Microglial autophagy is impaired by prolonged exposure to β -amyloid peptides: evidence from experimental models and Alzheimer's disease patients

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Abstract Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the presence of misfolded proteins, amyloid- β ($A\beta$) aggregates, and neuroinflammation in the brain. Microglial cells are key players in the context of AD, being capable of releasing cytokines in response to $A\beta$ and degrading aggregated proteins by mechanisms involving the ubiquitin-proteasome system and autophagy. Here, we present in vivo and in vitro evidence showing that microglial autophagy is affected during AD progression. PDAPPJ20 mice—murine model of AD—exhibited an accumulation of the autophagy receptor p62 and ubiquitin+ aggregates in Iba1+ microglial cells close to amyloid deposits in the hippocampus. Moreover, cultured microglial BV-2 cells showed an enhanced autophagic flux during a 2-h exposure to fibrillar $A\beta$,

which was decreased if the exposure was prolonged to 24 h, a condition analogous to the chronic exposure to $A\beta$ in the human pathology. The autophagic impairment was also associated with lysosomal damage, depicted by membrane permeabilization as shown by the presence of the acid hydrolase cathepsin-D in cytoplasm and altered LysoTracker staining. These results are compatible with microglial exhaustion caused by pro-inflammatory conditions and persistent exposure to aggregated $A\beta$ peptides. In addition, we found LC3-positive autophagic vesicles accumulated in phagocytic CD68+ microglia in human AD brain samples, suggesting defective autophagy in microglia of AD brain. Our results indicate that the capacity of microglia to degrade $A\beta$ and potentially other proteins through autophagy may be negatively affected as the disease progresses. Preserving autophagy in microglia thus emerges as a promising approach for treating AD.

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Abbreviations

Tg	Transgenic PDAPPJ20 mice.
NTg	Non-transgenic PDAPPJ20 mice.
$A\beta$	Amyloid- β peptides
f $A\beta$ 42	Fibrillar Amyloid- β 1–42 peptides
BAF	Bafilomycin A1
PA	Plaque associated

NPA Non-plaque associated
 Veh Vehicle

Introduction

Alzheimer's disease (AD) is the most common form of [dementia](#) and a neurodegenerative disease characterized by the presence of intra- and extracellular [protein aggregates](#) (Lane et al. 2018). In the extracellular milieu, amyloid deposits are composed mainly of amyloid- β peptides (A β) that contain 39–42 aminoacids and are generated by proteolytic processing of the amyloid precursor protein (APP) by β - and γ -secretases (Walter 2006). Amyloid plaques accumulate in the brain parenchyma as the pathology progresses, negatively regulating synaptic connectivity and inducing neuronal dysfunction. The other pathological hallmark associated with this disease is the accumulation of paired helical filaments/neurofibrillary tangles, formed by hyperphosphorylated tau protein that detaches from microtubules, resulting in microtubule destabilization. AD is also characterized by hippocampal atrophy and neuroinflammation, the latter being largely due to the overactivation of microglia (Beauquis et al. 2013; Pomilio et al. 2016; Verkhratsky and Nedergaard 2018).

Microglia have a key role in the cerebral innate immune response. These cells, evenly distributed throughout the parenchyma, constantly screen their milieu and are activated by pathogen- and cell damage-associated molecules. Microglia respond to these stimuli by releasing cytokines, chemokines, and growth factors and undergo morphological changes associated with their activation. Activated microglia become highly motile, migrating to the lesion area and phagocytosing damaged neurons and cell debris (Fu et al. 2014; Lenz and Nelson 2018).

Misfolded and aggregated amyloid peptides bind to pattern recognition receptors on astroglia and microglia, leading to cell reactivity and release of inflammatory mediators, which progressively contribute to the disease during chronic induction (Ahmad et al. 2019; Jiang and Bhaskar 2017). Our group among others demonstrated that astrocytes in AD show morphological changes depending on their proximity to amyloid deposits and are involved in protein homeostasis by taking up and degrading APP-derived peptides, regulating microglial activation (Beauquis et al. 2013; Gregosa et al. 2019;

Olabarria et al. 2010; Olabarria et al. 2011; Pomilio et al. 2016; Simonovitch et al. 2016). Microglia are phagocytic cells that can internalize and degrade A β , as demonstrated in in vivo and in vitro experiments. A plethora of cellular pathways have been implicated in the uptake of fibrillar or soluble A β into this cell type: TLRs, TREM2, complement (CR3 CD11b/CD18) scavenger (SR-A, SRBI CD36, RAGE), and LRP receptors (Bamberger et al. 2003; Bose and Cho 2013; Fu et al. 2014; Kiyota et al. 2009; Koenigsknecht-Talboo and Landreth 2005; Koenigsknecht and Landreth 2004; Lee and Landreth 2010; Liang et al. 2005; Paresce et al. 1997; Stalder et al. 2001; Tahara et al. 2006).

Intracellular A β has been proposed to be degraded by macroautophagy—referred simply as autophagy—a catabolic intracellular mechanism of bulk degradation where long-lived ubiquitinated proteins, aggregates, and dysfunctional organelles are engulfed by an isolation membrane to form a double-membrane vesicle called autophagosome, that subsequently fuses with lysosomes—vesicles containing acidic hydrolases including proteases as cathepsin-D, among others—to form autolysosomes, resulting in degradation of autophagic cargoes. This process is strongly dependent on lysosomal integrity, as shown by autophagosome accumulation in the cytoplasm in pathologies where lysosomes are affected. The machinery mediating autophagosome maturation is vastly complex providing monitoring nodes to integrate nutrient availability with autophagy activity (Zhang et al. 2017; Zhao and Zhang 2019).

Autophagy is subjected to an age-associated decline that impedes neuronal homeostasis and, consequently, contributes to the progression of neurodegenerative diseases due to the accumulation of toxic protein aggregates in neurons (Metaxakis et al. 2018). In this sense, mTOR inhibition by rapamycin treatment before amyloid deposition reduces neuropathological hallmarks in AD transgenic mice. Other evidence suggests that as dementia progresses, treatment with rapamycin exacerbates the damage because the brain's lysosomal system is already severely affected (Carosi and Sargeant 2019). These experimental approaches were mostly focused on neuronal autophagy in the hippocampus (Spilman et al. 2010; Van Skike et al. 2018).

Remarkably, autophagy in glial cells and specifically in microglia in the context of AD has not received enough attention. Evidence obtained by Cho et al. (2014) indicates that microglial cells in culture are able

to ingest and degrade extracellular A β fibrils after a 2-h exposure via the lysosomal system in an inflammasome-mediated manner. More recently, the interplay between autophagy machinery, lysosomal activity, inflammasome assembly, and neuroinflammation was considered a key event in AD progression (Houtman et al. 2019; Wani et al. 2019). In this context, it is imperative to know whether autophagy remains functional in the aged brain, where microglial cells are dystrophic due to chronic exposure to A β , pro-inflammatory cytokines, and damage-associated molecules.

In the present study, we evaluate the state of autophagy-related proteins in microglial cells physically associated to amyloid plaques in a validated murine model of AD at different ages. In addition, employing an *in vitro* approach, we compare the effects of a short and a long exposure to A β on the components of the microglial autophagy machinery. Our results support the hypothesis that autophagy is affected in microglial cells associated to amyloid plaques in AD mice. While autophagy is functional in cultured microglia during a short exposure to A β 1–42 peptide, the autophagic flux is impaired during a longer exposure, results that are in line with our *in vivo* findings, possibly due to lysosome dysfunction. Finally, we present evidence of LC3+ autophagosome accumulation in hippocampal microglial cells in the vicinity of amyloid deposits of post mortem AD human brains, suggesting that these alterations could also be present in AD.

