Isolation and characterization of neurotoxic astrocytes derived from adult triple transgenic Alzheimer's disease mice

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Abstract

Alzheimer´s disease has been considered mostly as a neuronal pathology, although increasing evidence suggests that glial cells might play a key role in the disease onset and progression. In this sense, astrocytes, with their central role in neuronal metabolism and function, are of great interest for increasing our understanding of the disease. Thus, exploring the morphological and functional changes suffered by astrocytes along the course of this disorder has great therapeutic and diagnostic potential.

In this work we isolated and cultivated astrocytes from symptomatic 9-10-months-old adult 3xTg-AD mice, with the aim of characterizing their phenotype and exploring their pathogenic potential. These “old” astrocytes occurring in the 3xTg-AD mouse model of Alzheimer´s Disease presented high proliferation rate and differential expression of astrocytic markers compared with controls. They were neurotoxic to primary neuronal cultures both, in neuronal-astrocyte co-cultures and when their conditioned media (ACM) was added into neuronal cultures. ACM caused neuronal GSK3β activation, changes in cytochrome c pattern, and increased caspase 3 activity, suggesting intrinsic apoptotic pathway activation. Exposure of neurons to ACM caused different subcellular responses. ACM application to the somato-dendritic domain in compartmentalised microfluidic chambers caused degeneration both locally in soma/dendrites and distally in axons. However, exposure of axons to ACM did not affect somato-dendritic nor axonal integrity.

We propose that this newly described old 3xTg-AD neurotoxic astrocytic population can contribute towards the mechanistic understanding of the disease and shed light on new therapeutical opportunities.

Keywords: Adult 3xTg-AD mice; Astrocytes; Neurodegeneration; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is a devastating neurological disorder characterized by an accumulation of senile plaques and neurofibrillary tangles (NFTs) containing the β-Amyloid (Aβ) peptide and the hyperphosphorylated microtubule-associated protein tau, respectively (Braak et al., 1993). Its main behavioural symptoms are progressive loss of memory and general cognitive function, while at the cellular level is characterized by synaptic dysfunction, axonal loss/degeneration and neuronal death (Adalbert and Coleman, 2013; Gómez-Isla et al., 1997; Jackson et al., 2019; Selkoe, 2002; Terry et al., 1991). Although multiple molecular mechanisms have been postulated among the list of disease-relevant processes (Francis et al., 1999; Markesbery, 1997; Wang and Reddy, 2017), the cellular events and functional consequences of neurotoxic forms of Aβ and tau have taken central attention in AD research, leading to a great understanding of these actors in disease aetiology and progression (Hardy and Higgins, 1992; Selkoe and Hardy, 2016). Despite these efforts, the translation of this growing knowledge into advances at the clinical level has been limited (Liu et al., 2019). Indeed, one of the main concerns of AD research is the relative lack of wider mechanistic insights beyond Aβ and tau, and the scarcity of relevant preclinical models that can be used in the initial stages of drug development (Mullane and Williams, 2019).

Beyond the important role recognized for both Aβ and tau, it is now acknowledged that AD is a multifactorial chronic disease that involves a

complex interplay between neurodegeneration, cerebrovascular pathology and inflammation, and where aging is its greatest risk factor (Heneka et al., 2015; Zlokovic, 2005). In this context, there has been a growing awareness about the need to expand neuron-centric viewpoints that have classically prevailed in experimental neurology, incorporating the important role of glia in physiological and pathological conditions. This need has been accompanied by increasing experimental data that highlighted glia involvement in neuro-inflammation and neuroprotection associated with the progression of diverse brain pathologies (Díaz-Amarilla et al., 2011; Liddelow and Barres, 2017; Siracusa et al., 2019).

Astrocytes are the glial cells responsible for brain homeostasis, with key roles in synapse formation/elimination, modulation of synapses and neuronal connectivity, antioxidant protection, blood flow regulation, energetic and trophic supply, as well as debris clearing (Santello et al., 2019; Verkhratsky and Nedergaard, 2018). Astrocytes can also contribute to brain protection in pathology (Kettenmann and Ransom, 2013), and in the case of AD, they show morphological and functional changes that can affect the course of the disorder (Heneka et al., 2015; Olabarria et al., 2010). Furthermore, evidence indicates that there is an early imbalance in astrocytic function in AD, characterized by a heterogeneous morphology that is dependent on brain region and disease state (Kulijewicz-Nawrot et al., 2012; Olabarria et al., 2010). Previously, Iram and co-workers found that cultured astrocytes derived from old (12–14 months) transgenic animals of the heterologous mouse line Tg6799, also named 5xFAD, were functionally impaired in Aβ uptake and in neuronal support, proposing that these astrocytes were permanently affected following long-term exposure to an AD brain milieu in the animal model (Iram et al., 2016). Although restricted by

the presence of only defined genetic modifications, transgenic animals play central roles as tools for improving our understanding of AD pathogenesis. The 3xTg-AD mouse model of AD is an attractive transgenic model that contains the Swedish mutation for human amyloid precursor protein (APPswe), plus mutated human presenilin 1 (Psen-1) and human tau mutation (tauP301L). Importantly, it develops both plaque and tangle pathology in AD-relevant brain regions, while showing age-dependent cognitive deficits, astrogliosis, and brain atrophy that are indicative of both synaptic and neuronal loss (Billings et al., 2005; Chiquita et al., 2019; Oddo et al., 2003). Previous evidence shows that immortalized hippocampal astrocytes from new-born 3xTg-AD mice had an impaired ability to support blood brain barrier integrity through paracrine mechanisms *in vitro* (Kriaučiūnaitė et al., 2021). Furthermore, astrocytes from new-born 3xTg-AD mice exhibited compromised vesicle trafficking *in vitro* (Stenovec et al., 2016). Recently, González-Molina and co-workers found that extracellular vesicles released by astrocytes from 8-9 months-old 3xTg-AD mice impaired cells from the neurovascular unit *in vitro* (González-Molina et al., 2021).

