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Astrocytic palmitoylethanolamide pre-exposure exerts neuroprotective effects in astrocyte-neuron co-cultures from a triple transgenic mouse model of Alzheimer's disease.

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Abbreviations: Alzheimer's disease (AD); β -amyloid peptide (A β); Days in vitro (DIV); Glial fibrillary acidic protein (GFAP); Microtubule-associated protein 2 (MAP2); Non-transgenic (non-Tg); Palmitoylethanolamide (PEA); Triple-transgenic murine model of AD (3xTg-AD).

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Sarah Beggiato: Conceptualization, Investigation, Reviewing and Editing. Tommaso Cassano: Validation; Software; Luca Ferraro: Supervision, Writing- Original draft preparation. Maria C. Tomasini: Conceptualization, Investigation, Data curation.

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Abstract

Palmitoylethanolamide (PEA) is an endogenous lipid mediator that, also by blunting astrocyte activation, demonstrated beneficial properties in several *in vitro* and *in vivo* models of Alzheimer's disease (AD).

In the present study, we used astrocyte-neuron co-cultures from 3xTg-AD mouse (*i.e.* an animal model of AD) cerebral cortex to further investigate on the role of astrocytes in PEA-induced neuroprotection. To this aim, we evaluated the number of viable cells, apoptotic nuclei, microtubule-associated protein-2 (MAP2) positive cells and morphological parameters in cortical neurons co-cultured with cortical astrocytes pre-exposed, or not, to A β ₄₂ (0.5 μ M; 24 h) or PEA (0.1 μ M; 24 h).

Pre-exposure of astrocytes to A β ₄₂ failed to affect the viability, the number of neuronal apoptotic nuclei, MAP2 positive cell number, neuritic aggregations/100 μ m, dendritic branches per neuron, the neuron body area, the length of the longest dendrite and number of neurites/neuron in 3xTg-AD mouse astrocyte-neuron co-cultures. Compared to neurons from wild-type (non-Tg) mouse co-cultures, 3xTg-AD mouse neurons co-cultured with astrocytes from this mutant mice displayed higher number of apoptotic nuclei, lower MAP2 immunoreactivity and several morphological changes. These signs of neuronal suffering were significantly counteracted when the 3xTg-AD mouse cortical neurons were co-cultured with 3xTg-AD mouse astrocytes pre-exposed to PEA. The present data suggest that in astrocyte-neuron co-cultures from 3xTg-AD mice, astrocytes contribute to neuronal damage and PEA, by possibly counteracting reactive astrogliosis, improved neuronal survival. These findings further support the role of PEA as a possible new therapeutic opportunity in AD treatment.

Keywords: 3xTg-AD mice; Cell viability, Hoechst 33258, MAP-2 immunoreactivity; Reactive astrogliosis.

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is a neurodegenerative disease affecting several million people worldwide. AD is clinically characterized by a progressive loss of intellectual abilities such as learning and memory associated with a functional deterioration and, depending on the pathology severity, with behavioral disorders and neuropsychiatric signs (Ballard et al., 2011; Cortés et al., 2018; Zvěřová, 2019). The two pathognomonic lesions in the brain of AD patients are senile plaques due to extracellular neuritic β -amyloid peptide ($A\beta$) aggregations and neurofibrillary tangles in the cytoplasm of pyramidal neurons due to tau hyperphosphorylation (Selkoe and Hardy, 2016). Neuroinflammation in AD was originally considered as a mere bystander activated by emerging senile plaques and neurofibrillar tangles. More recent findings, however, clearly supported the view that neuroinflammation contributes as much or more to the pathogenesis of AD. Accordingly, increased glial cell activation is reliably observed in both rodent models of AD and in AD patients (Ameen-Ali et al., 2017; Edison et al., 2018; Takashi and Takaomi, 2018; Chaney et al., 2019).

Astrocytes are glial cells possessing homeostatic, metabolic and defensive properties and playing an important role in the development of inflammatory responses in the brain (Sofroniew, 2014, 2015). These cells are more numerous than microglia in the CNS and their activation seems to be more prolonged than that of other glial cells. This supports the hypothesis that the role of astrocytes in sustaining neuroinflammation in AD is more relevant than that of microglia (Ballard et al., 2011; Osborn et al., 2016; Acosta et al., 2017). Astrocyte pathological responses comprise reactive astrogliosis, a complex and multistage process mainly characterized by astrocyte morphology changes and generally aimed at neuroprotection and rescue of damaged neurons (Heneka et al., 2015; Osborn et al., 2016). In spite of these beneficial effects of reactive astrocytes on surrounding neurons, it is well recognized that extreme and extended astrocyte activation contributes to neuroinflammation development and progress, altering the neuron/astrocyte interaction and leading to harmful neuronal effects (Henstridge et al., 2019). In fact, activated astrocytes induce the release of cytokines, interleukins, nitric oxide, along with other cytotoxic factors, thus exacerbating neurodegeneration process observed in AD (Jain et al., 2015; Heneka et al., 2015; Osborn et al., 2016). As a consequence, a therapeutic approach directing against not only neurodegeneration, but also astrocyte dysfunctions in AD may be effective in reducing the disease progression (Bronzuoli et al., 2017; Yi et al., 2017; Fakhoury, 2018). In this context, palmitoylethanolamide (PEA), an endogenous lipid mediator (Petrosino and Di Marzo, 2017), looks like a promising pharmacological agent. The compound has been proved to be effective in counteracting neuroinflammation and

neurodegeneration, as demonstrated by numerous *in vitro* and *in vivo* animal models of AD (Avagliano et al., 2016; Petrosino and Di Marzo, 2017; Beggiato et al., 2019). We previously reported that PEA acts as a protective agent against A β 1-42 fragment (A β ₄₂)-induced toxicity in primary cultures of cortical neurons or astrocytes from wild-type (non-Tg) and the triple-transgenic murine model of AD (3xTg-AD) mice (Tomasini et al., 2015). Furthermore, PEA demonstrated protective properties against the neurotoxic effects induced by astrocytic A β ₄₂ exposure in a mouse astrocyte-neuron co-culture preparation (Beggiato et al., 2018). Collectively, these data propose that PEA exerts favorable effects against A β -induced neurodegeneration either by exerting a direct neuroprotective action or by limiting the neurotoxic consequences of astrocyte activation. From a translational point of view, it became therefore significant to assess the effects of PEA on astrocyte-neuron co-cultures from an animal model of AD. Thus, in the present study we used astrocyte-neuron co-cultures from 3xTg-AD mice to further investigate on the possible role of astrocytes in PEA-induced neuroprotection. To this aim, we evaluated the number of viable cells, apoptotic nuclei, microtubule-associated protein-2 (MAP2) positive cells and the morphological parameters in primary cortical neurons co-cultured with primary cortical astrocytes pre-exposed, or not, to A β ₄₂ (0.5 μ M; 24 h) or PEA (0.1 μ M; 24 h).

2. Materials and Methods

2.1. Animals and Ethics statement

In the present study, colonies of 3xTg-AD mice and wild type littermates (non-Tg) were used. These animals were initially established at the animal facilities of the Puglia and Basilicata Experimental Zooprophyllactic Institute (Foggia, Italy) (Cassano et al., 2012), according to the procedures described previously (Oddo et al., 2003a, 2003b). The 3xTg-AD mice harboring APP_{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 (Oddo et al., 2003a; Oddo et al., 2003b). Genotypes were confirmed by polymerase chain reaction (PCR) after tail biopsies (Oddo et al., 2003a). 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. The experimental protocols performed in this study were in accordance with the European Communities Council Directive of September 2010 (2010/63/EU) and were approved by the Italian

Ministry of Health. Efforts were made to minimize the number of animals used and to reduce their discomfort.