Materials and methods

Animals

PDAPPJ20 [hAPP(J20)] mice, a recognized mouse model of AD, has been described elsewhere (Beauquis et al. 2014; Galvan et al. 2006; Hsia et al. 1999; Mucke et al. 2000; Roberson et al. 2007; Selkoe 2000). These mice carry the *hAPP* gene with the familial AD Swedish and Indiana mutations, developing behavioral alterations and amyloid pathology in a progressive fashion (Lin et al. 2013; Pomilio et al. 2016) and also showing evidence of Tau hyper-phosphorylation (Simon et al. 2009). Transgenic mice were maintained by heterozygous crosses with C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) in our animal facility (Institute of Biology and Experimental Medicine, UBA-CONICET;

OLAW-NIH Assurance Identification Number F16-00065-A5072-01) and were housed under controlled conditions of temperature (22 °C) and humidity (50%) with 12 h/12 h light/dark cycles (lights on at 7:00 a.m.). PDAPPJ20 mice were hemizygous with respect to the transgene, verified by PCR using hAPP primers. All animal experiments followed the NIH Guide for the Care and Use of Laboratory Animals (<https://www.ncbi.nlm.nih.gov/books/NBK54050>) and were approved by the Ethical Committee of the Institute of Biology and Experimental Medicine. All efforts were done to reduce the number of mice used in the study as well as to minimize animal suffering and discomfort.

Mouse tissue processing

Animals were anesthetized by intraperitoneal administration of ketamine (80 mg/kg BW, Holliday-Scott, Argentina) and xylazine (10 mg/kg BW, Bayer, Argentina) and then transcardially perfused with 30 mL of 0.9% saline followed by 30 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed, fixed overnight in 4% paraformaldehyde solution at 4 °C, and then coronally cut at 60 μ m in a vibrating microtome (PELCO easiSlicer, Ted Pella, USA). Sections were stored in a cryoprotectant solution (25% glycerol 25% ethylene glycol, 50% phosphate buffer 0.1 M, pH 7.4) at –20 °C until use. Histological techniques were performed in the free-floating mode, using six representative serial sections of the whole hippocampus.

Immunohistochemistry and immunofluorescence in mice brain slices

Microglial cells were histologically identified by immunodetection using Iba1-specific antibody (Wako Pure Chemical Industries, 019-19741), as it was previously described (Pomilio et al. 2016). Briefly, the sections were washed three times with PBS for 3 min, and non-specific antigenic sites were blocked using a solution of PBS containing 0.1% Triton X-100 and 10% normal goat serum. Primary antibodies were incubated in a solution containing 0.1% Triton X-100 and 10% normal goat serum overnight at 4 °C. Iba1-specific antibody (1:1500) were incubated alone or mixed with antibodies for Amyloid- β peptides (1:100, clone 4G8, Covance, SIG-39220), CD68 (1:1000, Wako, M0876), Ubiquitin (1:1000, Chemicon, USA, MAB1510) and

p62 (1:1000, Santa Cruz Biotechnology, SC-28359). For immunofluorescence, sections were incubated with anti-rabbit Alexa 488 (Invitrogen, A11008) and anti-mouse Alexa 555 (Invitrogen, A21424), placed on gelatin-coated slides, and mounted with PVA-DABCO (Sigma-Aldrich, USA). For Iba1 immunohistochemistry, a biotinylated secondary antibody (Vector Laboratories, BA1000) was used followed by ABC kit (Vector Laboratories) and incubation with 2 mM diaminobenzidine (Sigma, USA) and 0.5 mM H₂O₂ in 0.1 M Tris buffer. Sections were placed on gelatin-coated slides, air-dried overnight, and dehydrated in graded solutions of ethanol. Section were then incubated in the Congo red working solution containing 0.2% Congo red (Biopack, Argentina), 3% NaCl (to saturation), and 0.01% sodium hydroxide in 80% ethanol for 10 min, washed two times with ethanol 95% and then two times with ethanol 100% for 3 min each. After that, samples were cleared in xylene and mounted with Canada balsam.

Histological analysis

Image acquisition For immunohistochemistry, images from each section were obtained using a 518CU Micrometrics camera attached to a Nikon Eclipse E200 microscope. For immunofluorescence, a Nikon Eclipse E80 or an Olympus DSU-IX83 confocal microscope was used.

Iba1+ area For Iba1+ area calculation, images were obtained under a $\times 100$ magnification, and the area of the hilus was delimited using the Optimas 6.5 software (Media Cybernetics). The Iba1 immunopositive area was calculated as the ratio between the immunoreactive and the total area in the subfield, using a user-defined threshold. The area occupied by Congo red staining was filtered using Photoshop software (Adobe Systems Inc. San Jose, CA) to prevent parameter overestimation ($n = 5$ mice per group).

Iba1+ cell number Microglial cell number was estimated by randomly placing a $6 \times 10^5 \mu\text{m}^3$ counting probe on the hilus of the hippocampus. A minimum of 100 cells per animal were counted ($n = 5$ mice per group).

Iba1+ soma size Soma size was evaluated as an indirect and morphological indicator of microglial activation. Microphotographs from randomly chosen individual

cells were obtained, the soma delineated and its surface quantified using ImageJ software. At least 50 cells were quantified for each animal ($n = 5$ mice per group).

Activation stage of Iba1+ cells Three distinctive microglial phenotypes (ramified, intermediate, and ameboid) were discriminated in the hippocampus of PDAPPJ20 mice by their Iba1 immunoreactivity, as it was previously defined (Pomilio et al. 2016). Briefly, the ramified morphology is generally associated with non-activated microglia and was identified by a small rounded soma and multiple thin processes. Intermediate phenotype implied a series of modifications to the former, like a larger or more elongated soma and thicker processes, while ameboid morphology presented a larger soma, very few or absence of processes and was almost exclusively found near amyloid plaques. Using a randomly placed $6 \times 10^5 \mu\text{m}^3$ counting probe, the proportion of each phenotype was analyzed. At least 100 cells were classified on each animal ($n = 5$ mice per group).

Iba1+ cells containing amyloid peptides Confocal images were obtained under a $\times 600$ magnification from brain slices processed for Iba1 and Amyloid- β co-immunofluorescence. This quantification was performed considering only plaque-associated microglia, defined as the Iba1+ cells that have their nucleus at a maximum distance of 50 μm from the core of an amyloid plaque. The number of Iba1+ cells containing intracellular A β + signal was related to the total number of Iba1+ cells ($n = 5$ mice per group).

CD68+ area For CD68+ area calculation, images were obtained under a $\times 600$ magnification. The CD68 immunopositive area was calculated as the ratio between the immunoreactive and the total area in the dentate gyrus, using a user-defined threshold. ($n = 4$ mice per group).

CD68+ microglial cells Confocal images were obtained under a 600x magnification from brain slices processed for Iba1 and CD68 co-immunofluorescence. The number of CD68+/Iba1+ cells were related to total Iba1+ cells in $15.6 \times 10^{-3} \text{ mm}^2$ probes randomly placed on the hilus ($n = 5$ mice per group).

Ubiquitin+ microglial cells Percentage of Iba1+/ubiquitin+ related to total Iba1+ cells was quantified using

randomly placed $2.6 \times 10^2 \text{ mm}^2$ counting frames on confocal images of the CA1 region of the hippocampus obtained under a $\times 20$ objective. Plaque-associated cells were quantified placing the frame on amyloid deposits while in non-plaque-associated cells the frame was placed at least $50 \mu\text{m}$ away from any deposit. At least 100 cells were counted per animal ($n = 5$ mice per group).

p62+ microglial area Confocal images were obtained under a $\times 200$ magnification from brain slices processed for Iba1 and p62 co-immunofluorescence. Percentage of Iba1+ area that is also p62+ was estimated using M1 colocalization index by setting a user-defined threshold employing the JACOP plugin for ImageJ (NIH) ($n = 4$ mice per group).

p62+ microglial cells Percentage of Iba1+/p62+ related to total Iba1+ cells was quantified using randomly placed $2.6 \times 10^2 \text{ mm}^2$ counting frames on confocal images of the hilus of the hippocampus obtained under a $\times 20$ objective. Plaque-associated cells were quantified placing the frame on amyloid deposits while in non-plaque-associated cells the frame was placed at least $50 \mu\text{m}$ away from any deposit. At least 100 cells were counted per animal ($n = 5$ mice per group).

Cell culture

BV-2 murine microglial cells, immortalized by infection with v-raf/c-myc recombinant retrovirus (Bocchini et al. 1992), were elsewhere described as an in vitro model of brain microglial cells (Bussi et al. 2017; Porte Alcon et al. 2018) and gently provided by Dr. Guillermo Giambartolomei (Hospital de Clínicas, Buenos Aires, Argentina). Cells were maintained in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated FBS (Internegocios, Buenos Aires, Argentina), 2.0 mM glutamine, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 –95% air, and the medium was renewed three times a week.