In our study, we isolated and cultured astrocytes from 9-10 months-old 3xTg-AD mouse model of AD with a high proliferation rate, a differential expression of astrocytic markers, and the capacity to mediate neuronal degeneration. The results add evidence to the hypothesis that environmentally-induced changes in astrocyte function during the progression of AD may in turn promote/accelerate neurodegeneration.

2. Materials and Methods

2.1 Materials:

All reagents were acquired from Sigma unless otherwise stated. Commercial antibodies used in this study are detailed in table 1 of supplementary material (SM1).

2.2 Animals:

All procedures using laboratory animals were performed in accordance with the international guidelines for the use of live animals and were approved by the Institutional Animal Ethics Committee (CEUA-CUDIM) as established by the National Committee of Animal Experimentation (CNEA). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Mice homozygous 3xTg-AD (B6;129-Tg(APPSwe,tauP301L)1Lfa Psen1(tm1Mpm)/Mmjax strain) (Oddo et al., 2003) and wild-type non-transgenic (Non-Tg) (B6129SF2/J and/or C57BL/6J strains) (The Jackson Laboratory) were bred and housed in CUDIM SPF centralized animal facility with a 12-h light-dark cycle with *ad libitum* access to food and water. All experiments were performed on female animals since sex differences in the development of the pathology have been described in this AD murine model (i.e, expression of plaques and tangles, behavioral deficits) with a greater expression of transgenes in females (Carroll et al., 2010; Clinton et al., 2007).

2.3 Astrocyte cultures from adult and neonatal Mice:

Depending on the experiment, mixed cortical and hippocampal primary astrocyte cultures were isolated from adult (9-10 months; 3xTg-AD or non-Tg) or new-born (postnatal day 0-2, 3xTg-AD or non-Tg) mice. After euthanasia, cerebral cortices were aseptically dissected and meninges were removed. The

tissues were minced and then dissociated both enzymatically (0.25 % trypsin, 37 °C, 10 min) and mechanically to obtain single cell suspension. After filtration through a mesh, the cell suspension was spun and resuspended in complete medium [DMEM (Capricorn) containing 10% FBS (GIBCO), and penicillin (100 IU/mL)/streptomycin (100 μg/mL) solution (Capricorn)]. The cells were plated in tissue flasks and then cultured at 37 °C in a humidified 5% CO_2 incubator.

In the case of astrocytes isolated from neonatal mice, once plated, the medium change occurred once every 2-3 days. When cells grew to confluence after approximately 10 days in vitro (DIV), flasks were vigorously shaken to remove the loosely attached contaminated microglia and oligodendrocyte progenitor cells (OPCs), then incubated for another 48 hr with 10 μM cytosine arabinoside, and then amplified to flasks, 35 mm Petri dishes or 96-well plates for the different assays (Cassina et al., 2002). In the case of astrocytes isolated from adult mice, culture medium was removed after 7 DIV and then was replaced every 3-4 days. The flask was shaken to remove debris and possible cell contaminants when culture medium was changed. Cells were passaged for the first time at 21-28 DIV. To do so, the culture was incubated with trypsin (0.25 % trypsin, 37 °C, 4 min). Then, the cells were harvested in complete medium and spun at 250 × g for 10 min. The pellet was then resuspended in complete medium, and the cells were amplified to flasks, 35 mm Petri dishes or 96-well plates and maintained at 37 °C in a humidified 5% CO_2 incubator.

2.4 Primary neuronal cell cultures:

Neurons from cortex and hippocampus were isolated from (E16-18) C57BL/6J embryos as previously reported, with minor modifications (Dajas-Bailador et al.,

2014; Lucci et al., 2020). Embryo cortices were aseptically dissected in Hank's Balanced Salt Solution (HBSS) and meninges were removed. The tissues were minced and then enzymatically disaggregated (0.25% trypsin, 37 °C, 5 min), followed by mechanic disaggregation, to obtain single cells. Dissociated neurons were plated on poly-L-ornithine-coated 35 mm dishes ($57x10^3$ cells/cm^2) in complete medium [Neurobasal medium (GIBCO) supplemented with 2% B27 (GIBCO), GlutaMAX (GIBCO) and penicillin (100 IU/mL)/streptomycin (100 µg/mL) solution (Capricorn)], and maintained in a humidified incubator with 5% CO_2 at 37 ºC. For studies of axonal integrity, neurons were grown in compartmentalised microfluidic devices (MFD; Xona Microfluidics LLC, Temecula, CA, USA; Cat #SND450) at a density of $8x10^4$ cells per somatic compartment. By seeding the neuronal cultures in these devices, axons were fluidically isolated from the somato-dendritic compartment. For co-culture experiments, neurons ($32x10^3$ cells/cm^2) were plated on confluent monolayers of adult or neonatal astrocytes and maintained for 5 DIV in complete neuronal medium.

2.5 Astrocyte conditioned media (ACM) exposure in neuronal cultures:

For preparation of ACM, astrocyte monolayers were incubated in serum-free DMEM medium for 24 hr. Respective supernatants were centrifuged at 1.000 × g for 10 min and conserved at -80ºC overnight. After 24 hr, the ACM were lyophilized and conserved until its use at 4ºC.

Old 3xTg-AD ACM, neonatal 3xTg-AD ACM, and non-Tg ACM (previously lyophilized and reconstituted in sterile distilled water) were added (proportion 1:1 to the existing neuronal culture medium) at DIV12 to the neuronal cultures

for 24 hr. The effect of fresh (non-conditioned) medium, added at the same proportion, on neuronal cultures was also evaluated as control. In the case of neuronal cultures in microfluidic devices, at DIV12 Old 3xTg-AD ACM or neonatal non-Tg ACM was added either in the somatic compartment or in the axonal compartment for 24 hr. To ensure the isolation of fluids between the different compartments, a volume difference of 50 µL was generated in favour of the untreated side. After treatments, cultured cells were fixed and processed for immunocytochemistry.