2.2. Primary cultures of cerebral cortex neurons

Primary cerebral cortex neuron cultures were obtained from non-Tg and 3xTg-AD mouse embryos (embryonic day 18) according to the method described for rat cortical neuronal cultures (Antonelli et al., 2008). Concisely, after meninges removal, embryo cortices were dissociated in 0.025% (w/v) trypsin (37°C). Thereafter, fragments of tissue were dissociated by repetitive mild pipetting through wide- and narrow-bore fire-polished Pasteur pipettes in Neurobasal medium (Gibco, Grand Island, NY, USA) supplemented with glutamine (0.1 mM; Sigma Chemical Co., St. Louis, MO, USA), gentamicin (10 µg/ml; Sigma Chemical Co., St. Louis, MO, USA) and 2% B27® Supplement (50X), serum free (Gibco®). After the cell counting, the neurons were plated on glass coverslips in a poly-L-lysine (5µg/ml)-coated multiwells (24 wells; Nunc A/S, Roskilde, Denmark) at a density of 200,000 cells per well. Cultures were grown at a 37°C in a humidified atmosphere, 5% CO₂/95% air. After 8 days of *in vitro* incubation (days *in vitro*: DIV), the cultures were used for the experiments. The incubation of primary cultures of cerebral cortex neurons with an antibody against glial fibrillary acidic protein (GFAP), revealed no contamination of neurons with astrocytes (*data not shown*).

2.3. Primary cultures of cerebral cortex astrocytes

Primary cultures of cerebral cortex astrocytes were prepared from neonatal non-Tg and 3xTg-AD mice (1 or 2 days old) and cultured as previously described (Tomasini et al., 2015; Scuderi et al., 2012). After the removal of cerebral cortices, the tissue was dissociated by slight trypsinization (37°C) and then mechanically triturated to attain single cells. After their suspension 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)], the cells were seeded in 75-cm² flasks at a density of 3×10^6 cells/flask and then incubated (37°C) in a humidified atmosphere, 5% CO₂/95% air. The culture medium was replaced 24 h later and twice weekly until astrocytes formed a monolayer strictly attached to the bottom of the flask (12 or 14 days after dissection). At cell confluence, flasks were subjected to a vigorous shake in order to separate astrocytes (remaining adherent to the bottom of the flasks) from microglia and oligodendrocytes (floating on the supernatant). Finally, the astrocytes were counted and plated on poly-L-lysine (5 µg/ml)-coated Petri dish (35 mm diameter) at a density of 1,500,000 cells per dish (Nunc A/S, Roskilde,

Denmark). The monoclonal anti-gial fibrillary acidic protein (GFAP) was used to evaluate the cell culture purity; only cultures with more than 95% GFAP-positive cells were used for the experiments.

2.4. Astrocyte-neuron co-cultures and treatment procedure

Astrocyte-neuron co-cultures were prepared following the method described by Allaman (2010). All treatments were only applied to astrocytes cultured in dish containing DMEM. In particular, A β ₁₋₄₂ or PEA (0.1 μ M, Tocris Bioscience, Bristol, UK) were applied for 24 h. The treatment concentrations were chosen according to previous results (Scuderi et al., 2012; Tomasini et al., 2015; Beggiato et al., 2018). A β ₁₋₄₂ (Tocris Bioscience, Bristol, UK) was freshly prepared by dissolving the peptide, at the concentration of 0.5 μ M, in distilled water; by means of atomic force microscopy (AFM), a previous study (Morgese et al., 2015) indicates that following this procedure, the peptide solution contained many small particles (*i.e.* monomers) and very few larger particles (*i.e.* oligomers). After the treatment period, the medium was aspirated to remove A β ₄₂ or PEA and then the astrocytes were washed with fresh DMEM and then placed in supplemented Neurobasal medium for 4 h. Thereafter, the astrocyte-neuron co-cultures were created by gently transferring primary cortical neurons grown on coverslips on the top of the astrocyte layer cultured in 35 mm dish (neurons facing up; Fig. 1). Astrocytes were plated at a density of 1,500,000 cells/dish, while neurons were plated on glass coverslips at a density of 200,000 cells/well. Under these experimental conditions, neurons were never in direct interaction with A β ₄₂ and/or PEA. 24 h after the initiation of co-cultures, neuronal viability, MAP immunoreactivity, nuclear staining and different morphometric parameters have been assessed.

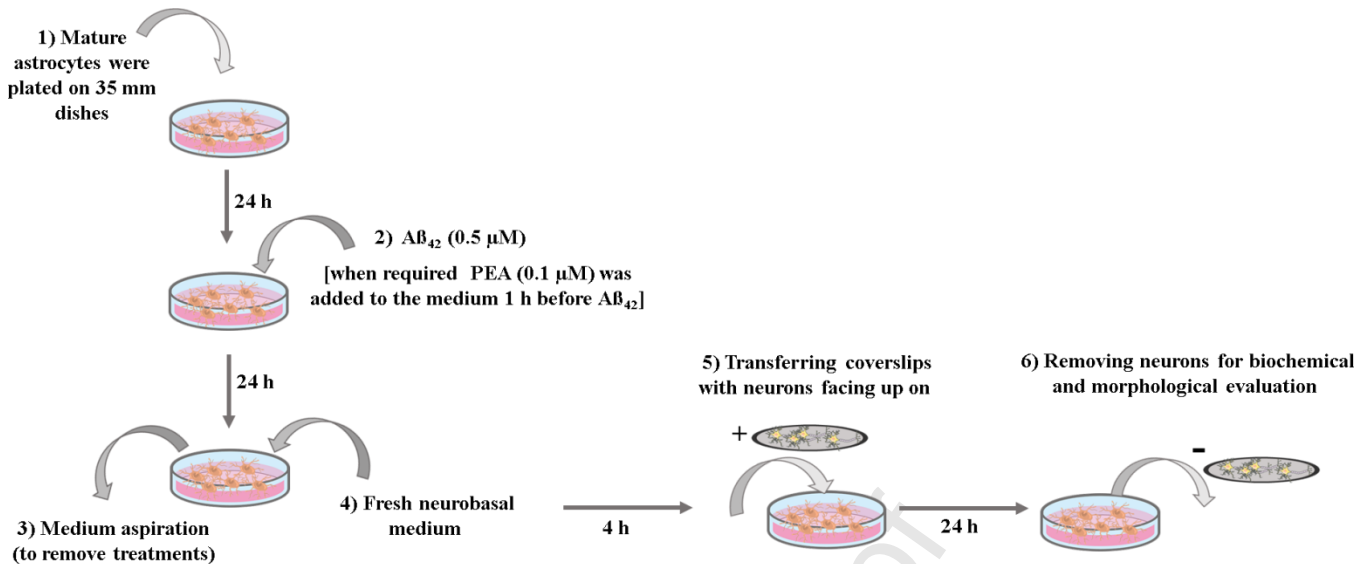


Figure 1. Schematic representation of PEA and $A\beta_{42}$ treatment protocol in cortical astrocyte-neuron co-cultures. Control co-cultures were subjected to the same protocol but treated with the respective $A\beta_{42}$ and/or PEA vehicle.

2.5. Neutral red assay

Neuronal viability was assessed with neutral red assay as previously described (Scuderi and Steardo, 2013; Repetto et al., 2008) with small adaptations. Shortly, 24 h after the creation of astrocyte-neuron co-cultures, the dishes were incubated for 2 h at 37°C with a Neutral red working solution [50 μ g/ml in phosphate-buffered saline (PBS) 1X without calcium and magnesium, Sigma-Aldrich, MO, USA]. A washing period with PBS was used to eliminate the extracellular remaining dye. Thereafter, the cortical neuron-holding glass coverslips were removed from the dishes containing the astrocytes and placed in 24-multiwell plates with neurons facing up. A destain solution (ethanol: deionized water: glacial acetic acid, 50:49:1 v/v) was used to remove the dye from each well. A microplate absorbance reader (Sunrise, Tecan) was used to read the absorbance (wave length = 540 nm); the absorbance values obtained from treated cells were compared to those measured in control non-exposed cultures, and expressed as % changes (Beggiato et al., 2018).