Reagents and treatments

A β 1–42 peptides were obtained from Sigma (Cat. No. A9810) and stored at -80°C dissolved in DMSO at a final concentration of 0.5 mM. Prior to treatment,

amyloid fragments were incubated for 72 h with distilled water at 37°C to allow fibrils formation. Cells were exposed for 2 or 24 h to vehicle or 0.5 μM fibrillized A β 1–42 peptides (fA β 42). Eventually, cells were co-incubated during the last 2 h of treatment with Bafilomycin A1 (Fermentek, Israel) at a final concentration of 0.1 μM . Nutrient restriction was performed by replacing maintenance medium with a similar one but containing 2% heat-inactivated FBS (Internegocios, Argentina) during 6 h, prior to exposition to fA β 42 or vehicle.

LC3 immunofluorescence in cells

After amyloid exposure, cultures were washed twice with PBS, fixed with 4% paraformaldehyde/4% sucrose in PBS 30 min at RT and washed five times with PBS. Fixed samples were permeabilized with Triton X-100 0.25% in PBS (10 min at RT), washed three times with PBS and blocked with 1% BSA in PBST (Tween 20 0.1% in PBS) overnight at 4°C . Coverslips were incubated with a solution of goat anti-LC3 (Novus, USA, NB100-2331) 1:100 and mouse anti-A β (clone 4G8, Covance, SIG-39220) 1:1000 for 1 h, washed three times with PBS, and incubated with a solution of Alexa 488 donkey anti-mouse (Life Technologies, A11008) 1:1000 in 1% BSA in PBST; 1 h. After three washes with PBS, samples were incubated with Alexa 555 goat anti-rabbit (Life Technologies, A27039) 1:1000, 1 h. Finally, coverslips were washed three times with PBS and mounted with PVA-DABCO (Sigma-Aldrich, USA). Representative images were obtained by Nikon Eclipse E80 confocal scanning laser microscope (Nikon Instech Co., Ltd., Karagawa, Japan). Controls were performed by incubating samples under the same conditions without one or both primary antibodies (data not shown).

LC3+ BV2 cells and amyloid content

BV-2 cells containing LC3+ vesicles were estimated by a user-defined threshold and counting the number of cells with LC3+ vesicles per field. The percentage of the total LC3+ area that co-localize with A β was estimated by calculating Manders M2 coefficient on individual cells employing the WCIF ImageJ Plugin Pack (NIH) ($n = 5$ per group).

Western blotting

BV-2 cell lysates were homogenized by sonication in supplemented RIPA buffer. Protein quantification was performed by the Bradford standardized method (Bradford 1976). After lysis with loading buffer, samples were loaded on 13% acrylamide gels, separated by SDS-PAGE, and then transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in TBS containing 0.5% Tween-20 (TBST). Specific antibody for LC3 (1:200, Cell signaling, 12,741) was employed in TBST. After initial probing with primary antibodies, membranes were washed in 0.5% TBST and TBS solutions, incubated for 1 h with 1:1000 dilutions of species-appropriate, HRP-conjugated secondary antibodies (Bio-Rad, USA). After washing, immunoreactivity was visualized by reaction in ECL detection reagents (luminol and p-coumaric acid; Sigma-Aldrich) for 3 min, followed by immediate exposure of the blot using Amersham Imager 600 RGB (GE Healthcare). Bands at the relevant molecular weights were quantified using the ImageJ plugin for gel analysis ($n = 4$ samples per group).

Lysosome analysis in cells Prior to fixation, BV-2 cells were incubated with LysoTracker Red DND-99 (Invitrogen) in maintenance medium without FBS at a final concentration of 500 nM during 30 min at 37 °C. Then, cells were fixed and coverslips were processed for immunofluorescence as it was previously described for LC3, omitting permeabilization with Triton X-100. Specific antibody against cathepsin-D (1:100, Santa Cruz Biotechnology sc 10,725) and the corresponding secondary antibody were employed. Finally, coverslips were mounted in PVA-DABCO (Sigma-Aldrich).

Lysosomal number and average size were estimated from confocal images employing ImageJ software and a user-defined threshold ($n = 5$ per group). Percentage of cathepsin-D+ signal co-localizing with LysoTracker was estimated by the Manders M2 coefficient employing the WCIF ImageJ Plugin Pack (NIH). At least 40 cells per treatment were considered for analysis. In parallel, the number of BV-2 with diffuse LysoTracker staining was related to the total number of cells in every field, considering at least six fields per treatment.

BV-2 cells transfection

BV-2 cells were transfected with a plasmid encoding GFP-mCherry-LC3B, gently provided by Dr. Juan

Bonifacio (Jia et al. 2017). It allows the expression of LC3 protein linked to GFP and mCherry fluorophores, where GFP is pH-sensitive. For transfections, cells were seeded on poly-D-lysine coated coverslips in 24-well plates at a density of 8×10^3 cells/well and allowed to grow for 48 h until 50–60% confluence. Transfection complexes were prepared in serum-free media in a ratio polyethyleneimine:DNA 3.5:1 (1 µg plasmid per well). Mixtures were vortexed, incubated 10 min at RT and then dropwise added to the cells in serum media. After 5 h, media was renewed. Treatments were conducted at 24 h post-transfection. Co-localization between fluorophores on individual cells was estimated by M1 and M2 coefficients using JACoP plugin for ImageJ ($n = 30$ cells per group).

Immunohistochemistry in human brain sections

Human brain samples were obtained from the Brain Bank of the Instituto de Investigaciones Neurológicas Dr. Raúl Carrea (www.fleni.org.ar/investigacion-educacion/investigacion-2/biobancos/ Buenos Aires, Argentina) by previous agreement. Paraffin sections containing hippocampus from control subjects and AD patients were used. The image shown in Fig. 7 corresponds to an 83-year-old male patient with the following characteristics. Clinical diagnosis: sporadic Alzheimer's disease. CDR > 1. Neuropathology: CERAD Score C, Braak VI, and Thal no less than A2. Final diagnosis: "High" AD neuropathologic change. The control subject employed in this analysis is 73 years-old, with no neurological diseases and normal cognitive status, according to Hyman et al. (2012).

Brain samples were fixed in 10% formalin and then processed for paraffin embedding. Consecutive 5-µm-thick sections were processed for immunohistochemistry, employing commercial kit from Leica (Leica Bond-Max, Leica Biosystems). Specific antibodies that recognize LC3 (Novus, NB100-2331) and CD68 (CD68-L-CE, Leica Biosystems) were incubated overnight. Finally, samples were cleared in xylene and mounted with Canada balsam.

Statistical analysis

In bar graphs, data are expressed as mean $\pm$ SEM. Analyses were performed under blind conditions to the experimental groups, using InfoStat software (Universidad Nacional de Córdoba, Argentina),

employing one-tailed Student's *t* test for two independent samples, one- or two-way ANOVA when required. For ANOVA, Tukey's, Dunnett's or Sidak's post hoc comparisons were used to compare experimental groups, depending on the biological question. In all cases, the required assumptions for each test were verified and significant differences were considered at the 5% level. The sample size was calculated using StatMate 2.0 (GraphPad Software Inc.), employing previous data and considering to obtain a 5% statistical significance with 80% statistical power in order to detect differences of at least 10% between groups.

