Avens Publishing Group
Journal of Parkinson's Disease & Alzheimer's Disease
October 2022 Vol.:9, Issue:1


Open Access Research Article

Journal of
Parkinson's Disease & Alzheimer's Disease

# Autophagy Monitoring in Cerebral Pericytes from Alzheimer's disease Mouse Model in an Inflammatory Environment

**Vérité J, Thoreau V, Rabeony H, Fournier B, Janet T‡ and Page G*‡**

*University of Poitiers, Neurovascular Unit and Cognitive Disorders, Pôle Biologie Santé, Poitiers, France*

*‡Janet T and Page G contributed equally to this work.*

***Address for Correspondence**

Page G, University of Poitiers, Neurovascular Unit and Cognitive Disorders, Pôle Biologie Santé, Poitiers, France; E-mail: guylene.page@univ-poitiers.fr

**Submission:** 01 August, 2022
**Accepted:** 27 September, 2022
**Published:** 03 October, 2022



**Keywords:** Pericytes; Alzheimer's disease; Inflammation; Autophagy; Interleukin-1β

## Abstract

**Background:** The blood-brain barrier (BBB) is a complex neurovascular unit involving pericytes as multi-functional cells that play a crucial role in maintaining homeostasis. In Alzheimer's disease (AD), platelet-derived growth factor receptor-β (PDGFRβ) immunostaining revealed significantly reduced pericyte coverage of brain capillaries as well as reduced pericyte numbers in AD cortex and hippocampus compared with control brains. However, the mechanisms of pericyte loss have yet to be completely defined. Moreover, we have previously shown that, in microglia, interleukin-1β (IL-1β)-induced inflammation blocks autophagic flow, a physiological process involved in the degradation of proteins including the β-amyloid peptide. Thus, we evaluated whether the inflammatory response in AD impaired autophagy in pericytes.

**Methods:** A longitudinal autophagic status monitoring was performed in pericytes purified from brains of AD and wild type (WT) mice at 3, 6 and 12 months. Furthermore, the impact of an inflammatory environment was studied not only in these primary pericytes but also in a pericyte cell line developed in the laboratory.

**Results:** Primary pericytes from AD mice displayed a significant increase of autophagic markers at 3 months whereas in later stages their expressions were like those of WT mice. In addition, IL-1β-induced inflammation did not modify the expression of autophagic markers and not those of mTOR signaling pathway in both primary and immortalized mouse pericytes.

**Conclusions:** For the first time, these data highlighted that autophagy is activated in primary pericytes from AD transgenic mice at 3 months. In addition, inflammation has no impact on autophagic flow under our experimental conditions.

