (stellate shape), that are typical of healthy astrocytes (Fig. 8). Following the exposure to $A\beta_{42}$ (0.5 μM; 24 h), the morphology of cultured astrocytes

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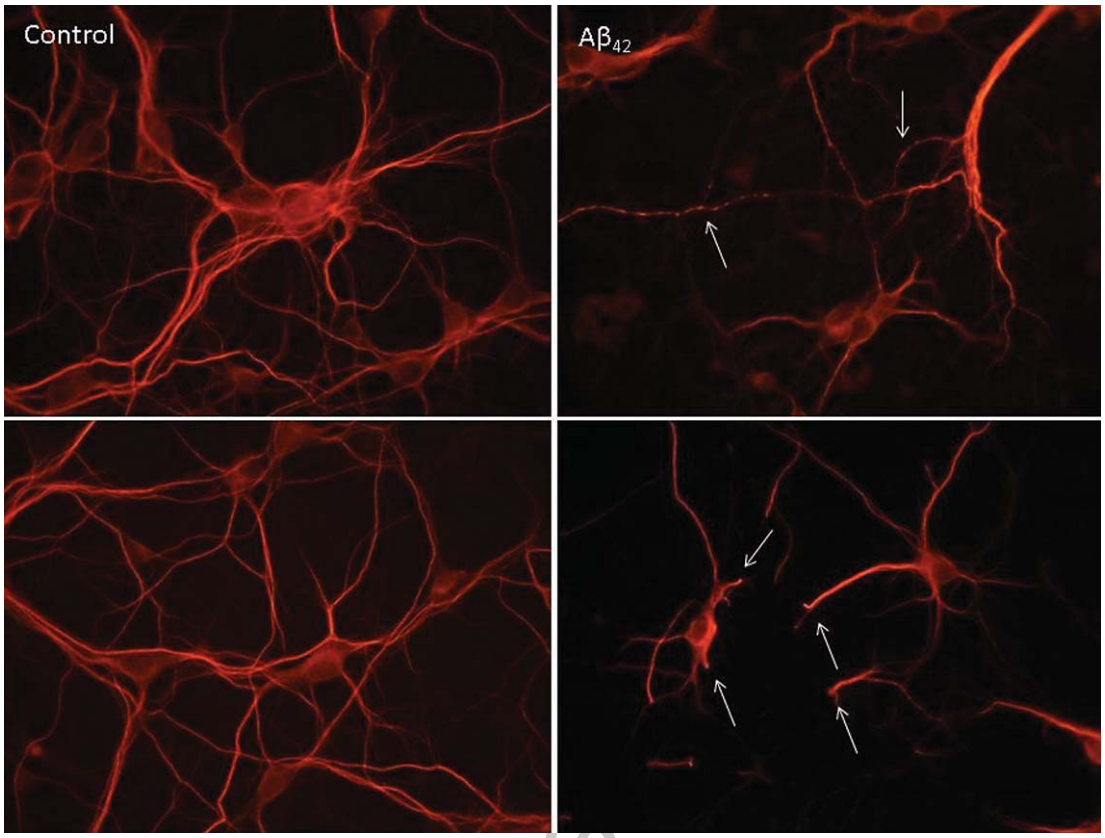

Fig. 3. Representative fluorescence photomicrographs of MAP2 immunoreactivity in primary cultures of cerebral cortical neurons from non-Tg mice not exposed (*left panel*) and exposed to $A\beta_{42}$ (0.5 μM; 24 h; *right panels*). Local neuronal network fragmentation was indicated by the white arrows (*right panels*). Neurons were stained with anti-MAP2 antibody and observed in sample fields under fluorescent microscope (magnification x40).

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from non-Tg mice resulted changed since convolutions and swellings in the terminal part of the processes were clearly evident (Fig. 9). 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. 10, 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. 10).