2.6 Astrocytes proliferation assay:

Astrocytes were plated in 96-wells plates at a density of $6x10^3$ cells/cm^2. Measurement of cellular protein content by the Sulforhodamine B assay (SRB), was used in order to assess proliferation rates of the different astrocyte cultures, according to Orellana and Kasinski with minor modifications (Orellana and Kasinski, 2016). The assay relies on SRB property for binding stoichiometrically to proteins under mild acidic conditions. Using basic conditions it can be extracted and the SRB bound can be used as an estimate for cell mass and thus be inferred to measure cell proliferation. After 5 hr (to obtain the values of cell density of 0 DIV), or 1, 2, 5, 6 or, 8 DIV, cells layers were fixed to the well bottoms by adding 10% TCA and plates were incubated at room temperature for 1 hr. Then, wells were rinsed twice with distilled water, and air dried. SRB (0,4% in 1% glacial acetic acid) was then added to the wells and incubated for 30min. After washing 3 times with 1% glacial acetic acid, plate was air dried and finally the dye was solubilized in Tris base (10 mM). Absorbance (570 nm) was measured on a microtiter plate reader

(Fisherbrand™ AccuSkan™ FC Filter-Based Microplate Photometer). Ten to twenty replicate wells of each astrocyte culture were run for each time point, and SRB assay was performed independently twice.

2.7 Western blotting:

Confluent monolayers of astrocytes were washed with PBS and then lysed with appropriate buffer containing 50 mM HEPES (pH 7.5), 50 mM NaCl, 1% Triton X-100 and complete protease inhibitor mixture. Protein quantification was achieved using a Bicinchoninic Acid (BCA) kit. Protein samples were prepared in Laemmli sample buffer 5x, resolved on 12% SDS-PAGE gels and transferred onto PDVF membranes (BioRad) using a wet transfer system. Membranes were blocked in 5% dried milk in TBS-T (0.5 mM Tris base, pH 8, 75 mM NaCl and 0.2% Tween-20) for at least 2 hr at room temperature. Membranes were incubated with primary antibody diluted in 2% BSA in TBS-T and then with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 2% BSA in TBS-T for 1 hr. Membranes were developed using chemiluminescent substrates (Thermo).

2.8 Immunocytochemistry:

Cultured cells were fixed with cold 4% PFA for 20 min (or with ice-cold MeOH for 5 min, in the case of astrocytic cultures), washed with PBS/glycine, and permeabilized with 0.2% Triton-X100 for 30 min at room temperature. Unspecific binding sites were blocked with 3% bovine serum albumin (BSA, MP Biomedicals) for 60 min, and labelling with primary antibodies was performed overnight at 4°C. After incubation, cells were extensively washed with

PBS/Triton-X100 and incubated with appropriate secondary antibodies at room temperature for 2 hr. After nuclear staining with Hoechst 33342 or DAPI, samples were mounted and images were acquired in a ZEISS LSM 800-AiryScan laser scanning confocal microscope provided with a Zen Blue Edition V2.6 program or a Zeiss LSM880 confocal microscope provided with a Zen Black Edition V2.3 program.

2.9 Image analysis:

All the analysis of images was performed using ImageJ software (http://rsb.info.nih.gov/ij) (Schneider et al., 2012).

2.9.1 Quantification of axonal integrity in microfluidic neuronal cultures exposed to ACM:

For microfluidic devices, we employed an ImageJ macro previously developed by Sasaki et al. (Sasaki et al., 2009) with minor modifications (Loreto et al., 2020). In order to statistically compare the status of the axons after the different ACM treatments, the Image J macro defines the following indexes: preservation, degeneration and fragmentation. The Index of Preservation (IP) is an index of the number of intact axons. It establishes a relationship between the total area occupied by all particles and the area occupied by particles larger than 1000 px with circularity between 0 and 1. The other two indexes, the Index of Degeneration (ID, particles size less than 1000 px, circularity 0-1) and the Index of Fragmentation (IF, particles size less than 1000 px, circularity 0,9-1) were utilized using the same ratio with respect to the total area. These indexes allowed us to statistically compare the level of degeneration and fragmentation of the axons after the different ACM treatments. Four random fields from axon

compartment were analysed for each condition in 3 independent experiments, performed in duplicates each time. Data were normalized and expressed as mean ±S.D percentage compared to control (microfluidic neuronal cultures exposed to non-Tg ACM).

2.9.2 Quantification of viability in microfluidic neuronal cultures exposed to ACM:

The viability after ACM treatments in the microfluidic devices was quantified with the Cell Counter plugin by visualization of Hoechst 33342-stained nuclei. Condensed nuclei were counted according the following criteria: nucleus size, presence or absence of nucleoli and cytoplasmic morphology. Four random fields from soma compartment were analysed for each condition in 3 independent experiments, performed in duplicates each time. For each condition, the ratio of condensed nuclei was normalized and expressed as mean ±S.D percentage respect to control (microfluidic neuronal cultures exposed to non-Tg ACM).

2.9.3 Quantification of caspase-3 activity in neuronal cultures exposed to ACM:

The Colocalization Threshold tool was used for the quantification of the active caspase-3, to identify Hoechst 33342-stained nuclei and active caspase-3 cells, followed by the Cell Counter plugin. Eleven to thirteen random fields from cell cultures were analysed for each condition in 2 independent experiments. The percentage of active caspase-3 was calculated by comparing the active caspase-3 nuclei with the total number of nuclei, and expressed as mean ±S.D.

2.9.4 Quantification of active GSK3β in neuronal cultures:

The GSK3β activation after ACM treatments in the neuronal cultures was quantified using the Cell Counter plugin with visualization of GSK3β in its active and inactive forms (GSK3β phosphorylated at Tyr^{216} and Ser^{21} residues, respectively). Random fields from cell cultures were analysed for each condition in 3 independent experiments. The percentage of active GSK3β was calculated by comparing the number of neurons labelled with anti- $pTyr^{216}$ GSK3β with the total number of neurons labelled with anti- $pSer^{21}$ and anti- $pTyr^{216}$ GSK3β, and expressed as mean ±S.D.