## Introduction

We have previously published the first results on the interaction between autophagy and inflammation in Alzheimer's disease (AD) [1-3]. We showed that the pro-inflammatory cytokine interleukin-1β (IL-1β) was responsible for blocking autophagy and that microglia was the most sensitive cell type in primary neuron/astrocyte/microglia tri-cultures [1]. These last results are consistent with recent data concerning the failure of these brain resident immune cells during AD [4]. However, other cells showed senescence and died in AD such as pericytes [5]. Recently, authors showed elevated soluble platelet-derived growth factor receptor-β (sPDGFRβ) levels in cerebrospinal fluid (CSF), indicating pericyte injury and blood brain barrier (BBB) breakdown [6,7]. So, sPDGFRβ could be a promising early biomarker of human cognitive dysfunction [7,8]. Pericytes are mural cells abundant in the microvasculature in the central nervous system and physically the closest cells to brain endothelial cells (EC) wrapping around them, joined by gap junctions, and interfacing bypeg-and-socket structures [9,10]. Their functions are largely explored from embryonic to adult periods of development [6,11-13]. Under physiological conditions, pericytes regulate BBB integrity, angiogenesis, phagocytosis, cerebral blood flow (CBF) and capillary diameter, neuro inflammation and multi potent stem cell activity [9-14]. The presence of pericytes is essential because knock-out mouse models by deletion and/or genetic manipulation of *PDGFRβ* and/or *PDGFB* genes result in blood vessel dilation, EC hyperplasia, and microaneurysm formation [15]. Furthermore, in double transgenic $APP^{sw/0}$/$PDGFR\beta^{+/-}$ mice, the loss of pericytes early increased brain Aβ-amyloid peptide (Aβ) levels, accelerated amyloid angiopathy and cerebral β-amyloidosis, led to the development of tau pathology and to an early neuronal loss that is normally absent in APP transgenic mice [16,17]. Many studies showed that pericyte dysfunction is associated with AD neuropathology [18]. In fact, pericyte degeneration led to BBB disruption and unrestricted entry and accumulation of blood-derived products in brain (erythrocyte-derived hemoglobin and plasma-derived proteins such as albumin, plasmin, thrombin, fibrin, immunoglobulins and others) [19]. In humans with cognitive decline, Aβ constricted brain capillaries at pericyte locations and generated reactive oxidative species (ROS) which led to release of endothelin-1 acting *via* endothelin receptors (ETA) on pericytes and increasing the constricting effects [20]. Neuroimaging studies in individuals with mild cognitive impairment (MCI) and early AD revealed BBB breakdown in the hippocampus before brain atrophy and dementia [21-23]. The mechanisms of pericyte loss have yet to be completely defined. AD is also characterized by a great inflammatory response and by a blockage of autophagy [24-29]. In AD, both neuroinflammation and systemic inflammation can disrupt the BBB by modifying the tight junctions, damaging the vascular endothelial cell, degrading glycocalyx, allowing peripheral hematopoietic cells to infiltrate neural tissue and become part of the parenchymal microglia/macrophage [23,24,30]. Notably, IL-1β contributes to BBB dysfunction [25,31]. Pericytes express several mediators (chemokines, cytokines) that can enhance leukocyte extravasation and contribute to a cerebral inflammatory phenotype [32,33]. At the same time, pericytes over

ISSN: 2330-2178

express adhesion molecules that guide and instruct innate immune cells after transendothelial migration [32]. Moreover, pericytes are implicated in shaping adaptive immunity, with several studies that point to an immunosuppressive role [34]. However, in AD, pericyte loss also correlates with inflammation-mediated disruption of BBB [32]. It is well known that inflammatory response in particular IL-1$\beta$ production impairs autophagy resulting in neurodegeneration and memory loss in AD [1-3,35]. However, the autophagic status of pericytes in AD is still poorly understood. Therefore, this study aims to explore the autophagic status of pericytes from AD mice *versus* wild type (WT) mice at 3, 6 and 12 months. The analysis focused on the expression of Beclin-1, a protein involved in autophagic initiation [36], p62 as a cargo protein of the material to be eliminated [37], and soluble LC3 I and membranaryunsoluble LC3 II isoforms as elongation and fusion signals with lysosomes [38]. Furthermore, a pericyte cell line developed in the laboratory was also used to study the impact of IL-1$\beta$ in autophagy and mTOR signaling pathway.