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

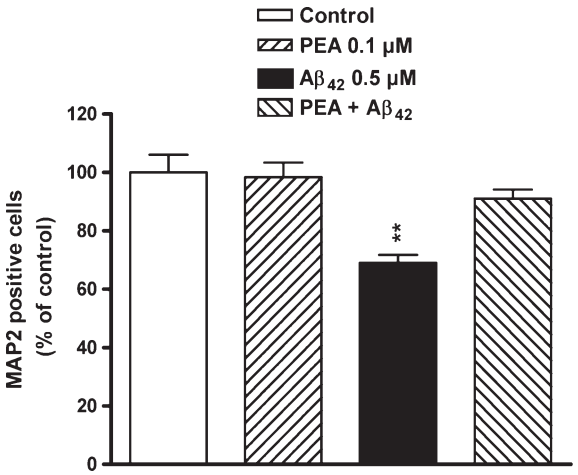

Fig. 4. Effects of Aβ42 exposure (0.5 μM; 24 h), alone or in combination with PEA (0.1 μM), on the number of MAP2 positive cells in primary cultures of cerebral cortical neurons from non-Tg mice. Neurons were stained with anti-MAP2 antibody and observed under fluorescent microscope. Each histograms represents the mean ± S.E.M. ($n = 8/12$). $^{**}p < 0.01$ significantly different from control, PEA and PEA + Aβ42 groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

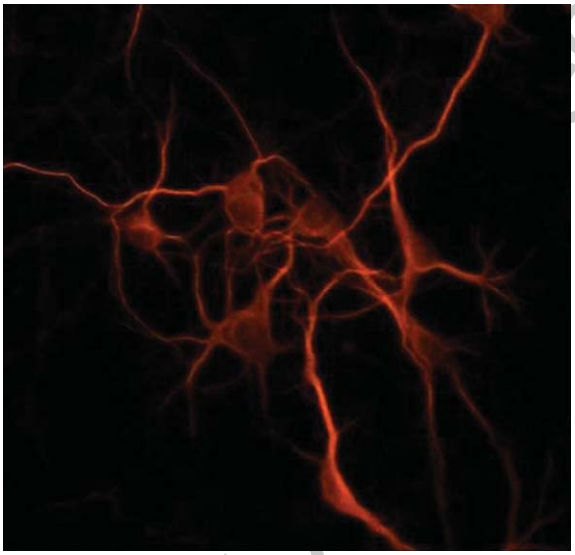

Fig. 5. Representative fluorescence photomicrographs of MAP2 immunoreactivity in primary cultures of cerebral cortical neurons from 3xTg-AD mice. Neurons were stained with anti-MAP2 antibody and observed in sample field under fluorescent microscope (magnification x20).

antibody in non-Tg cortical cell cultures exposed to Aβ42 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

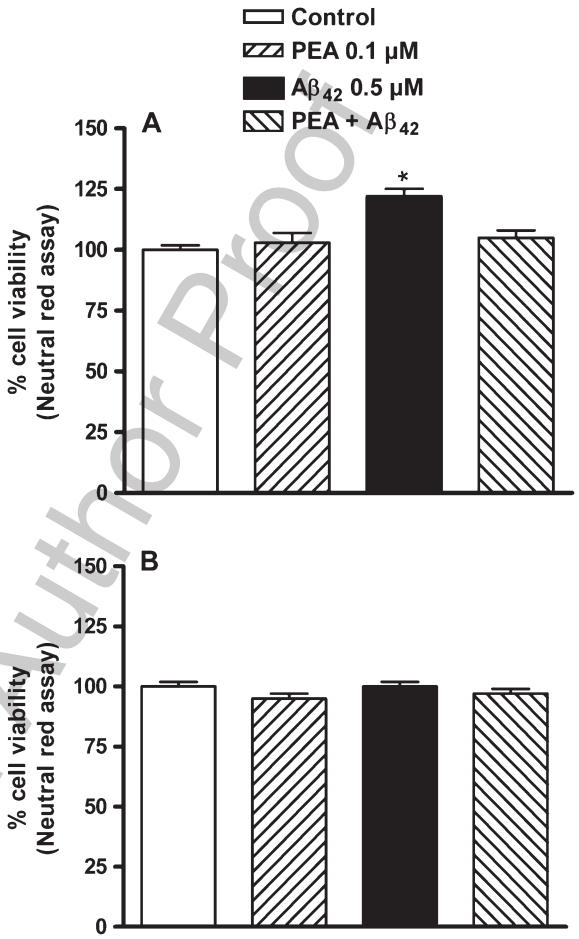

Fig. 6. Effects of Aβ42 exposure (0.5 μM; 24 h), alone or in combination with PEA (0.1 μM), on cell viability in primary cultures of cerebral cortical astrocytes from non-Tg (A) and 3xTg-AD (B) mice. PEA was added 1 h before Aβ42 and maintained in contact with the cells during Aβ42 exposure (24 h). Cell 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 + Aβ42 groups according to analysis of variance followed by the Newman-Keuls test for multiple comparisons.