2.10 Statistical analysis:

Data analysis was carried out using Prism v8.4.3 (GraphPad Software) and all data were expressed as mean ± S.D. All experiments were performed at least three independent times, unless otherwise stated (see specific section of each experimental design for details). The probability distribution of the data set was analysed using the Shapiro–Wilk normality test. Then the statistical evaluation between two groups was performed using unpaired two-tailed Student's t-test, while analyses of more than two groups were carried out using one-way ANOVA with Tukey's multiple comparisons post-hoc test. When the normality test failed, comparison between two groups was performed by Kolmogorov-Smirnov test, and multiple comparisons were analysed with Kruskal-Wallis test followed by Dunn post-hoc test.

3 Results

3.1 Isolation of astrocytes from symptomatic 3xTg-AD animals:

To investigate changes in astrocytes induced by brain milieu during the progression of AD, we isolated and cultured astrocytes derived from 9-10-months-old 3xTg-AD mice, a stage when glial reactivity has been already described for this model (Kreimerman et al., 2019; Oddo et al., 2003).

Mixed cortical and hippocampal astrocytic cultures were obtained from 4 different symptomatic 9-10-months-old adult 3xTg-AD mice. These isolated cultures produced clusters of cells, with some resembling elongated flat astrocytes that proliferated rapidly and continuously after the first passage *in vitro* (Figure 1A). In subsequent passages, these cultures yielded layers of an enriched cell subpopulation characterized by flat, fusiform to polygonal morphology with overlapping processes, which were successfully cultured and studied until passage 15. These cells showed a marked staining with the typical astrocytic marker S100β, were negative for Iba1 and Olig2 markers, and responded to Forskolin by inducing the growth of astrocytic processes, thus providing further confirmation of their astrocytic phenotype (Figure 1B, SM2). In the case of non-Tg mice, one astrocytic culture was obtained from a total of 7 adults (9-10 months-old), but this yielded cells with limited growth potential (Figure 1A).

3.2 Characterization of the old 3xTg-AD astrocytic population:

In order to further characterize the astrocyte cultures derived from these adult 3xTg-AD mice, we evaluated the expression of the astrocyte markers Glial fibrillary acidic protein (GFAP), S100 calcium-binding protein β (S100β) and Connexin 43 (Cx43). Due to the low yield of astrocytes from old non-Tg mice, it was not possible to analyse them at the morphological and functional level with

significant statistical power. Instead, astrocytic cultures derived from neonatal non-Tg animals were used as controls throughout the study. To provide a further comparison in our experimental model, we also isolated astrocytic cultures from neonatal 3xTg-AD mice, thus examining whether the morphological and functional alterations observed in astrocytes were present in the pre-symptomatic phase of the disease.

When compared to these two other astrocytic cultures (neonatal non-Tg and 3xTg-AD), we observed that old 3xTg-AD astrocyte cultures showed a marked difference in the levels and patterns of expression of the astrocyte markers (Figure 2). Importantly, neonatal 3xTg-AD astrocytic cultures didn´t show differences in the expression of these markers respect to astrocytes derived from control neonatal non-Tg mice (Figure 2).

As shown in figure 2A-B, we observed a notable reduction in GFAP expression in astrocytes derived from the old 3xTg-AD mice as compared with the two other astrocytic cultures, demonstrated by immunocytochemistry and western blot (Figure 2A and 2B). In addition, our old 3xTg-AD astrocytes exhibited a change in the pattern of staining of S100β protein compared with neonatal 3xTg-AD and non-Tg derived astrocytes (Figure 2A). As well, we observed a markedly different cell distribution of Cx43 in old 3xTg-AD astrocytes compared with the two other astrocytic cultures (Figure 2A). In the latter, punctiform distribution demonstrated the existence of sparse gap junctions at the cell surface in contrast with the intracellular localization visualized in the old 3xTg-AD astrocytes (Figure 2A).

Subsequently, we investigated the expression of the enzyme Monoamino oxidase B (MAO-B), which has been primarily located in activated astrocytes

(Ekblom et al., 1993; Saura et al., 1994). As shown in figure 2B, we found that the expression of MAO-B, although not significant, had a trend towards increase in old 3xTg-AD astrocytes compared with neonatal 3xTg-AD and non-Tg derived astrocytes (Figure 2B).

In addition to the clear differences in the distribution and abundance of the key markers described above, old 3xTg-AD astrocytes showed a significant increase in the rate of proliferation compared to neonatal 3xTg-AD and non-Tg astrocytes (Figure 2C).

3.3 Neurotoxic potential of old 3xTg-AD astrocytes:

To investigate the pathogenic potential of these newly characterised old 3xTg-AD astrocytes we decided to use a neuron-astrocyte co-culture model. For this, mixed cortical and hippocampal neurons isolated from E16-18 non-Tg mice embryos were plated on confluent monolayer of old 3xTg-AD astrocytes, or 3xTg-AD and non-Tg neonates' astrocytic cultures. As shown in figure 3, after 5 DIV primary neurons could develop on non-Tg, and 3xTg-AD neonatal astrocytic cultures, showing extensive dendritic and axonal growth. However, we found a massive loss of neuronal cells when grown on top of the old 3xTg-AD astrocytic monolayers (Figure 3). Importantly, passage number after initial culture did not significantly change the observed functional and morphological characterization of these old 3xTg-AD astrocytic cultures (from passages 2 to 15), with all showing similar results.

We next explored whether the effect exerted by these astrocytes was mediated via soluble factors secreted to the cellular environment. For this, we evaluated the effect on neuronal cultures following addition (24h) of conditioned medium

from old 3xTg-AD astrocytes (old 3xTg-AD ACM) or controls. We observed that exposure of neuronal cultures to fresh (non-conditioned) medium, ACM from non-Tg, or ACM from 3xTg-AD neonates control astrocytic cultures did not affect neuronal viability, whereas ACM from old 3xTg-AD dramatically impacted neuronal viability (Figure 4).