Results

Morphological changes of microglia from early to late stages in the hippocampus of PDAPPJ20 mice, an experimental model for AD

As we have previously reported, immunolabeling of the ionized calcium binding adaptor molecule 1 (Iba1), a microglia/macrophage-specific marker, allowed us to characterize microglial cells and study cell morphology parameters along AD progression in PDAPPJ20 animals (Pomilio et al. 2016). Figure 1a shows the different pattern of Iba1 labeling in young non-transgenic (NTg) compared with old PDAPPJ20 transgenic (Tg) mice, with the evident presence of deposits surrounded by reactive microglia. The disease progression is associated with an increase in microglial reactivity as it is shown in Fig. 1b, including representative images from Tg mice at 5, 9, and 15 months of age. The high-magnification images in Fig. 1c emphasize the morphological alterations found in microglial cells from young and old Tg and NTg mice. We studied the Iba1+ area in the hilus of the hippocampus from Tg and NTg mice from 5 to 15 months of age (m), in order to include the initial and subsequent stages of the disease (Fig. 1d). We focused on the hilus of the dentate gyrus, because this region is early affected in the brain of Tg mice, as evidenced by the presence of amyloid deposition. The percentage of Iba1+ area in the hilus from 9 and 15 m mice was significantly increased in Tg relative to age-matched NTg mice ($p < 0.05$ and $p < 0.0001$, respectively). Transgenic animals also showed increased soma size—associated with microglial reactivity—and cell density (Iba1+ cells) probably suggesting a neuroinflammatory reaction (Fig. 1d–f). However, the

soma size of Iba1+ cells also experiences an increase due to aging in NTg mice (Fig. 1e NTg 5 vs. 15 m, $p < 0.05$).

At 15 months, the cell distribution in the hippocampus drastically changed, displaying clusters of reactive microglial cells around amyloid deposits. We discriminated three microglial morphological categories: ramified, characterized by a small soma and enlarged and thin cell processes; intermediate, with fewer and shorter processes; and ameboid, typically showing few or no processes and a bigger soma, adopting a round shape in the proximity of amyloid deposits. At 5 m, we found a reduction in the number of ramified cells in the Tg animals compared with NTg littermates. In the older groups (9 and 15 m), the effect was even more pronounced ($p < 0.001$) (Fig. 1g). The Tg groups at 5 and 9 m showed a significant increment in the intermediate morphology (Fig. 1h). As expected, the ameboid morphology was detected only in the Tg mice, being more numerous in the older age, which is probably associated to the progression of amyloid deposition (Fig. 1i).

In aged PDAPPJ20 mice, Iba1+ cells are also A β + and exhibit loss of the phagocytic marker CD68

To explore the phagocytic capacity of microglia in PDAPPJ20 mice, we analyzed Iba1 and A β signals by confocal microscopy. Employing the 4G8 antibody, which recognizes residues 18–23 of the A β sequence, we detected not only extracellular deposits but also amyloid content inside the Iba1+ microglial cells at 9 and 15 m (Fig. 2a). Figure 2b illustrates the co-localization between the microglial marker and A β along the *z*-axis of a three-dimensional reconstruction in the orthogonal views. At 15 m, the co-localization index decreased compared with 9 m ($p < 0.05$) (Fig. 2c). Iba1+ cells exhibited the lysosomal marker for phagocytic activity mainly in microglial processes (Fig. 2d). In old animals, studying the CD68+ area in microglial cells we found no significant changes between genotypes. Tg mice showed a level slightly above the NTg in this parameter (Fig. 1e). However, the proportion of Iba1+ cells co-localizing with this marker was lower in Tg mice compared with NTg group (Fig. 2f), suggesting a dysfunction in the phagocytic capacity of AD microglia and also a potential lysosomal impairment.

◀ **Fig. 1** Histological evaluation of microglial alterations in the hippocampus of PDAPPJ20 mice associated to aging. **a** Representative images of Iba1 immunohistochemistry coupled to Congo red staining on brain section from young non-transgenic (NTg) (left) and aged transgenic (Tg) mice (right). Inset shows hypertrophied Iba1+ cells surrounding an amyloid plaque. Scale bar represents 300 μ m. **b** Images focusing on Iba1 immunoreactivity in the hilus of the dentate gyrus during pathological aging in Tg animals from 5 to 15 months of age. **c** High-magnification images showing Iba1 immunoreactivity from NTg and Tg mice at 5 and 15 months of age. Scale bar represents 50 μ m. **d** Iba1+ area in the hilus of the hippocampus expressed as a percentage of the subregion area immunopositive for this marker. **e, f** Microglial soma size (**e**) and number (**f**) quantified in the hilus of the hippocampus. **g–i** Frequency of microglial activation phenotypes assessed by morphological evaluation in the hilus of the hippocampus. Asterisks represent statistically significant differences between groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, non-significant differences (two-way ANOVA followed by Tukey post hoc comparisons), $n = 4$ –5 animals per group

Dysfunction of cell proteostasis in the hippocampus of PDAPPJ20 mice

We found ubiquitin+ signal in microglia in the hippocampus of aged PDAPPJ20 Tg mice. (Fig. 3a). Figure 3b illustrates orthogonal views that show the co-localization between the microglial marker and ubiquitin along the z -axis. The proportion of Iba1+/ubiquitin+ cells seemed to increase with aging, suggesting a potential accumulation of ubiquitinated proteins during physiological brain aging (NTg) in this cell type. In the hippocampus from Tg mice, we observed two separate cell phenotypes according to the proximity to amyloid deposits: plaque-associated microglial cells that were highly hypertrophied and non-plaque-associated microglial cells. In addition, we observed a remarkable increase of ubiquitin immunoreactivity in plaque-associated Iba1+ cells compared with cells located far from deposits, regardless of the age of AD mice (between 9 and 20 m) (Fig. 3c).

Ubiquitinated proteins can be present in different cell compartments, in the case of autophagosomes via interaction with the adaptor protein p62. In 20-month-old Tg mice, the ubiquitin signal co-localized with p62 on Iba1+ cells, suggesting that the accumulation of ubiquitin occurred mainly in autophagy-related vesicles (Fig. 3d). Moreover, as p62 is specifically degraded by autophagy (Spilman et al. 2010), the presence of p62 *puncta* on Iba1+ microglia near amyloid deposits from

old Tg mice suggests a dysfunction in this process (Fig. 3e). As it can be appreciated in the orthogonal view (Fig. 3f), we found an important increase in p62 immunoreactivity in microglial cells associated to amyloid plaques (PA) while far from deposits (NPA) the signal seems to be lower and similar to that found in young Tg mice (Fig. 3g, h).

Autophagic flux after short or long-term exposure to fibrillar A β 1–42 peptide

To further study the contribution of autophagy to the degradation of misfolded proteins in microglia, we employed the BV-2 murine microglia cell line that recapitulates the neuroinflammatory response of primary microglia with high fidelity (Bussi et al. 2017; Cho et al. 2014). To this end, we exposed cell cultures to vehicle or 0.5 μ M fA β 42 during 2 h (short exposure) or 24 h (long exposure) (Fig. 4a) and evaluated the autophagy progression by LC3 immunoblotting (Fig. 4b). Upon autophagy induction, LC3-I is converted to lipidated LC3-II and eventually degraded during autophagy progression. Therefore, the LC3-II/LC3-I ratio allows the evaluation of autophagic flux. As it can be appreciated in the blot quantification (Fig. 4c) after a short exposure to fA β 42, the LC3-II/LC3-I ratio was significantly reduced. To confirm that this decrease was due to LC3 degradation by autophagy, we added Bafilomycin A1 to the culture medium, which targets the V-ATPase ATP6V0C/V0 subunit and is widely used as a blocker for lysosomal activity and autophagic flux. The presence of Bafilomycin A1 inhibited the fA β 42-induced decrease of LC3-II/LC3-I ratio, suggesting that a 2 h exposure to fA β 42 increased the autophagy flux in these cells (Fig. 4c).

Next, we wanted to test whether this increase in the autophagic flux was preserved after a long-term exposure to fA β 42 (24 h), a condition similar to a chronic situation. In this case, the LC3-II/LC3-I ratio showed a significant increase as compared with vehicle-treated cells ($p < 0.01$). This increment was not affected in a significant way by the presence of the lysosomal inhibitor Bafilomycin A1 (Fig. 4d), suggesting that the autophagic flux was compromised under this chronic condition.