2.6. MAP2 immunoreactivity

The neuronal morphology was analyzed by using MAP2 immunocytochemistry. In fact, MAP2 is a neuronal marker that can be considered an index of the cytoskeleton integrity. 24 h after the

creation of astrocyte-neuron co-cultures, the glass coverslip containing the cortical neurons was removed from the dish with cortical astrocytes and positioned in 24-multiwell plate. Cells were rinsed (three 5 min-periods) in 0.1 M PBS and then fixed with 4% paraformaldehyde in Sorensen's buffer 0.1M, pH 7.4 for 20 min. Thereafter, the cells were incubated overnight at 4°C in 0.3% Triton X-100/PBS solution containing the primary antibody-MAP2 (1:1000 dilution Chemicon, Temecula, CA). Then the neurons were rinsed 3 times with PBS and incubated (60 min; room temperature) with the secondary antibody, rhodamine-conjugated anti-rabbit antibody (1:100 dilution Chemicon, Temecula, CA). At the end of this incubation period, the cells were washed in PBS and mounted in glycerol and PBS (3:1 v/v) containing 0.1% 1,4-phenylenediamine and examined using a Nikon Microphot FXA microscope. Five distinct and non-overlapping fields in each coverslip were randomly selected for cell counts; images were obtained by using a 20x objective.

The morphometric parameters evaluated in the cortical cultured neurons were: cell body area, length of the longest dendrite, number of dendritic branches per neuron, number of neurites per neuron and MAP2 dendrite aggregations. Morphometric measurements were accomplished by using a Nikon Microphot FXA microscope (x40 magnification objective) on 5 different microscopic fields in each coverslip. In particular, the MAP-2 aggregation number was calculated and referred to 100 µm of dendritic length. Morphometric neuronal analysis was performed utilizing the Image-Pro Plus 4.1 software (Antonelli et al., 2008; Beggiato et al., 2018).

2.7. Nuclear Staining with Hoechst 33258.

The investigation of apoptotic neuronal death was performed with nuclear staining by using Hoechst 33258. 24 h after the creation of astrocyte-neuron co-cultures, the coverslips with cortical neurons were removed and rinsed twice with PBS (0.1 M), and then cells were fixed in 4% paraformaldehyde, and incubated (20 min; room temperature) with Hoechst 33258 (1 g/ml in 0.1M PBS). The coverslips were rinsed with PBS and then mounted on slides with a solution containing 50% glycerol in 0.044 M citrate, 0.111 M phosphate buffer, pH 5.5. Cells were visualized by using a fluorescence microscope (Nikon Microphot FXA). The % of cells displaying condensed chromatin (fragmented nuclei) was calculated by counting >3000 cells under each experimental condition (5 randomly selected fields per well, 9-18 wells per condition per experiment, and 5 independent experiments; Beggiato et al., 2018).

2.8. Statistical analysis

Results are presented as means \pm standard error of mean. The statistical analysis was carried out by using Student's *t* test. $P < 0.05$ was the accepted level of significance.

3. Results

3.1. Cortical astrocyte-neuron co-cultures from 3xTg-AD mice: effects of astrocytic A β_{42} pre-exposure on neuronal health

These experiments were carried out to compare the impact of astrocytic A β_{42} pre-exposure on neuronal health in astrocyte-neuron co-cultures from 3xTg-AD mice with that previously observed in astrocyte-neuron co-cultures from non-Tg mice (Beggiato et al., 2018). In fact, in spite of the demonstration that in primary cultures of cerebral cortex neurons or in primary cultures of cerebral cortex astrocytes from 3xTg-AD mice, A β_{42} failed to affect cell viability and cell morphology (Tomasini et al., 2015), it cannot be ruled out the possibility that the peptide exposure may have resulted in increased levels of neurotoxic agents from astrocytes, thus leading to neuronal injury in astrocyte-neuron co-cultures. To verify this possibility, 3xTg-AD cortical astrocyte cultures were pre-treated with A β_{42} (0.5 μ M; 24 h) and, after changing the culture medium (to remove A β_{42}), 3xTg-AD cortical neurons were co-cultured for another 24 h-period. After this period neuronal viability, MAP2 immunoreactivity and neuronal morphometric parameters were assessed.

3.1.1. Effects of astrocytic A β_{42} pre-exposure on neuronal viability

In the presence of astrocytes pre-exposed to A β_{42} (0.5 μ M; 24 h), the viability of cortical neurons from 3xTg-AD mice was similar to that observed in control (*i.e.*, A β_{42} vehicle treated) astrocyte-neuron co-cultures (Fig. 2).

To exclude the possibility that this negative results might be due to the fact that, under these experimental conditions, the peptide may have not an active conformation to induce cytotoxicity, we repeated the experiments in astrocyte-neuron co-cultures from non-Tg mice. As previously demonstrated (Beggiato et al., 2018), the presence of astrocytes pre-exposed to A β_{42} (0.5 μ M; 24 h) induced a significant reduction ($-26 \pm 4\%$ of control values; $p < 0.05$) of neuronal viability in primary mouse astrocytes-neurons co-cultures from non-Tg mice.

Astrocyte-neuron co-cultures from 3xTg-AD mice

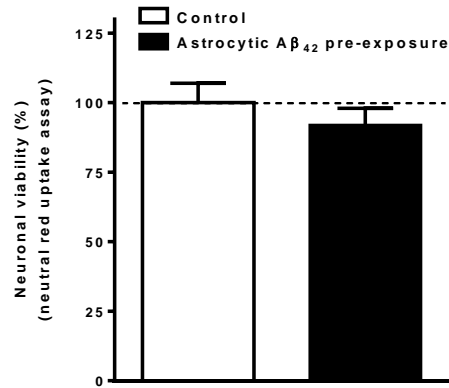


Figure 2. Effects of astrocytic Aβ₄₂ pre-exposure (0.5 μM, 24 h) on cell viability in primary cortical neurons from astrocyte-neuron co-cultures from 3xTg-AD mice. 3xTg-AD mouse cortical astrocyte cultures were pretreated with Aβ₄₂ and, after changing the culture medium (to remove Aβ₄₂), 3xTg-AD mouse neurons were co-cultured for another 24 h-period. Thereafter, cell viability was assessed by Neutral red assay and expressed as percentage of control values. Each histogram represents the mean ± S.E.M (n=10-20 wells; 4-5 animals per condition).

3.1.2. Effect of astrocytic Aβ₄₂ pre-exposure on apoptotic neuronal death

To evaluate apoptotic neuronal death we detected the chromatin structure alteration by using the specific DNA stain, Hoechst 33258. As shown in Fig. 3A and 3C, in the presence of astrocytes pretreated with Aβ₄₂ (0.5 μM; 24 h), the number of neuronal apoptotic nuclei was not significantly different from that observed in control (*i.e.* Aβ₄₂ vehicle treated) astrocyte-neuron co-cultures.

3.1.3. Effect of astrocytic Aβ₄₂ pre-exposure on neuronal MAP2 immunoreactivity

To evaluate the effect of astrocytic Aβ₄₂ pre-exposure (0.5 μM; 24 h) on neuronal MAP2 immunoreactivity, the number of vital MAP2-immunostained cultured cortical neurons in astrocyte-neuron co-cultures from 3xTg-AD mice was measured. The astrocytic Aβ₄₂ pre-exposure did not significantly modify the number of MAP2 positive co-cultured neurons as compared to control (*i.e.* Aβ₄₂ vehicle treated) co-cultures (Fig. 3B and 3C).