3.4 Cellular mechanisms underlying old 3xTg-AD astrocyte neurotoxic effects:

In order to see if old 3xTg-AD astrocyte neurotoxic effects were the result of their secretion of Aβ to the cellular environment, we analysed the effect of an inhibitor of γ-secretase, the pivotal enzyme that generates Aβ, on old 3xTg-AD ACM. Blocking Aβ generation *in vitro* by tert-Butyl (S)-{(2S)-2-[2-(3,5-difluorophenyl) acetamido] propanamido} phenylacetate (DAPT) did not prevent old 3xTg-AD ACM neurotoxic effects (SM3), suggesting that Aβ may not underlie neurotoxicity of these old 3xTg-AD astrocytes.

Furthermore, to provide evidence for the underlying cellular mechanisms of neurodegeneration occurring in our cellular model, we explored GSK3β activity. Interestingly, exposure of primary neurons to old 3xTg-AD ACM, produced changes in GSK3β phosphorylation state, when compared to neuronal cultures exposed to 3xTg-AD neonates control ACM (Figure 5A and B). Our results showed neuronal cultures with a significantly higher percentage of active GSK3β (pGSK3β-Y216) after addition of old 3xTg-AD ACM (Figure 5B). To further explore potential mechanisms of toxicity, we investigated the cytochrome c expression pattern and caspase-3 activity following old 3xTg-AD ACM application. We found that old 3xTg-AD ACM also caused changes in cytochrome c pattern and a significant increase in caspase-3 activity, when

compared to neuronal cultures treated with 3xTg-AD neonates control ACM (Figure 5C and D).

3.5 Differential local effect of old 3xTg-AD ACM in neuronal cultures:

Taking into account the polarized nature of neuronal organisation, at the morphological and functional level, and the importance that this has in degenerative mechanisms, it was important to explore whether subcellular compartments (somato-dendritic or axonal) might respond differently to the observed mediators of neuronal toxicity. For this, we used a compartmentalized microfluidic device which allows fluidic isolation of axonal and somato-dendritic compartments. In this way, we could compare the effect of old 3xTg-AD ACM and non-Tg control ACM, following 24 hr exposure at either somato-dendritic or axonal level. Our results demonstrate that old 3xTg-AD ACM caused radically different cellular responses that were specific to the sub-cellular compartment. As shown in Fig 6, addition of old 3xTg-AD ACM to the somato-dendritic surface caused complete neuronal degeneration, shown both locally in the somato-dendritic compartment and also in distal axons not exposed to the media. Surprisingly, when only axons were exposed to old 3xTg-AD ACM we observed no sign of degeneration and/or damage (both at axonal and retrogradely at somato-dendritic level), with fragmentation levels even lower than non-Tg control ACM (Figure 6).

4 Discussion

In pathological situations leading to neuronal degeneration as in AD, astrocytes acquire a reactive state characterized by molecular and cellular changes. Depending on the stage of the disease, gliosis can be a supportive or deleterious event for neuronal survival (Anderson et al., 2014; Sofroniew, 2020). In our study, we reported the morpho-functional characterisation of astrocytes from 9-10 months-old 3xTg-AD mouse model of AD, providing an important contribution towards the understanding of the changes adopted by astrocytes during the progression of AD.

In order to fully characterize these newly described astrocytes from old 3xTg-AD it was important to investigate their pattern of expression of key markers, including GFAP, S100β, Cx43 and MAO-B. As shown previously (Sofroniew, 2009) astrocytic expression of GFAP shows regional variability that is dynamically regulated in a context-dependent manner. Accordingly, in the 5xFAD AD mouse model, a continuous range of astrocytic subpopulations have been defined, ranging from GFAP-high to GFAP-low state (Habib et al., 2020), with even GFAP-negative astrocytes found in the adult CNS (Tatsumi et al., 2018). Interestingly, we found that our cultured old 3xTg-AD astrocytes show a notable reduction in GFAP expression combined with a high proliferative capacity (Figure 2). It has been previously reported that neither aging nor AD condition are associated with a change in the overall number of GFAP positive astrocytes in the 3xTg-AD mouse model (Olabarria et al., 2010), which has been previously argued as a lack of increased astrocytic proliferation in AD. Nevertheless, our results suggest that there could be astrocytic subpopulations with low GFAP expression but with high proliferative properties, and which could account for the progressive pathological events occurring in this mouse

model. In the case of the marker S100β, it is a small dimeric metal-binding astrocyte-derived protein and its over-expression is a prominent and consistent feature of AD (Cirillo et al., 2015; Marshak et al., 1992; Yeh et al., 2015). Furthermore, secreted S100β can act as a pro-inflammatory cytokine and a DAMP molecule, depending on its concentration, activating NF-κβ and RAGE (Leclerc et al., 2010). It regulates the intracellular levels of free calcium in neurons promoting Aβ formation and tau hyperphosphorylation, leading to an increase in cytosolic calcium levels and formation of calcium permeable pores. This can trigger neuronal apoptosis and the formation of free radicals through mitochondrial dysfunction (Cristóvaõ and Gomes, 2019). Interestingly, S100β modulates GFAP phosphorylation and promotes its disassembly (Bianchi et al., 1993). Although GFAP and S100β have been widely used as co-markers of astrocytes in brain injury, their pattern of expression can be shown to differ temporally and regionally at times. For example, previous studies in adult mice, animal models with brain pathologies, and human AD samples have shown opposite changes in GFAP and S100β labelling in astrocytes (Díaz-Amarilla et al., 2011; Kamphuis et al., 2014; Tatsumi et al., 2018; Yasuda et al., 2004). This is in line with our own findings from old 3xTg-AD astrocyte cultures. Cx43 is the major component of astrocytes´ gap junctions, and it has been involved in the regulation of cell proliferation as well (Dermietzel et al., 1989; Giaume and Theis, 2010; Huang et al., 1998; Zhu et al., 1992). Several brain inflammatory events and pathologies that affect neuronal survival, including AD, stroke, and seizures, among others, have been associated with changes in the expression of astrocytic connexins and the aberrant functioning of gap junctions. In this sense, it has been postulated that alterations of the astrocyte gap junctional