induced by Aβ42 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 Aβ42 exposure [42]. Furthermore, a microtubule deregulation after Aβ42 treatments has been observed in other studies. In particular, Mota et al. [43] documented Aβ42-induced decreases in total and polymerized levels of β-III tubulin along with polymerized α-tubulin, and these alterations were correlated with a reduced neurite length. Finally, Aβ42-induced microtubule depletion and loss of spines [44] as well as a retraction of synaptic contacts [45] were also observed.

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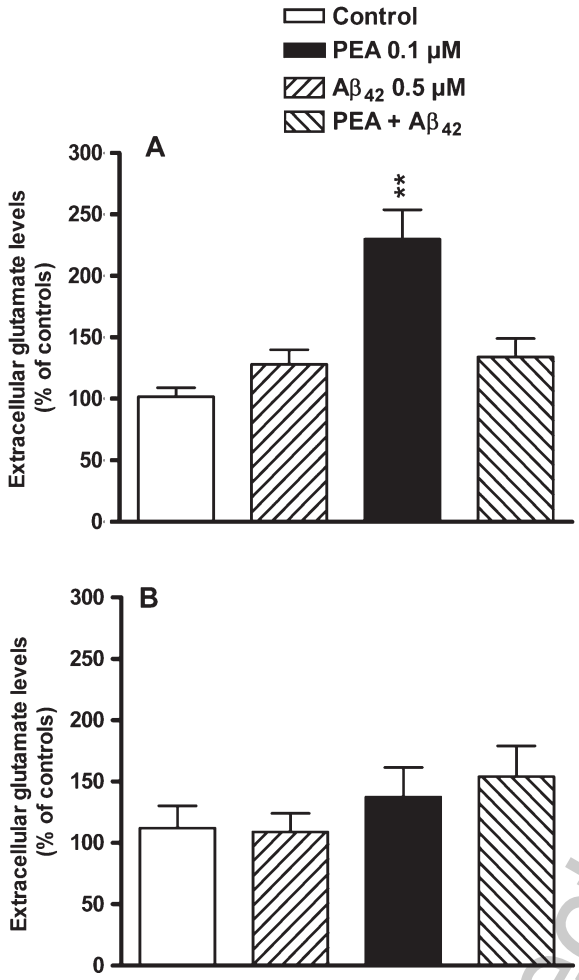

Fig. 7. Effects of $A\beta_{42}$ exposure (0.5 μM; 24 h), alone or in combination with PEA (0.1 μM), on extracellular glutamate levels in primary cultures of cerebral cortical astrocytes from non-Tg (A) and 3xTg-AD (B) mice. PEA was added 1 h before $A\beta_{42}$ and maintained in contact with the cells 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.

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 [46–50]. Deleterious effects of $A\beta_{42}$ on multiple steps of synaptic vesicle trafficking, leading to weaken synaptic transmission have also been reported [51]. $A\beta_{42}$-treated neurons also displayed reduced number of synaptic vesicles and a reduction in several presynaptic proteins [52]. 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 [53], suggesting a particular susceptibility of glutamatergic nerve terminals to $A\beta_{42}$-induced toxicity. In fact, intracellular accumulation of Aβ dramatically affects glutamatergic synaptic function at both presynaptic and postsynaptic levels [54]. Finally, there is strong evidence for $A\beta_{42}$-induced impairments in mitochondrial transport, dynamics and function that contribute to synaptic degeneration [47, 55, 56].