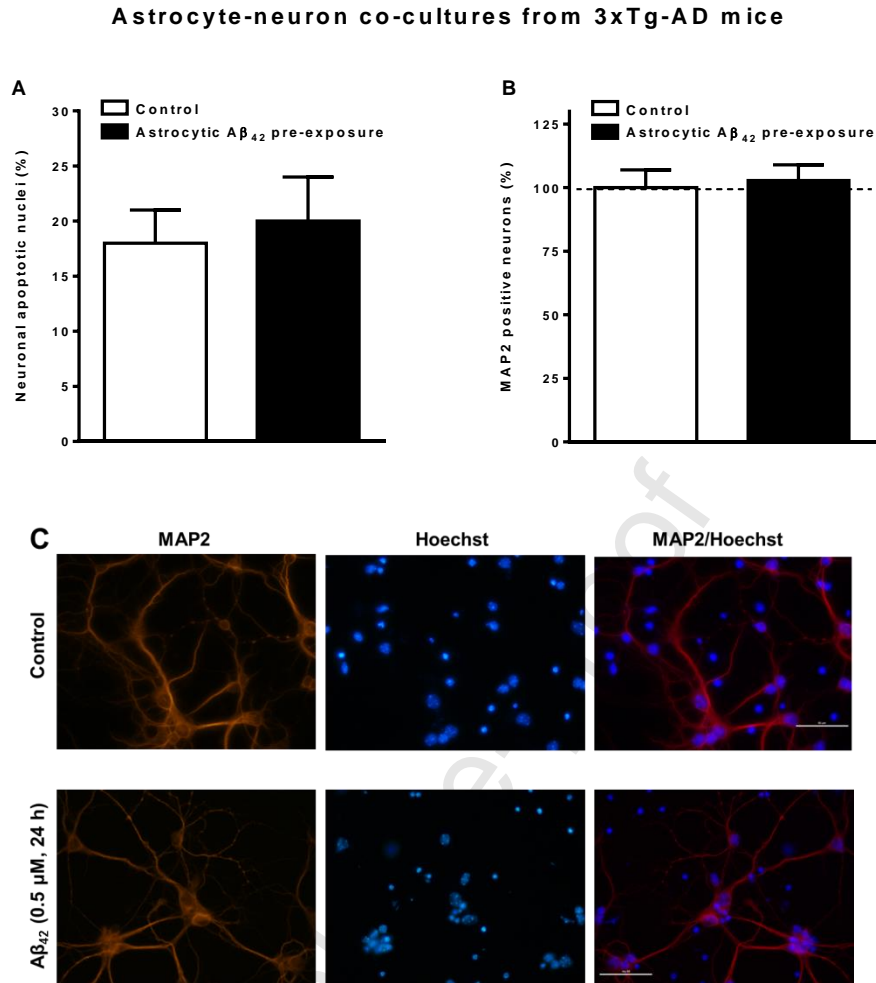


Figure 3. Effects of astrocytic A β_{42} pre-exposure (0.5 μ M, 24 h) on percentage of apoptotic nuclei (*Panel A*) and number of MAP2-positive cells (*Panel B*) in primary cortical neurons from astrocyte-neuron co-cultures from 3xTg-AD mice. 3xTg-AD mouse cortical astrocyte cultures were pretreated with A β_{42} and, after changing the culture medium (to remove A β_{42}), 3xTg-AD mouse neurons were co-cultured for another 24 h-period. The specific DNA stain Hoechst 33258 was used for the determination of apoptotic nuclei. The number of MAP2 positive cells was determined under fluorescent microscope by staining neurons with anti-MAP2 antibody. Each histogram represents the mean \pm S.E.M (n=10-20 wells; 4-5 animals per condition). *Panel C*: representative fluorescent photomicrographs (magnification x40; Scale bar = 50 μ m) of MAP2 immunoreactivity (red) and Hoechst 33258 staining (blue).

3.1.4. Effect of astrocytic A β_{42} pre-exposure on neuronal morphometric parameters

To characterize the healthy neuronal growth, a morphometric analysis was carried out on cortical neurons in astrocyte-neuron co-cultures from 3xTg-AD mice, by using MAP2 immunofluorescence. No significant differences in the number of neuritic aggregations/100 μm , number of dendritic branches per neuron, cell body area, length of the longest dendrite and number of neurites per neuron were observed between cortical neurons of 3xTg-AD mice co-cultured with astrocytes pre-exposed or not (*i.e.* control) to $\text{A}\beta_{42}$ (0.5 μM ; 24 h; Fig. 4).

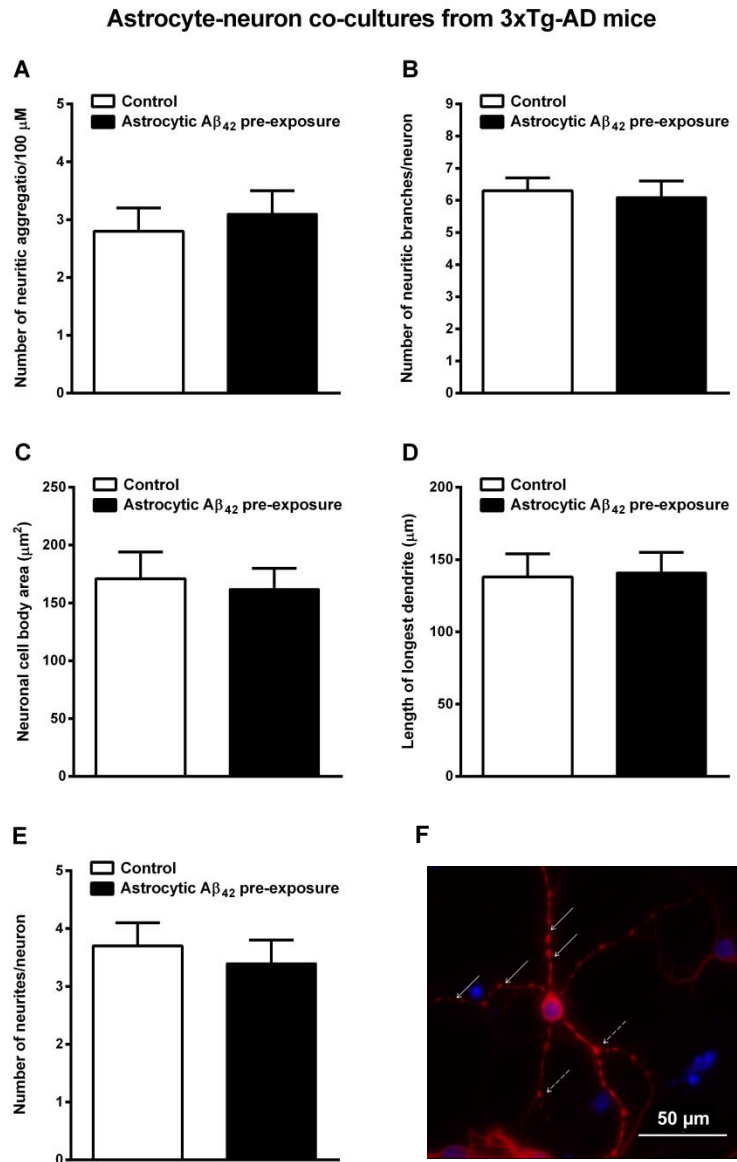


Figure 4. Effects of $\text{A}\beta_{42}$ pre-exposure on neuronal morphometric parameters in primary cortical neurons in astrocyte-neuron co-cultures from 3xTg-AD mice. 3xTg-AD mouse cortical astrocyte cultures were pretreated with $\text{A}\beta_{42}$ and, after changing the culture medium (to remove $\text{A}\beta_{42}$), 3xTg-AD mouse neurons were co-cultured for another 24 h-period. Thereafter, neurons were stained with

anti-MAP2 antibody and observed under fluorescent microscope. The number of aggregation/100 μm (*Panel A*), the number of dendritic branches per neuron (*Panel B*), the cell body area (*Panel C*), the length of the longest dendrite (*Panel D*) and the number of neurites per neuron (*Panel E*) were measured. Each histogram represents the mean \pm S.E.M (n=10/15 wells; 3-4 animals per condition). *Panel F*: representative fluorescent photomicrograph of MAP2 (red) immunoreactivity and Hoechst 33258 (blue) staining. Local neuronal network fragmentation (n $^\circ$ aggregation/100 μm) is indicated by the white arrows and dendritic branches are indicated by the dotted arrows (magnification x40; scale bar = 50 μm).

3.2. Astrocytic influence on apoptotic neuronal death and morphological parameters in cortical astrocyte-neuron co-cultures: comparison between 3xTg-AD and non-Tg mice

The above results suggested a different impact of astrocytic A β_{42} pre-exposure on neuronal viability, MAP2 immunoreactivity and morphometric parameters in cortical astrocyte-neuron co-cultures from non-Tg (Beggiato et al., 2018) and 3xTg-AD mice. In view of this observation, further experiments were carried out to compare the effects of astrocytic influence on neuronal health in cortical astrocyte-neuron co-cultures from non-Tg and 3xTg-AD mice.

3.2.1. Astrocytic influence on apoptotic neuronal death

As shown in Fig. 5A, the number of apoptotic nuclei in neurons from 3xTg-AD mouse co-cultures was significantly higher than that observed in non-Tg co-cultures.

3.2.2. Astrocytic influence on neuronal MAP2 immunoreactivity

The number of vital MAP2 positive cultured neurons obtained from astrocyte-neuron co-cultures was determined. In the 3xTg-AD mouse co-cultures, the number of MAP2 positive neurons was significantly reduced when compared to non-Tg mouse co-cultures (Fig. 5B).