communication may affect the neuroprotective role of astrocytes and account for a glial contribution to neurodegenerative processes (Kielian and Esen, 2004; Rouach et al., 2002). Even more, our findings showed different cell distribution of Cx43 in old 3xTg-AD astrocytes, with predominant intracellular localization (Figure 2). In the same way MAO-B had a tendency to increase in old 3xTg-AD astrocytes, although not significant (Figure 2B). MAO-B is a key synthesizing enzyme of glial GABA through the putrescine degradation pathway, and evidence showed that in the postmortem brain of AD patients, astrocytic GABA and MAO-B are significantly upregulated (Adolfsson et al., 1980; Garaschuk and Verkhratsky, 2019). As well, MAO-B has been postulated as responsible for the marked increase in GABA levels found in reactive astrocytes of APP/PS1 mice through the putrescine degradation pathway (Jo et al., 2014). Furthermore, previous studies have reported conflicting findings about reactive astrogliosis markers in human AD brain, and suggest that activated astrocytes assessed by GFAP may differ from those assessed using the MAO-B radiotracer ^{3}H-L-deprenyl, depending on the stage of disease progression (Gulyás et al., 2011; Marutle et al., 2013; Rodriguez-Vieitez et al., 2016). This is in line with our results, where the isolated old 3xTg-AD astrocytic subpopulation is characterized by low GFAP expression and a high, though not significant, MAO-B expression.

Another key feature of these isolated old 3xTg-AD astrocytes is their proliferative potential, when compared to their aged-matched non-Tg astrocytic culture (Figure 1A). Likewise, although they took longer than neonatal-derived astrocytic cultures to attain first passaging conditions following isolation, they subsequently proliferated rapidly and continuously *in vitro*, with a higher

proliferation rate, suggesting the progressive enrichment of a highly-proliferative astrocytic subpopulation (Figures 1A and 2C).

Under physiological conditions, astrocytes are fundamental for neuronal growth and viability. Nevertheless, it has been suggested that in diverse brain pathologies including ALS, ischemia and AD, astrocyte reactivity involves a gain of neurotoxic function and/or loss of their neuroprotective properties (Díaz-Amarilla et al., 2011; Iram et al., 2016; Liddelow et al., 2017; Perez-Nievas and Serrano-Pozo, 2018; Villarreal et al., 2016). In line with this, an additional functional feature of these old 3xTg-AD astrocytes was their capacity to mediate neuronal degeneration (Figure 3). Although we cannot specifically conclude whether the observed exacerbated neurotoxic effects of these old 3xTg-AD astrocytes are due to the "old" and/or "AD" characteristics, it should be noted that neonatal 3xTg-AD astrocytic cultures did not show such toxic profile. As a result, it is possible to speculate that, in spite of the presence of AD-related genes, the physiological astrocytic supporting role might be impacted mostly as a result of the brain milieu-induced compensatory changes during the pathology progression in the 3xTg-AD mice model.

The observed astrocyte reactivity may involve the release of deleterious soluble factors that can affect neurons (Bahniwal et al., 2017; González-Reyes et al., 2017). In accordance, we showed that ACM from old 3xTg-AD caused a massive decrease in neuronal viability (Figure 4). Understanding the mechanisms involved in this toxic profile remains critical for the search of putative targets for AD intervention. Evidence suggests that astrocytes may contribute to amyloid burden in the brain in AD by secreting significant amounts of Aβ (Frost and Li, 2017). Nevertheless, our results showed that Aβ production

may not underlie neurotoxicity of old 3xTg-AD astrocytes (SM3). Another important amount of data postulate Glycogen synthase kinase-3 beta (GSK3β) to play a central role in AD, since its deregulation results in several pathological hallmarks of the disease (Hooper et al., 2008). GSK3β function in the brain is associated with cell cycle, neural development, neuron polarization, and neurodegeneration (Bonda et al., 2010; Seira and Del Río, 2014). Due to its relevant role in fundamental processes including cell structure, metabolism, and gene expression, GSK3β activity is subject to tight regulation, where its enzymatic activity is mainly inhibited by phosphorylation of serine-9 (pGSK3β-S9) or enhanced by phosphorylation of tyrosine-216 residue (pGSK3β-Y216). Evidence suggests that GSK3β over-activity is involved in tau hyper-phosphorylation, memory impairment, increased Aβ production and in inflammatory responses; all of which are pathological processes occurring in AD (Hooper et al., 2008). Our results point in the direction of this role for GSK3β, demonstrated by the increase in active GSK3β in the neuronal cultures after addition of old 3xTg-AD ACM (Figure 5A and B). Furthermore, cell survival is supported by pro-survival apoptosis-related GSK3β substrates which, when GSK3β activity is low, avoid phosphorylation and hence proteasome-mediated degradation. Numerous studies have demonstrated that inhibition of GSK3β activity protects neurons from a wide range of environmental stresses, including amyloid toxicity, which may be relevant for treatment of AD (Jope et al., 2011). Besides, among the known mechanisms that may contribute to neuronal loss in AD brain, the intrinsic apoptotic signalling pathway has predominated in studies of Alzheimer's disease (Bijur et al., 2000; Jope et al., 2011; Lucas et al., 2001; Pap and Cooper, 1998). GSK3β promotes mitochondria-mediated intrinsic

apoptotic signalling pathway as a result of a wide array of insults including DNA damage, oxidative stress and endoplasmic reticulum (ER) stress, among others. This apoptotic process involves among others key actors, GSK3β, Cytochrome-c and caspase-3 participation (Beurel and Jope, 2006). In this sense, our results suggest that exposure to old 3xTg-AD ACM may cause intrinsic apoptotic pathway initiation in the primary neuronal cultures (Figure 5).