## Materials & Methods

### Chemical products

Sodium fluoride (NaF), phenylmethylsulfonyl fluoride (PMSF), protease and phosphatase inhibitor cocktails, dithiothreitol (DTT), mouse monoclonal Anti-$\beta$-Actin antibody, mouse anti-alpha smooth muscle actin antibody ($\alpha$SMA), and all reagent-grade chemicals for buffers were purchased from Sigma (St Quentin Fallavier, France), sodium pentobarbital from CEVA, Animal Health (Libourne, France), 4X Laemmli sample buffer, 4-15% mini-PROTEAN® TGX™ gels, Tris-glycine running buffer and Trans-Blot® Turbo™ Transfer System from Biorad (Marnes-la-Coquette, France), Quant-it® protein assay. For western blot, primary antibodies against Beclin-1, mTOR, p70S6K (total and phosphorylated forms), mouse anti-GFAP for immunocytofluorescence, secondary anti-rabbit or anti-mouse IgG antibody conjugated with Horseradish Peroxydase (HRP) and mouse recombinant Interleukin-1$\beta$ (IL-1$\beta$)were purchased from Cell Signalling (Ozyme, St Quentin Yvelines, France) except p62/SQSTM1 and LC3I/II from MBL (CliniSciences distributor, Nanterre, France), rabbit polyclonal anti-NG2 Chondroitin Sulfate Proteoglycan (NG2) and anti-platelet derived growth factor receptor beta (PDGFR$\beta$) antibodies from Proteintech France, a rabbit polyclonal antibody anti-von Willebrand Factor (vWF) from Merck Millipore (Molsheim, Alsace France), macrosialin or murine homologue of the human CD68 and secondary antibodies goat anti-rat R-Phycoerythrin (RPE) from AbDSerotec(Düsseldorf, Germany), IgG- and protease-Free Bovine Serum Albumin (BSA) and secondary anti-mouse-Alexa 488 or anti-rabbit-TRITC from Jackson Immuno Research Europe Ltd (Interchim distributor, Montluçon, France).

### Cell culture

For this work, mouse pericytes were extracted following experimental protocol described in our patents (FR17/57643 and US-2022-0010258-A1). Two cultures of mouse pericytes were used: a mouse primary culture of pericytes and a mouse cell line of pericytes developed in the laboratoryas indicated in the patent.

Pericytes came from brains of APPswePS1dE9 or wild type (WT) mice at 3, 6 and 12 months of age. These transgenic mice were purchased from Mutant Mouse Resources and Research Centers (Stock No: 34829-JAX, USA) displaying Alzheimer phenotype (Authorization from "Haut Comité de Biotechnologiefrançais" (HCB) to Pr Guylène Page, number 2040 for reproduction, treatment, behavioral tests and ex-vivo experiments). Wild type (WT) with B6C3F1 background and APPswePS1dE9 mice were obtained by crossing a male APPswePS1dE9 mouse with a WT female mouse (from Charles River, strain Code 031) as explained previously [39].The use of animals was approved by the Ethical and Animal Care Committee (N°84 COMETHEA, Ethical Committee for Animal Experimentation Poitou-Charentes, France). At weaning, all mice were genotyped by polymerase chain reaction (PCR) analysis of tail biopsies according to the manufacturer's recommended protocols. All animal care and experimental procedures conformed with the French Decree number 2013–118, 1 February 2013 NOR: AGRG1231951D in accordance with European Community guidelines (directive 2010/63/UE). All efforts were made to minimize animal suffering, as well as the number of animals used. The animals were housed in a conventional state under adequate temperature (23 ± 3°C) and relative humidity (55 ± 5%) control with a 12/12 h reversed light/dark cycle with access to food and water ad libitum.

As a method of immortalization, we used the transformation with oncoprotein of murine polyomavirus, Polyoma middle T antigen. Protocol was already described for murine endothelial cells [40]. Stable cerebral pericyte cell line has been obtained 2 months later. This pure cell line displayed features of pericytes with $\alpha$SMA, NG2, PDGFR$\beta$ and no vWF, ZO-1, GFAP nor CD68 (markers of endothelial cells, astrocytes and microglia, respectively) expressions as shown in additional file 1 (see Supplementary Information).

### Cell treatment

Primary pericytes and immortalized pericyteswere seeded in 24-well plates (150,000 and 50,000 cells/well, respectively) to study their autophagic status in control or inflammatory conditions. Inflammation was induced by IL-1$\beta$ at 400 pg/mL during 48 hours in a cell incubator. After 48hr-treatment, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 50 mM NaCl pH 6.8, 1% (v/v) Triton X-100, 1 mM PMSF, 50 mM NaF, 1% (v/v) protease inhibitor and 1% (v/v) phosphatase inhibitor cocktails). Lysates were sonicated for 10 sec and centrifuged at 15,000 × g for 15 min at 4°C. The supernatants were collected and analyzed for protein determination using a Quant-it® protein assay kit. Samples were frozen at -80°C until western blot experiments.