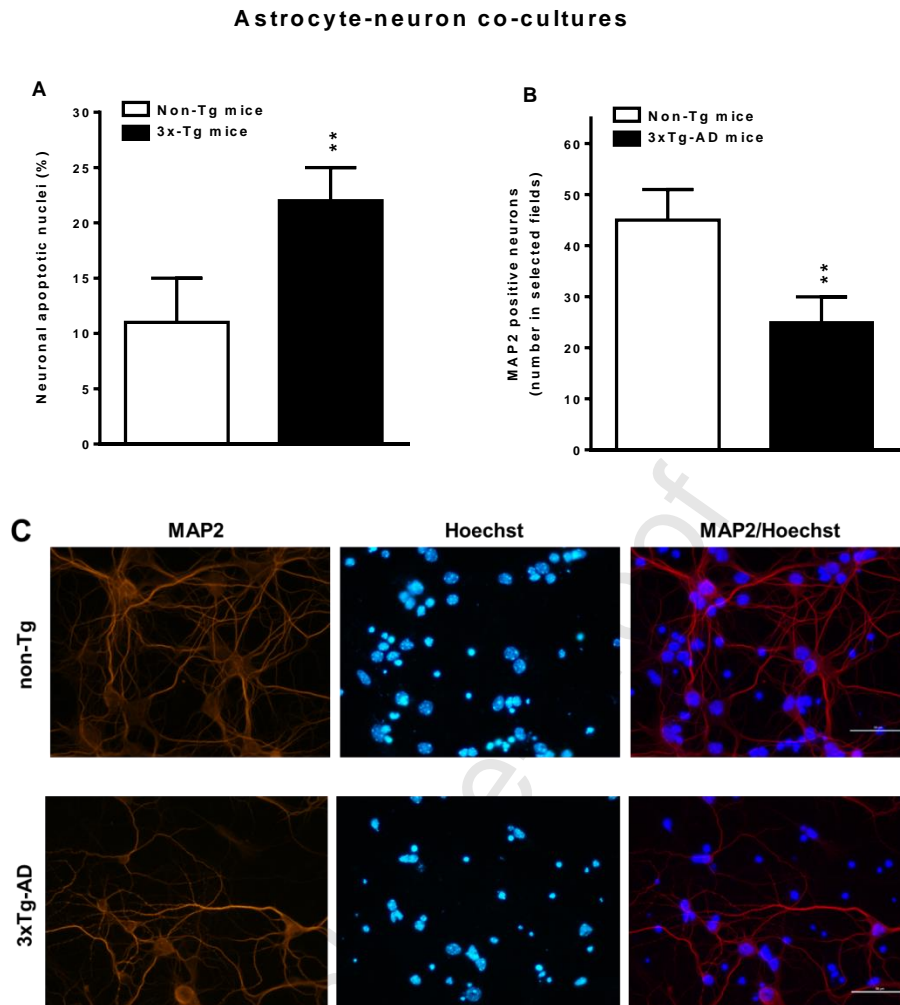


Figure 5. Astrocytic influence on apoptotic cell death (*Panel A*) and MAP2 immunoreactivity (*Panel B*) in primary cortical neurons from astrocyte-neuron co-cultures from 3xTg-AD mice and non-Tg (*i.e.* wild-type) mice. 3xTg-AD or non-Tg mouse neurons were co-cultured for 24 h with astrocytes from the respective genotype. Thereafter, the specific DNA stain Hoechst 33258 was used for the determination of apoptotic nuclei. At least five random microscopic fields were counted per sample, and the number of apoptotic nuclei was expressed as % of total nuclei. The number of MAP2 positive cells was determined under fluorescent microscope by staining neurons with anti-MAP2 antibody. Each histogram represents the mean + S.E.M (n=10-20 wells; 4-5 animals per condition). **p<0.01 significantly different from the respective non-Tg group (Student *t* test). *Panel C*: representative fluorescent photomicrographs of MAP2 (red) immunoreactivity and Hoechst 33258 (blue) staining (magnification x40; scale bar = 50 μ m).

3.2.3. Astrocytic influence on neuronal morphometric parameters

The morphometric analysis was applied to MAP2 immunostained cultured neurons in order to visualize and quantify the neurite maturation of neurons in co-cultures from non-Tg and 3xTg-AD mice. As shown in Fig. 6A, the number of neuritic aggregations/100 μm was significantly higher in the neurons from 3xTg-AD co-cultures, compared to those from non-Tg co-cultures. On the contrary, we observed significantly lower number of dendritic branches per neuron, neuronal body area, length of the longest dendrite and number of neurites per neuron in 3xTg-AD mouse co-cultures than in non-Tg mouse co-cultures (Fig. 6B-E).

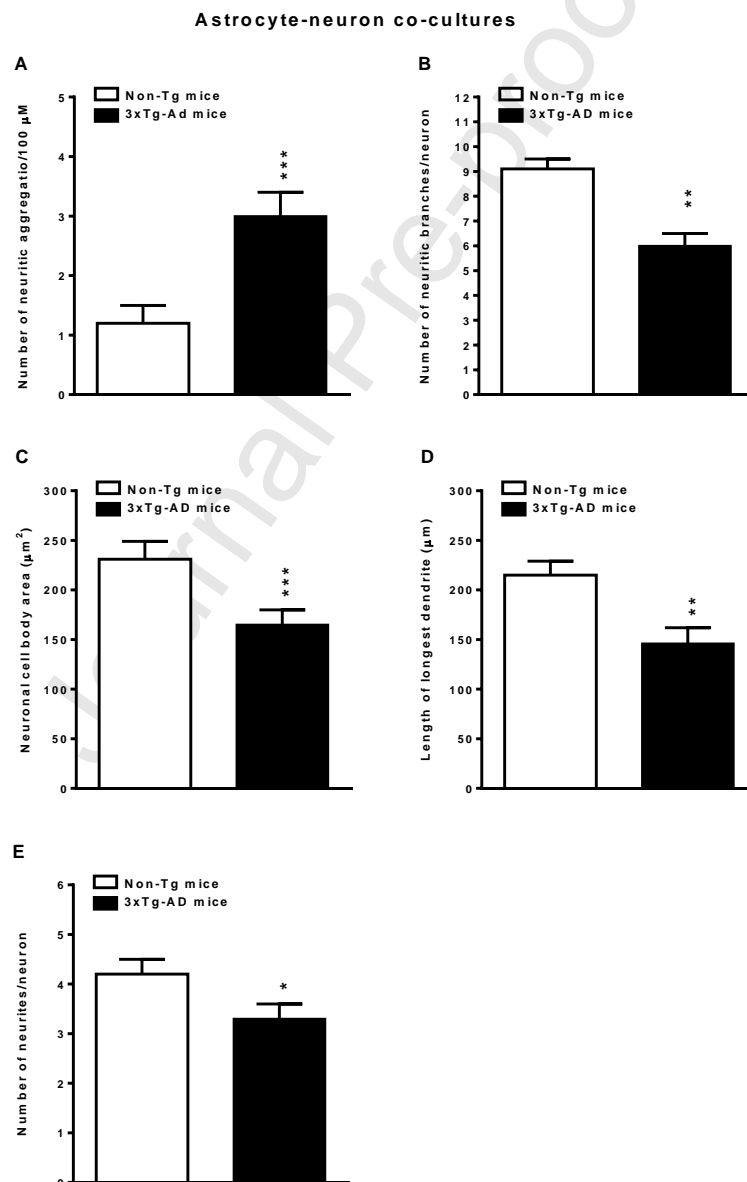


Figure 6. Astrocytic influence on morphological parameters in primary cortical neurons from astrocyte-neuron co-cultures from 3xTg-AD mice and non-Tg (*i.e.* wild-type) mice. 3xTg-AD or non-Tg mouse neurons were co-cultured for 24 h with astrocytes from the respective genotype. Thereafter, neurons were stained with anti-MAP2 antibody and observed under fluorescent microscope; the number of aggregation/100 μm (*Panel A*), the cell body area (*Panel B*), the length of the longest dendrite (*Panel C*), the number of dendritic branches per neuron (*Panel D*) and the number of neurites per neuron (*Panel E*) were measured. Each histogram represents the mean \pm S.E.M (n=10/15 wells; 3-4 animals per condition). *p<0.05, **p<0.01, ***p<0.001 significantly different from the respective non-Tg group (Student *t* test).