The impairment in the autophagic flux caused by a long-term exposure to fA β 42 was also indirectly

Fig. 2 Immunoreactivity for phagocytic marker CD68 and amyloid content in hippocampal microglia of aged PDAPPJ20 mice. **a** Representative confocal images of Iba1/A β immunofluorescence showing plaque-associated microglial cells containing intracellular amyloid content (arrowheads). Asterisk represents the core of an amyloid plaque. Scale bar indicates 40 μ m. **b** Z-project reconstructed from a Z-stack representative for the Iba1 and A β immunoreactivity indicating co-localization along z-axis (orthogonal views). Scale bar in Z-project represents 25 μ m. **c** Percentage of microglial cells containing intracellular A β related to total plaque-associated Iba1+ cells in the dentate gyrus of the hippocampus. **d** Representative images of Iba1/CD68 immunofluorescence showing microglial cells positive for CD68 (arrowheads). Scale bar indicates 40 μ m. **e, f** Immunoreactivity for CD68 expressed as a percentage of the area of the dentate gyrus that is positive for CD68 (**e**) and percentage of CD68+ microglial cells with respect to total Iba1+ microglial cells in the hilus of the hippocampus (**f**). In bar graphs, asterisks represent statistical significant differences between groups. * $p < 0.05$ (one-tailed t test), $n = 4$ –5 animals per group

evaluated by the accumulation of LC3+ vesicles. In addition, microglial cells were processed for double immunofluorescence against LC3 and A β (Fig. 5a) in an experimental design shown in Fig. 5b. The analysis by confocal microscopy showed a significant increase in the number of cells containing LC3+ vesicles (Fig. 5c). Moreover, most of the intracellular reactivity for A β co-localized with LC3 (arrowheads in Fig. 5a), suggesting that accumulated autophagosomes contained amyloid peptides. When autophagy was studied under nutrient

restriction condition (NR)—a well-recognized inducer of autophagy flux—previous to the treatment with fA β 42, the co-localization between A β and LC3 decreased (Fig. 5d). This last result supports the implication of this pathway in the amyloid degradation in microglial cells.

To confirm the hypothesis that microglial autophagy could be impaired after a 24-h exposure to amyloid peptides, in particular to analyze a potential dysfunction on the autolysosome formation, when the lysosome

binds to the autophagosome, we employed transfected BV2 cells with a plasmid carrying mCherry-GFP-LC3, as the illustration in Fig. 5e, f shows. In a normal condition, the pH-sensitive GFP fluorescent signal is lost during the formation of the autolysosome while the mCherry signal associated to LC3 protein is present, leading to a decrease in the co-localization of both signals. After transfection, BV2 cells were exposed to fA β 42 or vehicle during 24 h. Then, we performed a confocal analysis followed by quantification of mCherry and GFP fluorescence signal on each cell. When BV2-transfected cells were exposed to vehicle, this co-localization index (M2) was near to 0.1 while the 24-h exposure to fA β 42 induced a significant increase ($p < 0.01$), indicative of a deficient autophagic flux (Fig. 5g). The images corresponding to the fA β 42 condition evidenced the co-localization between mCherry and GFP in cytoplasmic vesicles located near the cellular membrane (Fig. 5h).

Evidence of lysosome dysfunction contributing to defective autophagic flux in microglial cells

Lysosomal dysfunction is usually associated to impaired autophagic flux as a cause or a consequence. Here, we evaluated the lysosomal stability after the exposure of microglial cells to amyloid peptides for 2 or 24 h. Lysosomes were detected in microglial cells by LysoTracker staining coupled to cathepsin-D—a lysosomal-specific protease—immunofluorescence (Fig. 6a). A 2-h exposure to fA β 42 caused an enlargement of lysosomes that is no longer noticeable at a longer exposure time (Fig. 6b). Diffuse staining for LysoTracker—indicative of higher lysosome permeability—was significantly enhanced after a long exposure, suggesting that lysosomal integrity could be compromised in this condition (Fig. 6c). In line with this, the co-localization between LysoTracker and cathepsin-D was significantly decreased in this experimental group, probably due to the presence of extralysosomal cathepsin-D (Fig. 6d).

Presence of LC3+ aggregates in microglial cells from AD patients' brain

With the aim to explore if human microglia could also experience compromised autophagy in AD patients, we studied the presence of LC3 on phagocytic cells in the hippocampus of diagnosed sporadic AD and control

subjects. Figure 7 shows a representative image of the preliminary immunohistochemical study on paraffin consecutive slices from post mortem human brains. Figure 7a, b (large image) shows the pattern for LC3 immunoreactivity at low magnification in the CA2 sub-field of the hippocampus from a representative control and an AD patient, respectively. As expected, the LC3 signal was mainly found in cells morphologically recognized as neurons in this region. In the AD hippocampus, the LC3 labeling was considerably enhanced. Notably, LC3+ signal was found not only located in neuronal somas but also in several neurites.

Compared with the control subjects, we noted an evident tissue distortion in the hippocampal architecture in AD patients. In particular, we observed disorganization in the area surrounding amyloid plaques that were clearly recognized due to an enhanced background from the immunohistochemistry (arrows in Fig. 7b).

Figure 7b (bottom) shows images at higher magnification obtained from the areas indicated by squares, where amyloid deposits were identified. Remarkably, in AD hippocampus, we recognized small non-neuronal cells containing LC3+ aggregates (arrowheads in lower images). The microglial CD68 immunohistochemistry—marker associated with phagocytic activity—performed on 5 μ m consecutive brain slices allowed us to establish the microglial identity of these non-neuronal LC3+ cells (Fig. 7b, lower panel).

So, in line with our mentioned results, employing an AD experimental model and the *in vitro* findings, our data suggest an autophagic dysregulation on hippocampal microglia from AD patients.

Discussion

In the first part of our study, the results emphasized the microglial changes accompanying the pathologic progression in the hippocampus of AD mice as it is illustrated in Fig. 1a. Iba1+ microglia showed early relevant changes in cell reactivity that anticipate the presence of amyloid deposits, supporting a premature and crucial involvement of this cell type in the disease. At 5 months of age, when plaques are not found yet in this transgenic model, we observed a clear decrease in the proportion of Iba1+ cells exhibiting a ramified morphology coincident with an increase in the intermediate form, characterized by shorter processes and reactive soma. Later, the

distribution in the parenchyma followed a specific pattern according to the location of amyloid deposits. Ameboid microglia—associated with phagocytic activity—was found exclusively in the adjacency of amyloid

deposits, supporting the idea of different microglial populations in the hippocampus. Importantly, intracytoplasmic amyloid was detected in microglia close to plaques.

Fig. 3 Accumulation of autophagy-related proteins in plaque-associated microglial cells in the hippocampus of aged PDAPP-J20 mice. **a** Representative low-magnification confocal images of Iba1/Ubiquitin immunofluorescence indicating ubiquitin+ puncta surrounding amyloid plaques, in co-localization with Iba1+ cells shown by arrowheads. Scale bar represents 70 μ m. **b** Z-project constructed from a Z-stack representative for the Iba1 and ubiquitin immunofluorescence indicating co-localization along z-axis (orthogonal views). Scale bar in Z-project represents 50 μ m. **c** Percentage of microglial cells containing Ubiquitin aggregates related to total Iba1+ cells in Tg and NTg PDAPPJ20 mice. For Tg, it was discriminated between plaque-associated (PA) and non-plaque-associated (NPA) microglial cells. $**p < 0.01$ (two-way ANOVA followed by Sidak post hoc comparisons), $n = 4$ –5 animals per group. **d** Representative confocal images of double immunofluorescence indicating that most of ubiquitin+ aggregates are also positive for the autophagosomal protein p62 in the hippocampus of aged Tg mice. Scale bar represents 30 μ m. **e** Representative confocal images obtained from Iba1/p62 immunofluorescence indicating the presence of p62+ aggregates (arrowheads) in microglial cells in Tg animals, what is enhanced in the vicinity of amyloid plaques (asterisks) in the hippocampus of aged Tg mice. **f** Z-projects constructed from a Z-stacks representative for the Iba1 and p62 immunofluorescence in young (5 months) and aged (15 months) Tg mice, indicating co-localization. **g, h** Quantitative analysis of p62+ aggregates in microglial cells, expressed as the percentage of Iba1+ area that is also p62+ (**g**), and the percentage of the number of Iba1+ cells containing p62+ aggregates (**h**). In (**h**), Iba1+ cells in aged Tg animals were discriminated by their proximity to amyloid plaques in plaque-associated (PA) and non-plaque-associated (NPA) microglial cells

However, the phagocytic machinery seemed to be impaired during aging and the disease progression in the hippocampus of AD mice. The amyloid content inside Iba1+ cells significantly decreased with aging as well as the phagocytic ability, suggesting an exhausted cell state. These results are in agreement with the in vitro findings reported by Caldeira et al., showing lower phagocytic activity in senescent microglia exposed to A β compared with 2 DIV cells (Caldeira et al. 2017; Clayton et al. 2017). Coincidentally, our data suggest a progressive loss of microglial phagocytic capability and a potential dysfunctional proteostasis. In this sense, we distinguished a microglial subpopulation associated to amyloid plaques clearly exhibiting a stronger ubiquitin labeling (Fig. 3c, PA) compared with the subpopulation located far from deposits (NPA). Remarkably, these intramicroglial ubiquitin+ puncta seemed to be also p62+—an autophagic substrate involved in the autophagy-mediated degradation of ubiquitinated proteins—indicating that these proteins could be accumulating in microglial cells due to impairment in their

degradation. Infiltrating macrophages could also be Iba1+, and even if their contribution is controversial in a neurodegenerative context, further analysis would be necessary to distinguish this population employing cell sorting and specific markers for resident microglia as TMEM119 and Sall1 (Li et al. 2018).