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 Aβ peptides in 3xTg-AD mouse-derived cell cultures. In fact, an early *in vitro* Aβ overexpression associated with increased $A\beta_{42}$ levels was evident in cultured cortical neurons of 6 DIV obtained from 3xTg-AD mice [34]. Furthermore, an altered calcium homeostasis and decreased glutamatergic response were also observed in cultured cortical neurons from 3xTg-AD mice [34, 57–59]. 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 Aβ 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 [34]. Furthermore, *in vivo* microdialysis studies reported a significant decrease

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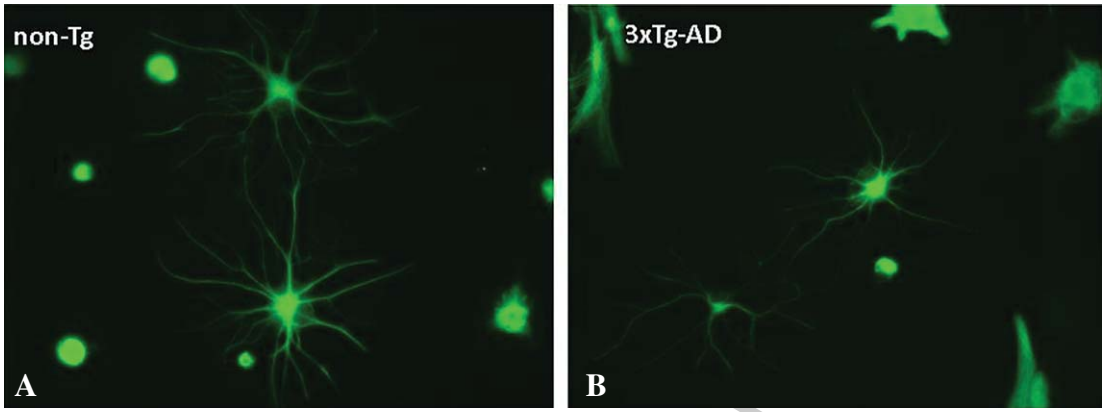

Fig. 8. Representative fluorescence photomicrographs of GFAP immunoreactivity in primary cultures of cerebral cortical astrocytes from non-Tg (A) and 3xTg-AD (B) mice. Astrocytes were stained with anti-GFAP antibody and observed in sample field under fluorescent microscope (magnification ×40).