It is noteworthy to mention that axonal degeneration is a common feature in neurodegenerative diseases including AD. Together with synaptic loss, impairment of axonal homeostasis is comprised as an early event in AD, and appears to precede somatic cell death (Adalbert and Coleman, 2013). Furthermore, it has been proposed that these degenerative events may occur along neuronal networks, and be spread through neuronal projections (Braak and Tredici, 2018; Saper et al., 1987; Vogel et al., 2020). In line with the diverse molecular mechanisms at play in specific sub-cellular compartments, we show important differences in neuronal outcome following localised exposure of neurons to old 3xTg-AD ACM in compartmentalised microfluidic chambers (Figure 6). Previous studies have demonstrated that Aβ axonal exposure is sufficient to trigger retrograde soma degeneration (Walker et al., 2018). Yet, Aβ somatic exposure can induce anterograde axonal degeneration (Deleglise et al., 2014). In the case of old 3xTg-AD ACM exposure, our results suggest that signalling molecules secreted by old 3xTg-AD astrocytes would have differential effects to neuronal subdomains. On one hand, ACM application to the somato-dendritic domain caused degeneration both locally in soma/dendrites and distally in axons (Figure 6). Rather surprisingly, exposure of axons to ACM did not affect somato-dendritic nor axonal integrity. Moreover, axons exposed to old

3xTg-AD ACM showed fragmentation levels significantly lower than non-Tg control ACM (Figure 6). The differential effects could be explained by a combination of both neurotoxic and neurotrophic molecules released by old 3xTg-AD astrocytes, with the latter being potentially interpreted as “growth-promoting” in isolated axons, but triggering cell death in post-mitotic neurons at the somatic level. In this way, a single type of old 3xTg-AD ACM component could have opposing effects depending on the subcellular location of its molecular target. This can be the case of neurotophins/pro-neurotrophins like nerve growth factor (NGF) whose release by astrocytes has been proposed to have a role in the progression of AD (Fahnestock and Shekari, 2019; Sáez et al., 2006; Sycheva et al., 2019). Analysis of ACM composition would undoubtedly help to better characterize the underlying mechanisms of old 3xTg-AD astrocytes´ neurotoxic/trophic effects. In line with this, ACM proteomic studies that are currently in progress will complement the morpho-functional features of the old 3xTg-AD astrocytes presented here.

From our results, some questions arise regarding the relevance of this astrocytic population in the AD brain. When do old 3xTg-AD astrocytes appear *in vivo*? For how long are they present? Additionally, does their function change during disease progression? Are old 3xTg-AD astrocytes a putative target option in therapeutic development, either the cells themselves, or a specific molecule expressed in these cells? Accordingly, on-going *in vivo* longitudinal studies in these mice as well as transcriptomic and metabolomic analysis will provide additional information about the old 3xTg-AD neurotoxic astrocytic population, and help us to obtain a more unified understanding of astrocyte functions throughout AD progression.

5 Conclusions

Overall, our findings revealed the existence of an astrocytic population during the symptomatic phase of the AD disease model, with a differential expression of astrocytic markers, high proliferation rate, and gain of toxic/degenerative effects to neurons in vitro. This highly neurotoxic population was only identified when astrocytes were isolated from adult symptomatic 3xTg-AD mice and not from neonates 3xTg-AD at a pre-symptomatic phase of the disease or non-Tg mice. This observation suggests that the appearance of this astrocytic sub-population is linked to the progression of the pathological events occurring in the 3xTg-AD mice model, in accordance with previous evidence from other ALS and AD animal models (Díaz-Amarilla et al., 2011; Gomes et al., 2020; Iram et al., 2016). Their generation may not be simply a reflection of the presence of AD-related genes. Instead it could be associated with the neuroinflammatory milieu that evolves during the progression of the disease in these animals, and promotes the phenotypic transition of precursors to the generation of these cells.

The demonstrated pathogenic profile of these old 3xTg-AD astrocytes provides a novel *in vitro* model of AD that would improve our understanding on the degenerative progression in AD. Further comprehension about their pathological potential may contribute to emerging cell-specific therapies of this neurodegenerative disorder.

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8 Figure legends

Figure 1: Isolation of an astrocytic population occurring in symptomatic 3xTg-AD animals. A. Representative phase-contrast microphotographs of mixed cortex and hippocampal astrocytic cultures obtained from symptomatic 3xTg-AD 9-10-month-old mice (old 3xTg-AD astrocytes). Cells plated in culture flasks and observed at 7 days in vitro (7 DIV) and after replating at passage 1, 3, 5 and 10. Control "old" astrocytic cultures obtained from 1 adult 9-10-months-old non-Tg mouse yielded cells with limited growth potential (old non-Tg astrocytes). Scale bar: 50 µm. **B.** Representative microphotographs of phase-contrast old 3xTg-AD astrocytes and non-Tg neonatal astrocytes (I and III) showing response to Forskolin (10µM, 24h) (II and IV). Scale bar: 50 µm.

Figure 2: Protein expression in old 3xTg-AD astrocytic cultures (old 3xTg-AD) compared with non-Tg neonates (non-Tg) and 3xTg-AD neonates (3xTg-AD neo) astrocytic cultures. A. Astrocytes in culture were analyzed by confocal immunofluorescence for the expression of GFAP (red), S100β (green) and Connexin-43 (red). Nuclei were stained with Hoechst 33342 (blue). Scale bar: 50 µm. **B.** Western blotting analysis of GFAP and MAO-B proteins in old 3xTg-AD astrocytic cultures and corresponding controls (Non-Tg and 3xTg-AD neo). β-actin was used as loading control. Representative figures and relative quantification of almost 3 independent immunoblots. Data are mean ± SD; One-way ANOVA with Tukey's multiple comparisons post-hoc test. **C.** Growth of old 3xTg-AD astrocytes, non-Tg and 3xTg-AD neonatal astrocytes was assessed over 8 days by SRB assay (10-20 replicates of each time point, performed in 2 independent experiments). Data are mean± SD; Two -way ANOVA with Tukey's multiple comparisons post-hoc test. a: $p<0.05$, 3xTg-AD

neo significantly different from old 3xTg-AD; b: $p<0.05$, non-Tg and 3xTg-AD neo significantly different from old 3xTg-AD.