3.3. Astrocytic influence on apoptotic neuronal death and morphological parameters in cortical astrocyte-neuron co-cultures from 3xTg-AD mice: effects of PEA treatment

Our previous (Tomasini et al., 2015; Beggiato et al., 2018) and the present results led to hypothesize that endogenous deleterious factors spontaneously released by cultured astrocytes from 3xTg-AD mice might contribute to neuronal damages in the present experimental model. To verify this possibility, in a final set of experiments the effects of PEA on the impact of astrocytes on neuronal conditions in cortical astrocyte-neuron co-cultures from 3xTg-AD mice, have been evaluated. To this aim, 3xTg-AD mouse cortical neurons were co-cultured with 3xTg-AD mouse astrocytes pre-exposed to PEA (0.1 μM ; 24 h); apoptotic neuronal death and morphological parameters were then evaluated.

3.3.1. Effect of PEA pre-exposure on astrocytic influence on apoptotic neuronal death

The number of apoptotic nuclei in 3xTg-AD mouse cortical neurons co-cultured with 3xTg-AD mouse astrocytes pre-exposed to PEA (0.1 μM ; 24 h) was significantly lower than that observed in control co-cultures (*i.e.* 3xTg-AD mouse cortical neurons co-cultured with 3xTg-AD mouse astrocytes not treated with PEA; Fig. 7A).

3.3.2. Effect of PEA pre-exposure on astrocytic influence on neuronal MAP2 immunoreactivity

As shown in Fig. 7B, the number of MAP2 positive neurons was significantly higher in 3xTg-AD cortical neurons co-cultured with 3xTg-AD astrocytes pre-exposed to PEA (0.1 μM ; 24 h) than in

control co-cultures (*i.e.* 3xTg-AD mouse cortical neurons co-cultured with 3xTg-AD mouse astrocytes not treated with PEA).

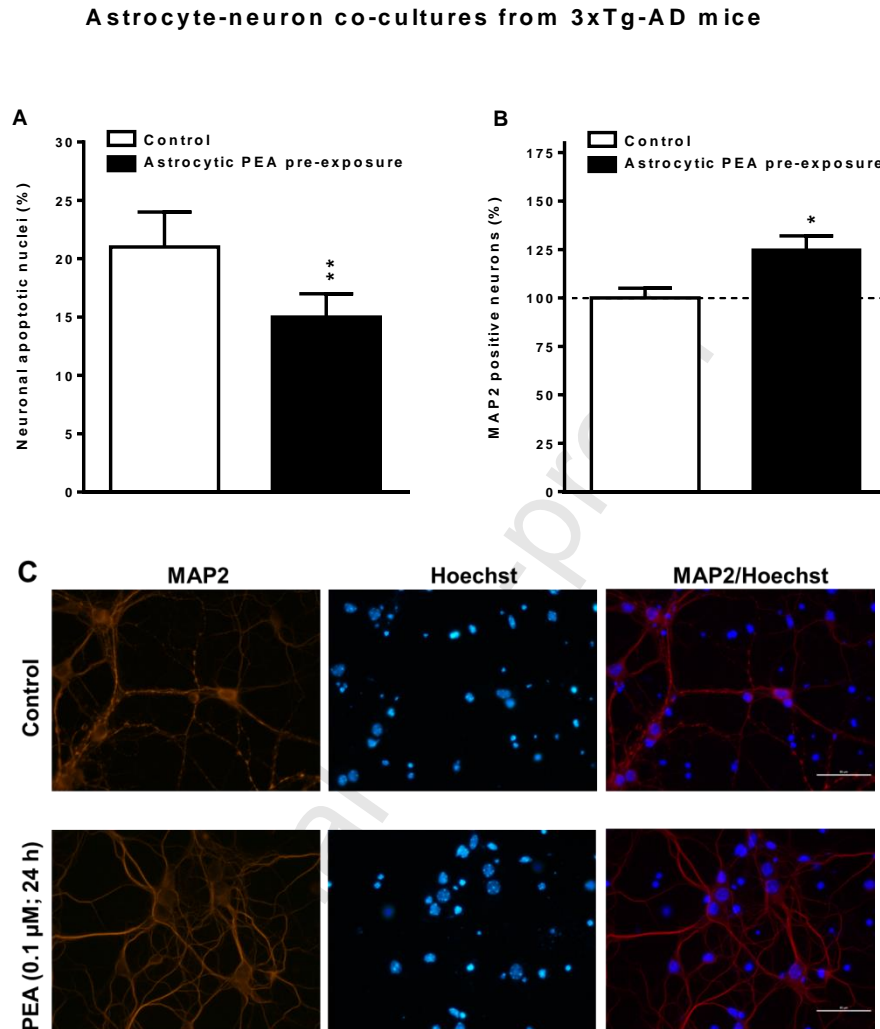


Figure 7. Effects of PEA (0.1 μM ; 24 h) pre-exposure on astrocytic influence on apoptotic cell death (*Panel A*) and MAP2 immunoreactivity (*Panel B*) in primary cortical neurons obtained from astrocyte-neuron co-cultures from 3xTg-AD mice. 3xTg-AD mouse cortical astrocyte cultures were pretreated with PEA and, after changing the culture medium (to remove PEA), 3xTg-AD mouse neurons were co-cultured for another 24 h-period. Thereafter, the specific DNA stain Hoechst 33258 was used for the determination of apoptotic nuclei. At least five random microscopic fields were counted per sample, and the number of apoptotic nuclei was expressed as % of total nuclei. The number of MAP2 positive cells was determined under fluorescent microscope by staining neurons with anti-MAP2 antibody. Each histogram represents the mean \pm S.E.M (n=10-20 wells; 3-4 animals per condition). **p<0.01, significantly different from the respective Control group (*i.e.*

neurons co-cultured with astrocytes not pre-exposed to PEA; Student *t* test). *Panel C*: representative fluorescent photomicrographs of MAP2 (red) immunoreactivity and Hoechst 33258 (blue) staining (magnification x40; scale bar = 50 μ m).

3.3.3. *Effect of PEA pre-exposure on astrocytic influence on neuronal morphometric parameters*

As shown in Fig. 8A, the number of neuritic aggregations/100 μ m was significantly lower in 3xTg-AD cortical neurons co-cultured with 3xTg-AD astrocytes pre-exposed to PEA (0.1 μ M; 24 h) than in control co-cultures (*i.e.* 3xTg-AD mouse cortical neurons co-cultured with 3xTg-AD mouse astrocytes not treated with PEA). The astrocytic PEA pre-exposure also induced an increase of the number of dendritic branches per neuron (Fig. 8B) and the length of the longest dendrite (Fig. 8D) in respect to control co-cultures. No significant differences in cell body area and number of neurites per neuron were observed in 3xTg-AD mouse cortical neurons co-cultured with 3xTg-AD mouse astrocytes pre-exposed or not to PEA (0.1 μ M; 24 h; Fig. 7C and 7E).