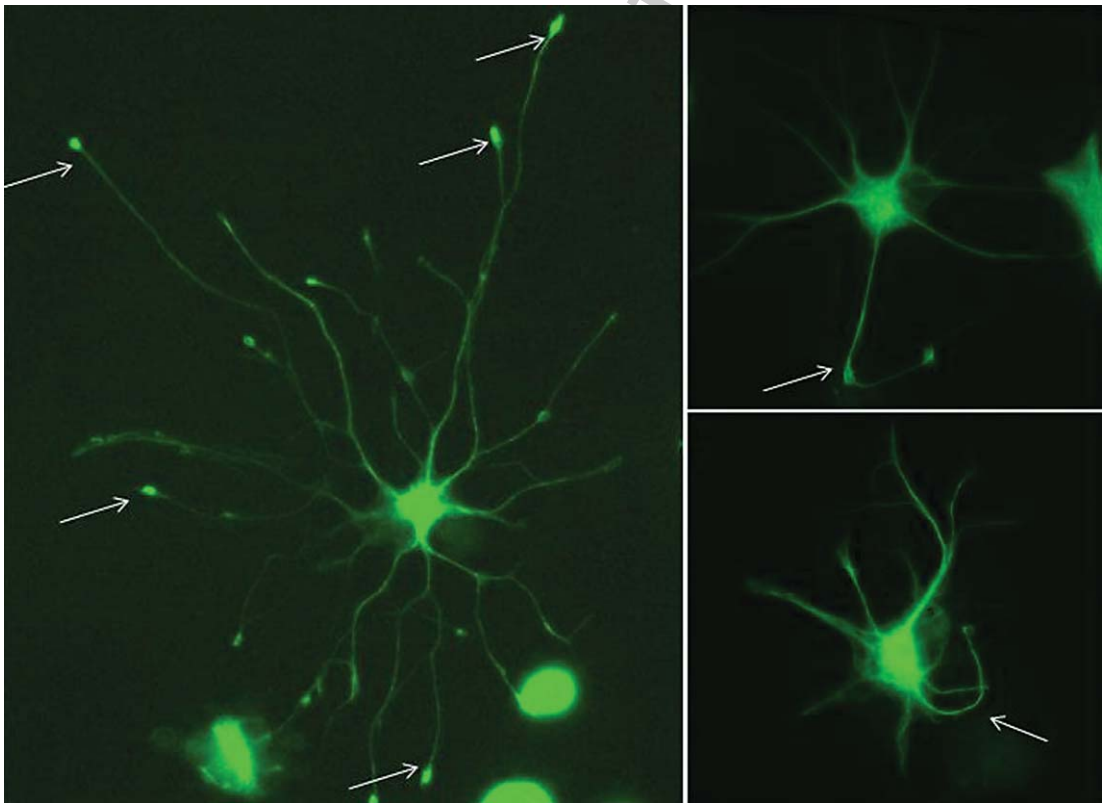

Fig. 9. Representative fluorescence photomicrographs of GFAP immunoreactivity in primary cultures of cerebral cortical astrocytes from non-Tg mice after 24 h of $A\beta_{42}$ (0.5 μM) exposure. Alterations of the morphology are indicated by the white arrows. Astrocytes were stained with anti-GFAP antibody and observed in sample fields under fluorescent microscope (magnification ×40).

of basal glutamate release in the frontal cortex and hippocampus of 18-month-old 3xTg-AD-mice [36] and a reduction of KCl-stimulated glutamate release in the hippocampus of 17-month-old APdE9 mice [60]. Interestingly for the possible translational aspects of the present findings, there are data in literature reporting reduced glutamate tissue levels in AD brains [60–62].

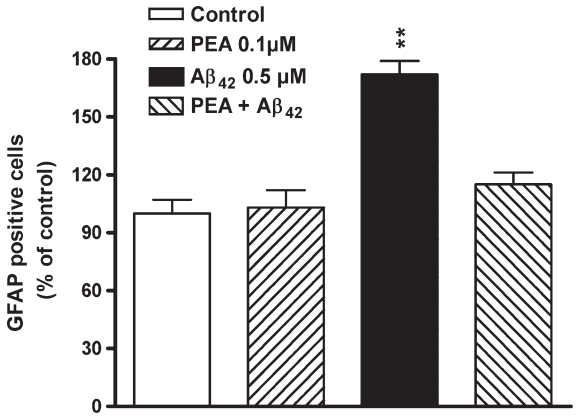

Fig. 10. Effects of $A\beta_{42}$ exposure (0.5 μM; 24 h), alone or in combination with PEA (0.1 μM), on the number of GFAP positive cells (expressed as percentage of control value) in primary cultures of cerebral cortical astrocytes from non-Tg mice. Astrocytes were stained with anti-GFAP antibody and 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.

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 [63]. 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 [64] as previously observed in human AD tissues [64] and in cultured animal astrocytes [29, 30, 66]. 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 [67]. Interestingly, similar alterations have been observed in 1–12 months 3xTg-AD mouse astrocytic morphology [68, 69], 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 [70]. 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 $A\beta_{25\text{-}35}$ exhibit increased glutamate release [71]. 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 [29, 67, 71] along with a consequent increase of intracellular Ca^{2+} levels leading to exocytotic glutamate release. Secondly, $A\beta_{42}$ could decrease glutamate uptake [72] and compromise the activity of glutamate transporters GLT-1 and GLAST [73, 74], thus reducing glutamate reuptake. Previous studies reported a significant reduction in the activity of glutamate transporters in human AD tissues [75, 76] and in animal models of AD [77, 78]. 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 [36]. Differently, Kulijewicz-Nawrot et al. [79] 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 [63]. In particular, these authors observed significant effects of

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$A\beta_{42}$ 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 [63]. 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 [22, 23, 26, 32, 80]. 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 [24, 31]. 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 $A\beta_{42}$ [30]. 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 [29]. 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 [30]. 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 Aβ excess [32].

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 [34]. Furthermore, previous studies have demonstrated that the expression of fatty acid amide hydrolase (FAAH) enzyme is elevated in astrocytes in AD [81] and in Down's syndrome, sometimes referred to as a human model of AD-like Aβ deposition [82]. 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 find-

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752 ings suggest that the compound may be effective in the
753 early AD or when Aβ is accumulating and initiating
754 damage in the central nervous system. In this context,
755 it will be relevant to evaluate the effects of PEA on cel-
756 lular viability and glutamate release *in vitro* choosing
757 a period of exposure of the 3xTg-AD mouse neurons
758 to the toxic peptide preceding the development of Aβ
759 accumulation and tau hyperphosphorylation [34, 83].

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