An in vitro system employing microglial cells exposed to fA β 42 allowed us to confirm that the autophagic flux experienced a decline after a long incubation time, suggesting that fibrillar amyloid can represent a chronic-like condition analogous to the in vivo scenario. The addition of the specific lysosomal activity blocker Bafilomycin A1 verified the dysfunction. In contrast, a short-term exposure to fA β 42 caused an increase in the autophagic flux of these cells, which is in line with the previous reports by Cho et al. working with primary microglia and BV2 cells, though exposed to a higher A β concentration and a different scheme of Bafilomycin A1 addition (Cho et al. 2014). Our in vitro results in BV2 cells are in line with the in vivo findings, both suggesting that a prolonged exposition to aggregated A β peptides (or by close association to amyloid plaques in PDAPPJ20 mice) negatively affects microglial autophagy, which is a key mechanism involved in A β degradation as it was previously reported by Cho and collaborators. Our findings emphasize that the mechanism for microglial autophagy impairment in the context of AD is A β —but also time dependent. Yet, this effect does not seem to be limited to microglial cells. Our group has recently reported that the duration of A β exposure is also relevant for the regulation of astroglial autophagy (Gregosa et al. 2019). In a similar way as it is presented in this study for microglia, exposure to fA β 42 during 24 h—but not 2 h—caused an accumulation of LC3+ vesicles in astrocytes.

Furthermore, our experiments using mCherry-GFP-LC3-transfected microglia provided extra evidence of autophagosome accumulation after longer A β exposure, strongly suggesting an impairment in the fusion between lysosome and autophagosome to form the autolysosome (Jia et al. 2017). We also found that the A β - and time-mediated impairment in microglial autophagy is also associated to the loss of lysosomal integrity. These organelles are critical cellular digestive compartments responsible for degradation in addition to recycling of a variety of intracellular and extracellular molecules, depending on their soluble acidic hydrolases. It was suggested that lysosomal failure triggers the pathogenesis of several neurodegenerative disorders

Fig. 4 In vitro autophagic flux is enhanced in microglial cells by a short exposure to Aβ fibrils but impaired after a longer exposure. **a** Experimental design for microglial BV2 cells exposed to fibrillar Aβ (fAβ42) during 2 or 24 h in presence/absence of bafilomycin-A1. **b–d** Representative blot (**b**) and optical density quantification of LC3 signal (related to control levels) at 2 h (**c**) and 24 h (**d**), as

an autophagic flux indicator. “+” and “-” in (**d**) indicate presence or absence of bafilomycin A1 (Baf A1), respectively. Asterisks represent statistically significant differences between groups. **p* < 0.05, ***p* < 0.01; ns, “non-significant differences” (one-way ANOVA followed by Tukey post hoc test), *n* = 4–7 samples

such as AD, Parkinson’s and Huntington’s diseases (Schultz et al. 2011). Remarkably, lysosomal failure has been reported in primary fibroblasts from patients with Down syndrome or trisomy 21, with an extra gene copy of APP, in combination with altered pH and LC3 turnover (Ying et al. 2019). In the present study, after a short-term exposure to Aβ, the lysosome staining co-localized with cathepsin-D, which is a lysosomal-specific aspartic endo-protease (Stoka et al. 2016). Our analysis indicated a preserved structure of this organelle in microglia. However, after a longer exposure to Aβ,

we verified a loss of lysosomal integrity given by diffuse cytoplasmatic LysoTracker staining and decreased co-localization with cathepsin-D. Our finding of extralysosomal cathepsin-D by confocal analysis is indicative of lysosomal membrane permeabilization. This is a notable result underlying lysosomal failure associated with Aβ uptake in microglia. The data included in this work provides evidence about the involvement of the autophagic pathway in microglia exposed to amyloid, taking into account that extracellular mechanisms could also contribute to this complex setting.

Fig. 5 Evidence of autophagosome accumulation and lysosomal alterations in microglial cells after a long exposure to fibrillar Aβ (fAβ42). **a** Representative confocal images from LC3/Aβ immunofluorescence performed on BV-2 microglial cells exposed during 24 h to fAβ42, indicating the presence of LC3+ aggregates that co-localize with amyloid (arrowheads). **b–d** Experimental design (**b**) and quantitative analysis of LC3/Aβ immunofluorescence, showing the percentage of microglial cells containing LC3+ aggregates (**c**) and the percentage of Aβ-immunoreactive area that co-localize with LC3 signal after fAβ42 exposure in cells that were previously exposed (or not) to nutrient restriction (**d**). * $p < 0.05$ (one-tailed t test), $n = 6–7$ fields per group. **e**

Experimental design for transfection of microglial cells with GFP-mCherry-LC3 plasmid. **f** Illustration representing autophagic vesicles of transfected microglial cells. Vesicles present both GFP and mCherry signal in autophagosomes, but only mCherry in autolysosomes. **g** Percentage of co-localization between GFP and mCherry signals in transfected cells exposed to vehicle/fAβ42 during 24 h. Asterisks represent statistically significant differences between groups. ** $p < 0.01$ (one-tailed t test), $n = 35–40$ cells. **h** Representative confocal images of microglial cells transfected with GFP-mCherry-LC3 plasmid and exposed to vehicle/fAβ42 during 24 h. Scale bar represents 5 μm

Fig. 6 Evidence of lysosomal alterations during a long exposure to fibrillar A β (fA β 42). **a** Representative confocal images of cathepsin-D immunofluorescence coupled to LysoTracker staining in microglial BV-2 cells exposed to vehicle/fA β 42 during 2 or 24 h. Top, the general pattern of LysoTracker+ signal; bottom, the localization of cathepsin-D in relation to lysosomes. Scale bar represents 15 μ m. **b** Lysosomes mean size in square micrometers. *** p < 0.001 (one-way ANOVA followed by Dunett post hoc test), n = 35–50 cells per group. **c** Percentage of microglial cells presenting diffuse staining for LysoTracker. *** p < 0.001 (one-way ANOVA followed by Dunett post hoc test), n = 4–7 samples per group. **d** Percentage of cathepsin-D+ area that co-localized with LysoTracker signal. *** p < 0.001 (one-way ANOVA followed by Dunett post hoc test), n = 35–50 cells per group

There is a consistent body of evidence supporting that neuroinflammation contributes to the autophagic flux blockage in neurons (Du et al. 2017). Furthermore, inflammasome-forming NLRP3 has been shown to negatively regulate autophagy (Sun et al. 2017). However, the impact on glial autophagy remains unclear. The novelty of this study is also founded on the evidence of impaired and defective autophagic flux in microglia from aged AD mice, coincidentally with a growing neuroinflammation. Microglia seemed to be exhausted and not able to promote an effective autophagy when fibrillar amyloid is continually present in vitro. In line with our contribution, recent reports focused on the pro-inflammatory cytokine TNF α promoting a microglial pro-inflammatory M1 phenotype concurrent with an altered autophagic flux (Jin et al. 2018), and on the other

hand, IL-4 inducing an M2 alternative phenotype and autophagy enhancement (Tang et al. 2019).