Astrocyte-neuron co-cultures from 3xTg-AD mice

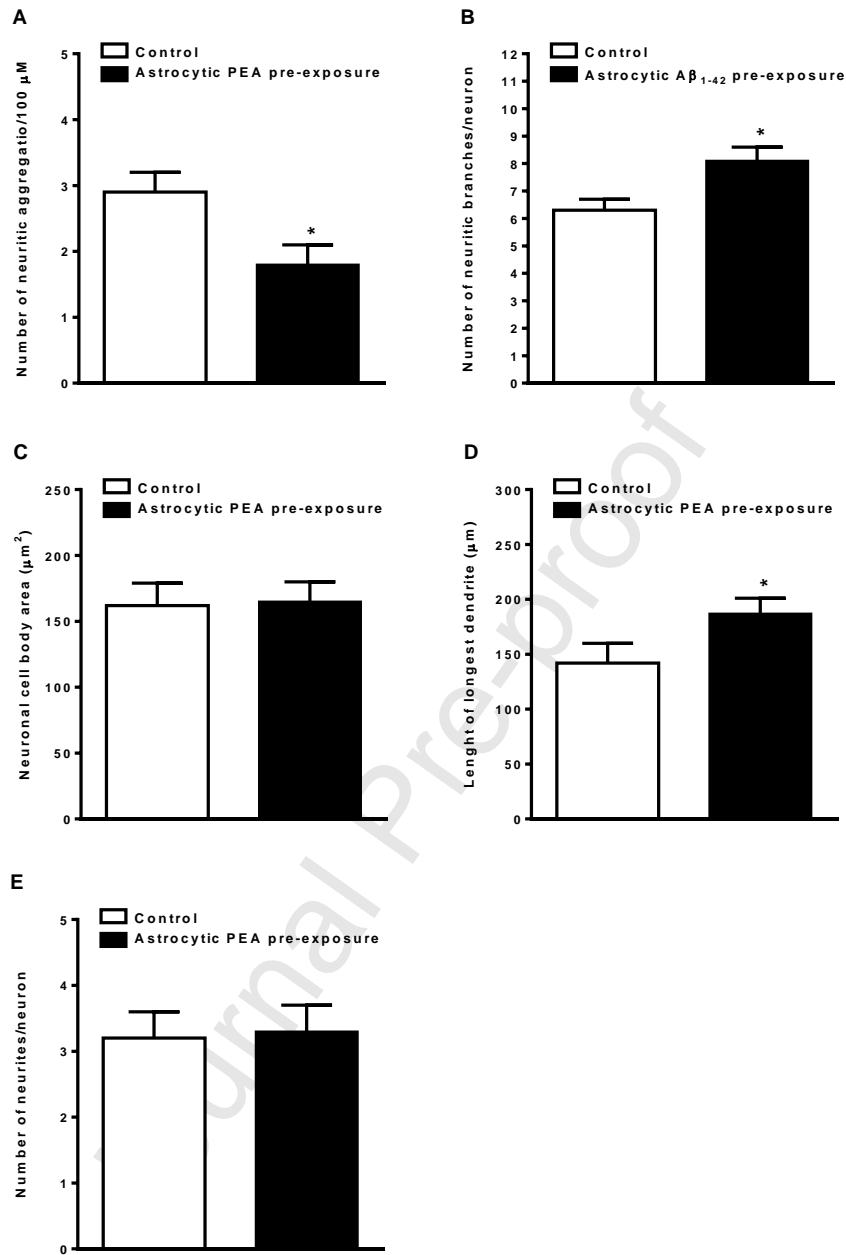


Figure 8. Effects of PEA (0.1 μM ; 24 h) pre-exposure on astrocytic influence on morphological parameters in primary cortical neurons obtained from astrocyte-neuron co-cultures from 3xTg-AD mice. 3xTg-AD mouse cortical astrocyte cultures were pretreated with PEA and, after changing the culture medium (to remove PEA), 3xTg-AD mouse neurons were co-cultured for another 24 h-period. Thereafter, neurons were stained with anti MAP2 antibody and observed under fluorescent microscope. The number of aggregation/100 μm (*Panel A*), the number of dendritic branches per neuron (*Panel B*), the length of the longest dendrite (*Panel C*), the cell body area (*Panel D*) and the number of neurites per neuron (*Panel E*) were measured. Control groups represent co-cultures obtained from astrocytes pretreated with the PEA vehicle. Each histogram represents the mean \pm

S.E.M (n=10/15). * $p < 0.05$ significantly different from the respective Control group (*i.e.* neurons co-cultured with astrocytes not pre-exposed to PEA; Student *t* test).

4. Discussion

It is now generally accepted that activated microglia and astrocytes are integral players in the pathogenesis of AD (Osborn et al., 2016; Chun and Lee, 2018; Kinney et al., 2018; Henstridge et al., 2019). Reactive astrogliosis is mainly characterized by astrocytic dysfunctions, increased expression of both GFAP and S100B, also associated with an increased production of pro-inflammatory mediators (Osborn et al., 2016; Bronzuoli et al., 2017). This phenomenon has been detected in proximity of plaques and A β deposits; by this way astrocytes contribute to neurodegeneration in AD, thus becoming a possible relevant target for the development of innovative therapies (Bronzuoli et al., 2016; 2017; Acosta et al., 2017).

Using the same experimental preparation as in the present study, we previously demonstrated that wild-type (*i.e.* non-Tg) mice astrocytes are involved in A β_{42} -induced neurotoxicity and PEA, by dampening A β_{42} -induced astrocyte activation, enhanced neuronal survival (Beggiato et al., 2018). In particular, in the presence of astrocytes pre-exposed to A β_{42} , a significant decrease in neuronal cell viability, a significant increase in apoptotic neuronal death and in neurite aggregation number were observed, thus suggesting that astrocytes are able to perpetuate/sustain the neurotoxic effects of the peptide (Beggiato et al., 2018). Interestingly, these results have not been replicated in 3xTg-AD mouse co-cultures (present study), where astrocytic A β_{42} -pre-exposure did not induce any significant effect on neuronal viability, apoptotic neuronal death and neuron morphology. These results are in line with our previous findings demonstrating that in primary cultures of cerebral cortex neurons or in primary cultures of cerebral cortex astrocytes from non-Tg, but not 3xTg-AD mice, the treatment with A β_{42} significantly affected glutamate levels, cell viability and cell morphology (Tomasini et al., 2015). Furthermore, Grolla et al., (2013) reported significant effects of A β_{42} in entorhinal cortex and hippocampal astrocytes derived from non-Tg mice, while these effects were not observed in entorhinal cortex and hippocampal astrocytes derived from 3xTg-AD mice. Thus, a negative effect of A β_{42} in the co-cultured cells from 3xTg-AD mice may be expected based on our previous results. However, it cannot be ruled out the possibility that A β_{42} exposure may have resulted in increased levels of neurotoxic agents from astrocytes, thus leading to neuronal injury in astrocyte-neuron co-cultures, and that PEA pre-treatment could counteract this effect. In this context, using this model we provide further information to support the hypothesis that cultured

astrocytes or neuron from 3xTg-AD mice seem to be less sensitive to the exogenous A β_{42} -induced insult, when compared to non-Tg mouse derived cells. The discrepancies between the results observed in cultured cells from 3xTg-AD and non-Tg mice have been associated with the demonstrated expression of endogenous intra- and extra-neuronal A β peptides in the transgenic mouse derived cell cultures (Vale et al., 2010; Tomasini et al., 2015), although other explanations have not been ruled out. Thus, it has been hypothesized that exogenous A β_{42} was ineffective in 3xTg-AD mice-derived cortical cultures as this cells at 8 DIV were already exposed to endogenous A β fragments (Tomasini et al., 2015). The exposure to endogenous A β fragments could also explain the evidence that, in the present study, astrocytic A β_{42} pre-exposure did not influence neuronal viability and morphology in astrocyte-neuron co-cultures from 3xTg-AD mice. On the other hand, our previous data demonstrating that under the same experimental conditions as used in the present study, A β_{42} directly induced toxicity in non-Tg mouse co-cultures (Beggiato et al., *J. Alzheimer's Dis.*, 2018, 61, 389-399) suggest that the peptide may have an active conformation able to induce cytotoxicity. Thus, it seems likely that the negative results we obtained cannot be due to the strong aggregation propensity of A β_{42} .