### Western blot

Samples (40 $\mu$g proteins) were prepared for electrophoresis by adding 4X Laemmli sample buffer containing 0.05 M DTT and loaded into 4-15% mini-PROTEAN® TGX™ gels with Tris-glycine SDS running buffer. Systems ran at 200 V for 35 minutes. Then, gels were transferred to nitrocellulose membranes using Trans-Blot® Turbo™ Transfer System (25V, 3 min for 0.2 µm nitrocellulose MINI gel). Membranes were washed for 10 min in Tris-buffered saline/Tween (TBST: 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.05% Tween 20) and aspecific antigenic sites were blocked 2h in TBST containing 10% BSA for detection of LC3 and in TBST containing 5% non-fat milk for other proteins. Antibodies used were rabbit anti-Beclin-1, anti-p62, anti-LC3, anti-total mTOR, anti-total p70S6K, anti-$P_{S2448}$-mTOR,

ISSN: 2330-2178

anti-$P_{T389}$-p70S6K all at a dilution 1:500 in TBST containing 5% BSA overnight at 4°C. Membranes were washed twice with TBST and then incubated with the HRP-conjugated secondary anti-rabbit IgG (1:1000), during 1 hour at RT. Membranes were washed again and exposed to the chemiluminescence Luminata Forte Western HRP Substrate (Millipore, Saint-Quentin-en-Yvelines, France) followed by signal's capture with the Gbox system (GeneSnap software, Syngene, Ozyme distributor). After 2 washes in TBST, membranes were probed with mouse antibody against $\beta$-actin (1:1000) overnight at 4°C. They were then washed with TBST, incubated with HRP-conjugated secondary antibody anti-mouse (1:1000 in blocking buffer) for 1h, exposed to the chemiluminescence Luminata Classico Western HRP Substrate (Millipore, Saint-Quentin-en-Yvelines, France) and signals were captured. Automatic image analysis software is supplied with Gene Tools (Syngene, Ozyme distributor). Protein/$\beta$-actin*ratios* were calculated and showed in the corresponding figures.

### CYTO-ID® Autophagy Detection Kit

This live cell analysis kit provides a convenient approach for the analysis of the regulation of autophagic activity at the cellular level. Cell treatment was performed in 96-well plates (1,25,000 cells/well). According to the supplier's recommendations, cell medium was gently removed after treatment and cells were washed with 200 $\mu$L of 1X assay buffer containing 5% NBCS. Then, 100$\mu$L of 1X Assay Buffer/5% NBCS containing 2$\mu$L/mL of CYTO-ID® green detection reagent and 2$\mu$L/mL Hoechst 33342 nuclear stain were added in each well. Plates were incubated 30 min at room temperature in a black chamber. Then, cells were washed twice with 100 $\mu$L of 1X assay buffer/5% NBCS and 100 $\mu$L of this buffer were added for measurement of fluorescent signals ($\lambda$Excitation: 480nm/$\lambda$Emission: 530nm for CYTO-ID® green detection reagent and $\lambda$Excitation: 340nm/$\lambda$Emission: 480nm for Hoechst 33342 nuclear stain) by using a ThermoVarioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, Illkirch, France). Rapamycin (1 $\mu$M)as an inducer of autophagy and Chloroquine (10 $\mu$M) as a lysosomal inhibitor were included as positive controls. Ratios of fluorescent intensity for CYTO-ID® green detection reagent /fluorescent intensity for Hoechst 33342 nuclear stain were calculated and results were normalized to control.