In addition, our preliminary data from sporadic Alzheimer's disease patients, the most common form of this pathology with a prevalence near to 95% of cases, suggested by the first time the increased LC3 labeling on microglial cells surrounding amyloid plaques. Moreover, microglia showed an immunophenotype associated to dementia: the presence of CD68+ microglia has been positively associated with loss of cognitive function assessed by the Mini-Mental State Examination in patients (Minett et al. 2016). Furthermore, the glial senescence could also contribute to homeostatic loss associated with cognitive decline in pathophysiological aging. Senescent human astrocytes showed a particular phenotype characterized by an

Fig. 7 Human post mortem AD brains exhibit evidence of LC3+ vesicles accumulation in microglial cells. **a, b** Representative images of LC3 immunohistochemistry performed on consecutive brain sections containing hippocampus from a control subject (**a**) and a sporadic AD patient (**b**). Arrows in (**b**) represent amyloid plaques evidenced by distortion in tissular architecture and denser background. Scale bar represents 100 μ m. Bottom, high-magnification images from four areas containing amyloid plaques (represented by asterisks), including consecutive sections processed for immunohistochemistry against LC3 (upper image) and CD68 (lower image). Some LC3+ cells are also positive for CD68 immunoreactivity (arrowheads)

altered cytokine profile and changes in splicing factor expression (Lye et al. 2019). Also, cerebrovascular alterations can also contribute to synaptic dysfunction. The deposition of blood protein fibrinogen in the parenchyma induces spine elimination mediated by microglia activation (Merlini et al. 2019).

Our results suggest that along with the pathology progression, microglial cells could lose their capability to promote the clearance of amyloid and damaged cellular material. With recent several large-scale genetic studies involving microglial molecules in AD, the implication of these cells has become even more relevant than ever before. Mosher and Wyss-Coray enumerated six distinct functions of microglia and proposed the progressive loss of some of them according to brain aging and neurodegeneration (Mosher and Wyss-Coray 2014). Our data showing altered cell morphology,

failed proteostasis and phagocytic activity, impaired autophagy, and lysosome dysfunction associated to A β exposure emphasizes the novel concept of a microglial exhaustion in AD.

This study, employing brain tissue from aged AD mice together with an extensive *in vitro* experimental approach counting diverse and specific molecular tools, contributes to highlight (1) the crucial role of microglial cells in AD, (2) the possible microglial cell exhaustion state due to synergic senescence and chronic exposure to amyloid, and (3) the microglial autophagy impairment, in association with lysosome dysfunction. Furthermore, our results on human brain tissue reveal AD-associated changes on microglial autophagy.

Our findings joint recent data emphasizing the potential role of glia as attractive targets for therapies addressing the complexity of AD brain pathological aging.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Ahmad MH, Fatima M, Mondal AC (2019) Influence of microglia and astrocyte activation in the neuroinflammatory pathogenesis of Alzheimer's disease: rational insights for the therapeutic approaches. *J Clin Neurosci* 59:6–11
- Bamberger ME, Harris ME, McDonald DR, Husemann J, Landreth GE (2003) A cell surface receptor complex for fibrillar beta-amyloid mediates microglial activation. *J Neurosci* 23(7):2665–2674
- Beauquis J, Pavia P, Pomilio C, Vinuesa A, Podlutska N, Galvan V, Saravia F (2013) Environmental enrichment prevents astroglial pathological changes in the hippocampus of APP transgenic mice, model of Alzheimer's disease. *Exp Neurol* 239:28–37
- Beauquis J, Vinuesa A, Pomilio C, Pavia P, Galvan V, Saravia F (2014) Neuronal and glial alterations, increased anxiety, and cognitive impairment before hippocampal amyloid deposition in PDAPP mice, model of Alzheimer's disease. *Hippocampus* 24(3):257–269
- Bocchini V, Mazzolla R, Barluzzi R, Blasi E, Sick P, Kettenmann H (1992) An immortalized cell line expresses properties of activated microglial cells. *J Neurosci Res* 31(4):616–621
- Bose S, Cho J (2013) Role of chemokine CCL2 and its receptor CCR2 in neurodegenerative diseases. *Arch Pharm Res* 36(9):1039–1050
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Bussi C, Peralta Ramos JM, Arroyo DS, Gaviglio EA, Gallea JJ, Wang JM, Celej MS, Iribarren P (2017) Autophagy down regulates pro-inflammatory mediators in BV2 microglial cells and rescues both LPS and alpha-synuclein induced neuronal cell death. *Sci Rep* 7:43153
- Caldeira C, Cunha C, Vaz AR, Falcao AS, Barateiro A, Seixas E, Fernandes A, Brites D (2017) Key aging-associated alterations in primary microglia response to Beta-amyloid stimulation. *Front Aging Neurosci* 9:277
- Carosi JM, Sargeant TJ (2019) Rapamycin and Alzheimer disease: a double-edged sword? *Autophagy* 15(8):1460–1462
- Clayton KA, Van Enoo AA, Ikezu T (2017) Alzheimer's disease: the role of microglia in brain homeostasis and Proteopathy. *Front Neurosci* 11:680
- Cho MH, Cho K, Kang HJ, Jeon EY, Kim HS, Kwon HJ, Kim HM, Kim DH, Yoon SY (2014) Autophagy in microglia degrades extracellular beta-amyloid fibrils and regulates the NLRP3 inflammasome. *Autophagy* 10(10):1761–1775
- Du D, Hu L, Wu J, Wu Q, Cheng W, Guo Y, Guan R, Wang Y, Chen X, Yan X, Zhu D, Wang J, Zhang S, Guo Y, Xia C (2017) Neuroinflammation contributes to autophagy flux blockage in the neurons of rostral ventrolateral medulla in stress-induced hypertension rats. *J Neuroinflammation* 14(1):169
- Fu R, Shen Q, Xu P, Luo JJ, Tang Y (2014) Phagocytosis of microglia in the central nervous system diseases. *Mol Neurobiol* 49(3):1422–1434
- Galvan V, Gorostiza OF, Banwait S, Ataie M, Logvinova AV, Sitaraman S, Carlson E, Sagi SA, Chevallier N, Jin K, Greenberg DA, Bredesen DE (2006) Reversal of Alzheimer's-like pathology and behavior in human APP transgenic mice by mutation of Asp664. *Proc Natl Acad Sci USA* 103(18):7130–7135
- Gregosa A, Vinuesa A, Todero MF, Pomilio C, Rossi SP, Bentivegna M, Presa J, Wenker S, Saravia F, Beauquis J (2019) Periodic dietary restriction ameliorates amyloid pathology and cognitive impairment in PDAPP-J20 mice: potential implication of glial autophagy. *Neurobiol Dis* 104:542
- Houtman J, Freitag K, Gimber N, Schmoranz J, Heppner FL, Jendrach M (2019) Beclin1-driven autophagy modulates the inflammatory response of microglia via NLRP3. *EMBO J* 38(4)
- Hsia AY, Masliah E, McConlogue L, Yu GQ, Tatsuno G, Hu K, Kholodenko D, Malenka RC, Nicoll RA, Mucke L (1999) Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc Natl Acad Sci USA* 96(6):3228–3233
- Hyman BT, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Carrillo MC, Dickson DW, Duyckaerts C, Frosch MP, Masliah E, Mirra SS, Nelson PT, Schneider JA, Thal DR, Thies B, Trojanowski JQ, Vinters HV, Montine TJ (2012) National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. *Alzheimers Dement* 8(1):1–13
- Jia R, Guardia CM, Pu J, Chen Y, Bonifacino JS (2017) BORC coordinates encounter and fusion of lysosomes with autophagosomes. *Autophagy* 13(10):1648–1663
- Jiang S, Bhaskar K (2017) Dynamics of the complement, cytokine, and chemokine systems in the regulation of synaptic function and dysfunction relevant to Alzheimer's disease. *J Alzheimers Dis* 57(4):1123–1135
- Jin MM, Wang F, Qi D, Liu WW, Gu C, Mao CJ, Yang YP, Zhao Z, Hu LF, Liu CF (2018) A critical role of autophagy in regulating microglia polarization in Neurodegeneration. *Front Aging Neurosci* 10:378
- Kiyota T, Yamamoto M, Xiong H, Lambert MP, Klein WL, Gendelman HE, Ransohoff RM, Ikezu T (2009) CCL2 accelerates microglia-mediated Abeta oligomer formation and