Based on the present findings, we hypothesized that endogenous deleterious factors spontaneously released by cultured astrocytes from 3xTg-AD mice might contribute to neuronal damages in the present experimental model. Indeed, activated astrocytes in AD become a part of the inflammatory process when, in addition to microglia, they start to secrete pro-inflammatory cytokines and other mediators, thus contributing to neurodegeneration (Heneka et al., 2010; Osborn et al., 2016). To verify this possibility, in the present study we also compared the effects of astrocytic influence on neuronal viability and morphology in cortical astrocyte-neuron co-cultures from non-Tg and 3xTg-AD mice. The results demonstrated that, in the absence of any exogenous treatment, astrocytes from 3xTg-AD mice have a negative impact on the number and morphology of co-cultured neurons. In fact, when compared to non-Tg mouse co-cultures, a reduced number of neurons, an increased number of apoptotic neuronal death and alterations of neuronal morphology have been observed in 3xTg-AD mouse co-cultures. Interestingly, a previous study demonstrated that 3xTg-AD mouse primary astrocyte cultures present signs of reactive astrogliosis (Bronzuoli et al., 2018). In particular, in the absence of any exogenous treatment, 3xTg-AD primary astrocyte cultures displayed a basal reactive and pro-inflammatory phenotype (Bronzuoli et al., 2018). Furthermore, in cultured hippocampal astrocytes from 3xTg-AD mice, transforming growth factors beta (TGF- β) TGF- β_2 and TGF- β_3 , but not TGF- β_1 , were up-regulated, pointing to astroglial TGF- β_2 and TGF- β_3 as potential early contributors to AD pathogenesis (Tapella et al., 2018). In view of these results and on the fact that in the astrocyte-neuron co-culture model we used, the two different cell

populations were not in direct contact with each other, it seems likely that endogenous mediators released from astrocytes reached the neighboring neurons, thus potentiating neuronal damage. Taken together, the present data suggest that in this transgenic animal model of AD, astrocytes contribute to neuronal degeneration, as indicated by the reduction of viable neurons and the increased neurite aggregation, which is considered a sign of neuronal damage often observed under neurodegenerative conditions. Interestingly, in our previous study on non-Tg mouse co-cultures we observed that astrocytic pre-exposure to A β ₄₂ increased neuronal aggregations, but failed to affect other morphological parameters (Beggiato et al., 2018). Differently, in the present study, significantly lower length of the longest dendrite and number of dendritic branches per neuron were also observed in astrocyte-neuron co-cultures from 3xTg-AD mice. These results suggest that the contribution of astrocytes to neurodegeneration in this animal model of AD could be particularly relevant, thus strengthening the role of these cells as possible relevant target for the development of innovative therapies against AD progression. In this context, it becomes relevant to investigate, in further studies, the effects of 3xTg-AD mouse astrocytes on non-Tg mouse cultured neurons. PEA is a non-endocannabinoid lipid mediator and is included in the class of N-acylethanolamine phospholipids (Petrosino and Di Marzo, 2017). Numerous *in vitro* and *in vivo* investigations demonstrated that PEA exerts its biological activities through various molecular targets in both central and peripheral nervous systems. In particular, PEA directly activates at least two different receptors: the peroxisome proliferator-activated receptor alpha (PPAR- α ; Lo Verme et al., 2005) and the orphan GPCR 55 (GPR55; Pertwee, 2007); the first one appears as the main molecular target involved in the anti(neuro)inflammatory effects of PEA. Besides these direct mechanisms of actions, PEA could also induce some indirect receptor-mediated actions, through the so-called “*entourage effect*” (Mattace Raso et al., 2014; Petrosino and Di Marzo, 2017). Specifically, PEA can indirectly activate cannabinoid receptors as well as the transient receptor potential vanilloid type 1 (TRPV1) channel, through different indirect mechanisms (Petrosino and Di Marzo, 2017; Beggiato et al., 2019 *for reviews*). PEA, therefore, display quite exclusive anti(neuro)inflammatory properties by acting on different molecular targets, contrarily to classic anti-inflammatory drugs. Accordingly, various preclinical and some clinical studies suggested PEA as an effective therapeutic agent in pathologies characterized by neurodegeneration and neuroinflammation (Calabrò et al., 2016; Brotini et al., 2017; Holubiec et al., 2018; Impellizzeri et al., 2019). Concerning AD, PEA exerted neuroprotective/anti-inflammatory effects in numerous *in vitro* and *in vivo* experimental models of the pathology (Beggiato et al., 2019; 2020). Among other studies, we previously demonstrated, by using astrocyte-neuron co-cultures from non-Tg mice, that PEA fully prevented the neuronal cell death, the reduced number of MAP2 positive cells, the neurite

fragmentation, and the increase of apoptotic nuclei induced by astrocytic A β_{42} pre-exposure (Beggiato et al., 2019). Furthermore, data from astrocyte-neuron co-cultures indicated that PEA treatment resulted in a massive reduction in astrocyte number and such an effect was associated with a significant decrease in the number of apoptotic nuclei in MAP2-positive neurons induced by A β challenge (Scuderi et al., 2012). Interestingly, the PEA-induced antigliosis and neuroprotective effects were inescapably due to PPAR α activation, since they were almost completely abolished by the selective PPAR α antagonist (Scuderi et al., 2012). To further evaluate the protective role of the compound against neurodegeneration in AD, in the present study the effects of PEA on astrocytic-induced neuronal damages have been investigated in astrocytes-neuronal co-cultures from 3xTg-AD mice. Overall, the results indicate that astrocytic PEA pre-exposure counteracts the reduced number of MAP2 positive cells, the increase of apoptotic nuclei and the neuron morphological changes induced by astrocytes when co-cultured with cortical neurons from 3xTg-AD mice. Based on these findings, it can be hypothesized that PEA exerts at least part of its neuroprotective effects by counteracting the production and/or release of deleterious mediators by astrocytes. This hypothesis is supported by the demonstration that PEA reduced A β -induced neuroinflammation by significantly reducing either the enhanced expression of pro-inflammatory molecules [cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS)], or the increased release of prostaglandin PGE₂, nitric oxide, interleukin-1 β , and tumor necrosis factor-alpha (TNF- α) (Scuderi et al., 2011). Additionally, PEA induced a significant decrease in the number of apoptotic nuclei in MAP2-positive neurons induced by A β challenge in mixed neuroglial co-cultures, reduced the expression and release of some pro-inflammatory cytokines and mediators in organotypic hippocampal slices (Scuderi et al., 2013) and displayed anti-inflammatory and neuroprotective activities in adult male rats given intrahippocampal injection of A β_{42} (Scuderi et al., 2014). Finally, a previous study (Bronzuoli et al., 2018) demonstrated that PEA was able to significantly reduce iNOS expression in transgenic-derived primary astrocytes.

As stated above, in our previous study on primary cortical neurons we observed that untreated (*i.e.* control) cortical neurons obtained from 3xTg-AD mice presented morphological modifications similar to those observed in A β_{42} -exposed cultured cortical neurons obtained from non-Tg mice (Tomasini et al., 2015). This finding has been explained as due to the release of endogenous A β fragments by 3xTg-AD mouse cultured neurons (Tomasini et al., 2015). This raises also the possibility that, in the present study, the neuronal damages observed in 3xTg-AD mouse co-cultures, when compared with the non-Tg ones, could be simply due to the release of endogenous deleterious factors by neurons and not by the influence of astrocyte-released modulators. However, the evidence that astrocytic pre-exposure to PEA counteracts the reduced number of MAP2 positive

cells, the increase of apoptotic nuclei and the neuron morphological changes observed when untreated astrocytes were co-cultured with cortical neurons from 3xTg-AD mice, strongly support the role of these cells in contributing to neurodegeneration. Based on the present results, it became relevant to evaluate, in future studies, the consequences of co-culturing 3xTg-AD astrocyte with non-Tg neurons, as well as non-Tg astrocytes with 3xTg-AD neurons to definitively confirm that astrocyte contribute to neuronal degeneration in the 3xTg-AD model.

In conclusion, the results obtained in the present study indicate that in mouse astrocyte-neuron co-cultures from 3xTg-AD mice, astrocytes contribute to neuronal damage and that PEA, by possibly counteracting reactive astrogliosis, improved neuronal survival. Together with previous data on experimental AD models (Tomasini et al., 2015; Beggiato et al., 2018; 2019; 2020), these data indicate that PEA could exert therapeutic properties by reducing the deleterious consequences of reactive astrogliosis. However, it seems likely that also direct neuroprotective actions or a reduction of microglial activation can concur to the beneficial effects of PEA in AD (Petrosino and Di Marzo, 2017; Skaper et al., 2018; Beggiato et al., 2019). Considering the extreme safety and tolerability of PEA in humans (Nestmann, 2016; Petrosino and Di Marzo, 2017; Skaper et al., 2018; Beggiato et al., 2019), the present findings confirm PEA as a possible new therapeutic opportunity in AD treatment.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Astrocytic palmitoylethanolamide pre-exposure exerts neuroprotective effects in astrocyte-neuron co-cultures from a triple transgenic mouse model of Alzheimer's disease.

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Declaration of Competing Interest

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Journal Pre-proof

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