### LYSO-ID® Red Cytotoxicity Kit

This GFP certified* live cell kit provides a rapid and quantitative approach for determining drug- or toxic agent-induced lysosome and lysosome-like organelle perturbations, for detecting phospholipidosis and also the accumulation of autophagosomes by blocking the downstream lysosomal pathway and/or intracellular trafficking of autophagosomes also lead to increase in the accumulation of intracellular LYSO-ID® red dye signal. A lysosome-perturbation agent, verapamil (10 $\mu$M), is provided as a positive control for monitoring changes in vacuole number and volume. A blue nuclear counter stain is integrated into the detection reagentto identify cell death or loss. As indicated for CYTO-ID®, cells were seeded in 96-well plates (1,25,000 cells/well) and treated with vehicle or IL-1$\beta$. After treatment, medium was carefully aspirated and 100µl of 1X Assay Buffer containing 2% of NBCS were added in each well. Then, the buffer was aspirated and 100$\mu$l of the 1X dual color detection reagent containing 1mL Dual Color Detection Reagent, 8.8 mL Detection Buffer and 2% of NBCS for 10 mL of solution was added in each well. Plates were incubated for 1 hour at room temperature and protected from light. At the end of incubation, plates were gently washed twice with 100 $\mu$L of 1X Assay Buffer/2% of NBCS and 80 $\mu$L of 1X Assay Buffer/2% of NBCS were added before measurement of fluorescence intensity. The red lysosome stain can be read witha $\lambda$Excitation: 540nm/$\lambda$Emission:680 nm and the blue nuclear counterstain can be read with a $\lambda$Excitation: 340nm/$\lambda$Emission: 480nm. Ratios of fluorescent intensity for LYSO-ID® red dye signal /fluorescent intensity for the blue nuclear counterstain were calculated and results were normalized to control.

### Statistical analysis

For biochemical analysis, results are expressed as means ± SEM. To compare quantitative variables between primary pericytes prepared from WT and APPswePS1dE9 mice treated or not with IL-1$\beta$ and between immortalized pericytes treated or not with IL-1$\beta$, Mann-Withney's tests were used. Longitudinal changes in parameters occurring during the life were analysed with a Kruskal-Wallis test followed by a post-hoc with Dunns test (GraphPad Instat, GraphPad Software, San Diego, CA, USA). The level of significance was $p < 0.05$.

## Results

### Monitoring of autophagy in primary mouse pericytes

To determine whether autophagy changes occurred in pericytes prepared from brains of APPswePS1dE9 mice at 3, 6 and 12 months of age, immunoblottings of Beclin-1, p62, LC3 I and LC3 II were performed. The levels of expression of Beclin-1 which is a key component in the initiation of autophagosome formation significantly increased by 31-fold in pericytes of 3-months old APPswePS1dE9 mice compared to age-matched WT mice [41]. On the contrary, a robust decrease by 36- and 10.8-fold in pericytes of APPswePS1dE9 mice was observed at 6 and 12 months, respectively compared to 3-months old APPswePS1dE9 mice (Figure 1).