Figure 3: Old 3xTg-AD astrocytes in co-culture showed neurotoxic effects. A: Diagrammatic representation of the experimental design used in B. **B:** Representative confocal immunofluorescence microphotographs of co-cultures at 5DIV. Embryonic non-Tg cortico-hippocampal (CxH) neurons were seeded on top of confluent feeder layers of neonatal non-Tg astrocytes (I), neonatal 3xTg-AD astrocytes (II), or old 3xTg-AD astrocytes (3rd passage) (III), and after 5 days cultures were fixed with PFA 4% and processed for immunocytochemistry. Fixed co-cultures were immunostained against GFAP (orange) / β-tubulin III (green) and nuclei were stained with Hoechst 33342 (blue). Scale bar: 20µm.

Figure 4: Conditioned medium from old 3xTg-AD astrocytes (old 3xTg-AD ACM) induced primary cortico-hippocampal neurons death. A: Diagrammatic representation of the experimental design used in B. **B:** Representative confocal immunofluorescence microphotographs of neuronal cultures at 13DIV. ACM from neonatal non-Tg astrocytes (II), neonatal 3xTg-AD astrocytes (III), or old 3xTg-AD astrocytes (IV) was added (proportion 1:1 to the existing neuronal culture medium) to embryonic non Tg CxH neurons at 12DIV for 24hs. The effect of fresh (non-conditioned) medium on neuronal cultures was also evaluated as control (I). After ACM exposure, cells were fixed with PFA 4% and processed for immunocytochemistry. Fixed cultures were immunostained against β-tubulin III (green) and nuclei were stained with Hoechst 33342 (blue). Scale bar: 20µm.

Figure 5: Exposure of primary CxH neurons to old 3xTg-AD ACM induced an increase in the percentage of active GSK3β, in cytochrome c pattern, and an increase in caspase 3 activity, indicative of intrinsic apoptotic pathway activation. A-C: Representative confocal immunofluorescence microphotographs of neuronal cultures treated with ACM from neonatal 3xTg-AD astrocytes (3xTg-AD neo ACM), or from old 3xTgv astrocytes (old 3xTg-AD ACM) for 1h 30min (A) or for 3h (C). After ACM exposure, cells were fixed with PFA 4% and processed for immunocytochemistry. Fixed co-cultures were immunostained against pGSK3β(S9) (inactive, green) and against pGSK3β(Y216) (active, orange), or against Cytochrome c (Cyt c, green) and against active Caspase 3 (Casp3, orange) (A and C, respectively). Nuclei were stained with Hoechst 33342 (blue). Scale bar: 20µm. **B:** Quantitative analysis of active GSK3β immunofluorescence in neuronal cultures exposed to ACM, n=3. Data are mean ± SD; Kolmogorov-Smirnov test, $^{*}p < 0.001$. **D:** Quantitative analysis of active caspase 3 immunofluorescence in neuronal cultures exposed to ACM, n=2. Data are mean ± SD; Kolmogorov-Smirnov test, $^{*}p < 0.01$. **E.** Diagrammatic representation of the experimental design used in A and C.

Figure 6: Old 3xTg-AD ACM induced a differential effect in primary CxH neurons depending on the site of exposure. A. Diagrammatic representation of the experimental design used in C. **B.** Microfluidic Neuron Device diagram (MFD, SND450 standard neuron device, Xona Microfluidics). The wells on the left (light pink, soma compartment) are connected by a main channel as are the wells on the right (grey, axon compartment). Each compartment is connected by a 450 µm microgroove barrier (space). 3 neurons are represented. Note that only axons can reach the right chamber after 12 DIV (Taylor et al., 2005). **C.**

Representative images of soma and axonal sides of the microfluidic chambers 24h after Non-Tg or old 3xTg ACM exposure. Schematics of the microfluidic chambers (above the graphs) depict where ACM were added (soma exposure in left, axonal exposure in right). ACM application is illustrated in orange. After ACM exposure, cells were fixed with PFA 4% and processed for immunocytochemistry. Fixed cultures were immunostained against β-tubulin III (green) and nuclei were stained with Hoechst 33342 (blue). Scale bar: 20μm. Quantification of condensed nuclei and indexes of axonal preservation, degeneration and fragmentation after ACM application in the soma (left) or axon side (right) of microfluidic chambers, n=3. Data are mean ± SD; Student's *t*-test.

SM1: Table with primary antibodies used for immunofluorescence (IF) and western blot (WB) assays (at dilutions recommended by the manufacturers).

SM2: Old 3xTgv astrocytes in culture were negative for Iba1 and Olig2 markers: Old 3xTgv astrocytes in culture (5th passage) were analyzed by confocal immunofluorescence for the expression of Iba1 and Olig2 (green). Nuclei were stained with DAPI (blue). No Primary Antibody Control was included to show that the observed green signal is due to non-specific binding of secondary antibody in the culture sample. Scale bar: 50 μm.

SM3: Aβ may not be involved neurotoxicity of old 3xTg-AD astrocytes. A. Diagrammatic representation of the experimental design used in B. Old 3xTg-AD astrocytic cultures were incubated with vehicle (DMSO) or with DAPT (tert-Butyl (S)-{(2S)-2-[2-(3,5-difluorophenyl) acetamido] propanamido} phenylacetate) (20μM), an inhibitor of γ-secretase, the pivotal enzyme that generates Aβ, for 24h. After that, their ACM was added to neuronal cultures (DIV12). The effects of fresh (non-conditioned) medium treated with DAPT

20μM or vehicle on neuronal cultures were also evaluated as controls, in order to see if they impact directly on neuronal viability. After 24h exposure, neuronal viability was analysed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, after experimental treatments, cells were incubated for 45 min at 37 C with MTT (0.5 mg/mL final concentration) that was reduced by metabolically active cells to purple formazan. Formazan crystals were dissolved with DMSO, and the absorbance was measured on a microtiter plate reader (Fisherbrand™ AccuSkan™ FC Filter-Based Microplate Photometer) at a test wavelength of 570 nm. **B.** Data are mean ± SD (presented as percentage of cell viability, assuming that absorbance of untreated control cells was 100 %); results of three independent experiments were analysed by multiple comparisons with Kruskal-Wallis test followed by Dunn post-hoc test, ****$p < 0.0001$.