- progression of neurocognitive dysfunction. *PLoS One* 4(7): e6197
- Koenigsknecht-Talboo J, Landreth GE (2005) Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines. *J Neurosci* 25(36):8240–8249
- Koenigsknecht J, Landreth G (2004) Microglial phagocytosis of fibrillar beta-amyloid through a beta1 integrin-dependent mechanism. *J Neurosci* 24(44):9838–9846
- Lane CA, Hardy J, Schott JM (2018) Alzheimer's disease. *Eur J Neurol* 25(1):59–70
- Lee CY, Landreth GE (2010) The role of microglia in amyloid clearance from the AD brain. *J Neural Transm* 117(8):949–960
- Lenz KM, Nelson LH (2018) Microglia and beyond: innate immune cells as regulators of brain development and behavioral function. *Front Immunol* 9:698
- Li Q, Lan X, Han X, Wang J (2018) Expression of Tmem119/Sall1 and Ccr2/CD69 in FACS-sorted microglia- and monocyte/macrophage-enriched cell populations after intracerebral hemorrhage. *Front Cell Neurosci* 12:520
- Liang X, Wang Q, Hand T, Wu L, Breyer RM, Montine TJ, Andreasson K (2005) Deletion of the prostaglandin E2 EP2 receptor reduces oxidative damage and amyloid burden in a model of Alzheimer's disease. *J Neurosci* 25(44):10180–10187
- Lin AL, Zheng W, Halloran JJ, Burbank RR, Hussong SA, Hart MJ, Javors M, Shih YY, Muir E, Solano FR, Strong R, Richardson AG, Lechleiter JD, Fox PT, Galvan V (2013) Chronic rapamycin restores brain vascular integrity and function through NO synthase activation and improves memory in symptomatic mice modeling Alzheimer's disease. *J Cereb Blood Flow Metab* 33(9):1412–1421
- Lye JJ, Latorre E, Lee BP, Bandinelli S, Holley JE, Gutowski NJ, Ferrucci L, Harries LW (2019) Astrocyte senescence may drive alterations in GFAPalpha, CDKN2A p14(ARF), and TAU3 transcript expression and contribute to cognitive decline. *Geroscience* 41(5):561–573
- Metaxakis A, Ploumi C, Tavernarakis N (2018) Autophagy in age-associated neurodegeneration. *Cells* 7(5)
- Merlini M, Rafalski VA, Rios Coronado PE, Gill TM, Ellisman M, Muthukumar G, Subramanian KS, Ryu JK, Syme CA, Davalos D, Seeley WW, Mucke L, Nelson RB, Akassoglou K (2019) Fibrinogen induces microglia-mediated spine elimination and cognitive impairment in an Alzheimer's disease model. *Neuron* 101(6):1099–1108 e1096
- Minett T, Classey J, Matthews FE, Fahrenhold M, Taga M, Brayne C, Ince PG, Nicoll JA, Boche D, Mrc C (2016) Microglial immunophenotype in dementia with Alzheimer's pathology. *J Neuroinflammation* 13(1):135
- Mosher KI, Wyss-Coray T (2014) Microglial dysfunction in brain aging and Alzheimer's disease. *Biochem Pharmacol* 88(4): 594–604
- Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L (2000) High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J Neurosci* 20(11):4050–4058
- Olabarria M, Noristani HN, Verkhratsky A, Rodriguez JJ (2010) Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia* 58(7): 831–838
- Olabarria M, Noristani HN, Verkhratsky A, Rodriguez JJ (2011) Age-dependent decrease in glutamine synthetase expression in the hippocampal astroglia of the triple transgenic Alzheimer's disease mouse model: mechanism for deficient glutamatergic transmission? *Mol Neurodegener* 6:55
- Paresce DM, Chung H, Maxfield FR (1997) Slow degradation of aggregates of the Alzheimer's disease amyloid beta-protein by microglial cells. *J Biol Chem* 272(46):29390–29397
- Pomilio C, Pavia P, Gorjod RM, Vinuesa A, Alaimo A, Galvan V, Kotler ML, Beauquis J, Saravia F (2016) Glial alterations from early to late stages in a model of Alzheimer's disease: evidence of autophagy involvement in Abeta internalization. *Hippocampus* 26(2):194–210
- Porte Alcon S, Gorjod RM, Kotler ML (2018) Regulated necrosis orchestrates microglial cell death in manganese-induced toxicity. *Neuroscience* 393:206–225
- Roberson ED, Searce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, Gerstein H, Yu GQ, Mucke L (2007) Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. *Science* 316(5825):750–754
- Schultz ML, Tecedor L, Chang M, Davidson BL (2011) Clarifying lysosomal storage diseases. *Trends Neurosci* 34(8):401–410
- Selkoe DJ (2000) Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. *Ann NY Acad Sci* 924:17–25
- Simon AM, Schiapparelli L, Salazar-Colocho P, Cuadrado-Tejedor M, Escribano L, Lopez de Maturana R, Del Rio J, Perez-Mediavilla A, Frechilla D (2009) Overexpression of wild-type human APP in mice causes cognitive deficits and pathological features unrelated to Abeta levels. *Neurobiol Dis* 33(3):369–378
- Simonovitch S, Schmukler E, Bepalko A, Iram T, Frenkel D, Holtzman DM, Masliah E, Michaelson DM, Pinkas-Kramarski R (2016) Impaired autophagy in APOE4 astrocytes. *J Alzheimers Dis*: JAD 51(3):915–927
- Spilman P, Podlitskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, Richardson A, Strong R, Galvan V (2010) Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS One* 5(4):e9979
- Stalder M, Deller T, Staufenbiel M, Jucker M (2001) 3D-reconstruction of microglia and amyloid in APP23 transgenic mice: no evidence of intracellular amyloid. *Neurobiol Aging* 22(3):427–434
- Stoka V, Turk V, Turk B (2016) Lysosomal cathepsins and their regulation in aging and neurodegeneration. *Ageing Res Rev* 32:22–37
- Sun Q, Fan J, Billiar TR, Scott MJ (2017) Inflammasome and autophagy regulation—a two-way street. *Mol Med* 23:188–195
- Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K (2006) Role of toll-like receptor signalling in Abeta uptake and clearance. *Brain* 129(Pt 11):3006–3019
- Tang RH, Qi RQ, Liu HY (2019) Interleukin-4 affects microglial autophagic flux. *Neural Regen Res* 14(9):1594–1602
- Van Skike CE, Jahrling JB, Olson AB, Sayre NL, Hussong SA, Ungvari Z, Lechleiter JD, Galvan V (2018) Inhibition of

- mTOR protects the blood-brain barrier in models of Alzheimer's disease and vascular cognitive impairment. *Am J Physiol Heart Circ Physiol* 314(4):H693–H703
- Verkhatsky A, Nedergaard M (2018) Physiology of Astroglia. *Physiol Rev* 98(1):239–389
- Walter J (2006) Control of amyloid-beta-peptide generation by subcellular trafficking of the beta-amyloid precursor protein and beta-secretase. *Neurodegener Dis* 3(4–5):247–254
- Wani A, Gupta M, Ahmad M, Shah AM, Ahsan AU, Qazi PH, Malik F, Singh G, Sharma PR, Kaddoumi A, Bharate SB, Vishwakarma RA, Kumar A (2019) Alborixin clears amyloid-beta by inducing autophagy through PTEN-mediated inhibition of the AKT pathway. *Autophagy*:1–19
- Ying J, Sato Y, Im E, Berg M, Bordini M, Darji S, Kumar A, Mohan PS, Bandyopadhyay U, Diaz A, Maria Cuervo A, Nixon RA (2019) Lysosomal dysfunction in down syndrome is APP-dependent and mediated by APP-betaCTF (C99). *J Neurosci* 39(27):5255–5268
- Zhang Y, Chen X, Zhao Y, Ponnusamy M, Liu Y (2017) The role of ubiquitin proteasomal system and autophagy-lysosome pathway in Alzheimer's disease. *Rev Neurosci* 28(8):861–868
- Zhao YG, Zhang H (2019) Autophagosome maturation: an epic journey from the ER to lysosomes. *J Cell Biol* 218(3):757–770

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