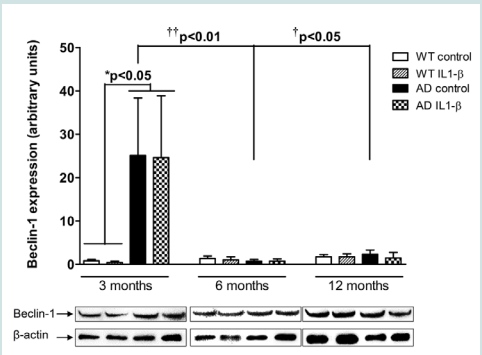


**Figure 1: Monitoring of Beclin-1 levels in primary pericytes of APPswePS1dE9 *versus* wild-type mice.** Pericytes prepared from brains of AD transgenic and WT mice were incubated in control (sterile water as vehicle) and inflammatory conditions. Inflammation was induced by IL-1$\beta$ at 400 pg/mL during 48 hr. Western blots were performed with cell lysates of pericytes to detect Beclin-1 at 55 kDa and $\beta$-actin at 42 kDa as a loading control in Western blot analysis. Beclin-1/$\beta$-actin ratio were calculated to determine the level of Beclin-1 expression in our experimental conditions. Results were expressed as mean ± SEM with arbitrary units with 6 to 13 mice per group. For WT and AD at 3M, by Kruskal-Wallis test p = 0.0055 and *p<0.05 compared to WT mice at 3 months by a Dunn's multiple comparison test. For AD control at 3, 6 and 12M, Kruskal-Wallis test p = 0.0093 and †p<0.05, ††p<0.01 compared to vehicle-treated AD mice at 3 months by a Dunn's multiple comparison test.

ISSN: 2330-2178

The protein p62 is an autophagic receptor which recognizes ubiquitinylated proteins and interacts with LC3 II at the forming autophagosome [37,42]. As for Beclin-1, results showed a great increase of its expression levels (6.8-fold) in pericytes from 3-months old APPswePS1dE9 mice compared to age-matched WT mice, whereasp62 levels were significantly decreased by 18.5- and 14-fold in pericytes prepared from brains of 6 and 12 months old APPswePS1dE9 mice, respectively *versus* transgenic mice at 3 months old age (Figure 2).

LC3, which is present in free cytoplasmic form as LC3 I and in LC3 II form when it is associated to phosphatidylethanolamine in the membrane of autophagosome, represents a useful marker of autophagy [38,42]. However, in our experimental conditions, we did not detect either isoforms in pericytes from brains of WT and transgenic mice at 6 and 12 months. At 3 months, levels of LC3 II increased in pericytes of APPswePS1dE9 mice compared to WT mice (3.65-fold, n=6 mice/group) with no modification of LC3 I expression (1.32 ± 0.60 and 1.70 ± 0.58 arbitrary units for WT and AD mice, respectively) and of the LC3 II/LC3 I ratio (data not shown).

Contrary to primary microglia [1], an inflammatory environment induced by IL-1$\beta$ did not influence the levels of autophagic parameters in primary pericytes (Figures 1 and 2). To complete these results, we performed CYTO-ID® and LYSO-ID® assays to determine if there is an autophagosome accumulation (CYTO-ID® and LYSO-ID® responses) or lysosome and lysosome-like organelle perturbations and phospholipidosis (LYSO-ID® response) at 3 months. No difference of the fluorescent intensity was observed in primary WT pericytes exposed to 400 pg/mL IL-1$\beta$ compared to vehicle-treated pericytes (Tables 1 and 2).

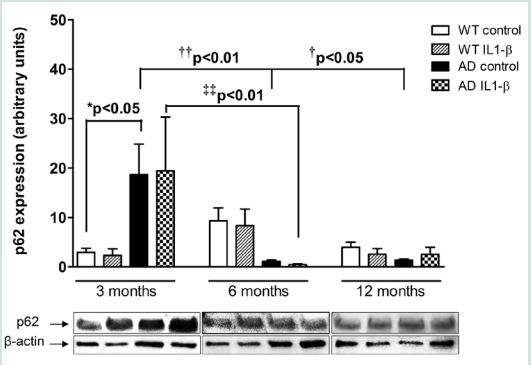


**Figure 2: Monitoring of p62 levels in primary pericytes of APPswePS1dE9 versus wild-type mice.** Pericytes prepared from brains of AD transgenic and WT mice were incubated in control (sterile water for injection as vehicle) and inflammatory conditions. Inflammation was induced by IL-1$\beta$ at 400 pg/mL during 48 hr. Western blots were performed with cell lysates of pericytes to detect p62 at 62kDa and β-actin at 42 kDa as a loading control in Western blot analysis. p62/$\beta$-actin ratios were calculated to determine the level of p62 expression in our experimental conditions. Results were expressed as mean ± SEM with arbitrary units with 4 to 16 mice per group. For WT and AD at 3 months, Kruskal-Wallis test p = 0.0062 and *p<0.05 compared to WT mice at 3 months by a Dunn's multiple comparison test. For AD control at 3, 6 and 12 months, Kruskal-Wallis test p = 0.0012 and †p<0.05,††p<0.01 compared to vehicle-treated AD mice at 3 months. For ADIL-1$\beta$ at 3, 6 and 12 months, Kruskal-Wallis test p = 0.0133‡‡ and p<0.01 compared to IL-1$\beta$-treated AD mice at 3 months by a Dunn's multiple comparison test.

**Monitoring of autophagy in mouse immortalized pericytes**

As indicated in the section of materials and methods, a murine WT pericyte line was obtained from primary pericytes extracted from the brains of 3-month-old B6C3F1 mice. This line allows us to obtain more biological material than primary cells and to reduce the number of animals in the experiments. It seemed useful to us to check whether this line responded similarly in their autophagic components, as mouse primary pericyte cultures in the same inflammatory environment. As shown in figure3, IL-1$\beta$ induced no modification of p62 (panels A,B) norLC3 I and LC3 II (panels A, C to E) expression levels. These results are also reinforced by the absence of changes in Beclin-1 expression and in mTOR and p70S6K activations in IL-1$\beta$-treated pericytes compared to control pericytes (Figure 4).

We also performed CYTO-ID® and LYSO-ID® experiments with the cell line. The fluorescent intensities were similarwhatever conditionto primary pericyte results (Tables 1 and 2).

## Discussion

Autophagy dysfunction was detected in brains of AD patients in 2005 [27]. Many authors then published data validating this alteration with a decrease in Beclin-1 level and its role in AD [36,43,44], a blockage of autophagic flow with dysfunction of lysosomal activities during AD in various experimental models of the disease [28,29,45]. Previous results showed that IL-1$\beta$ led to a blockage of autophagy in microglia, highly sensitive to this inflammatory stress [1]. Other authors demonstrated the relationship between autophagy and IL-1$\beta$ stress in AD [35]. Thus, chemical modulators of autophagy as well as gene therapy targeting autophagy related proteins offer great potential for AD treatment. A number of mTOR-dependent and independent autophagy modulators have been demonstrated to have positive effects in AD animal models and patients [28,46,47]. However, no data are available in the literature about autophagy at the level of BBB during aging or in AD, while the BBB dysfunction in AD is largely

**Table 1**: CYTO-ID® fluorescent intensity in mouse primary and immortalized pericytes.

| | Primary pericytes | Immortalized pericytes |
|---|---|---|
| **WT control** | 1.00± 0.01 | 1.00 ± 0.11 |
| **WT IL-1$\beta$** | 1.01± 0.02 | 0.95 ± 0.04 |

Pericytes were exposed to 400 pg/mL IL-1$\beta$ for 48hours and CYTO-ID® dual color reagent was added for 30 min at room temperature before being read by fluorescence using a Varioskan Flash microplate reader. Rapamycin (1 $\mu$M) as an inducer of autophagy and Chloroquine (10 $\mu$M) as a lysosomal inhibitor were included as positive controls. Ratios of fluorescent intensity for CYTO-ID® green detection reagent / fluorescent intensity for Hoechst 33342 nuclear stain were calculated and results were normalized to control. For Rapamycin and Chloroquine mixture, ratio was 1.32 ± 0.04.

**Table 2**: LYSO-ID® fluorescent intensity in mouse primary and immortalized pericytes.

| | Primary pericytes | Immortalized pericytes |
|---|---|---|
| **WT control** | 1.00± 0.20 | 1.00 ± 0.05 |
| **WT IL-1$\beta$** | 1.02± 0.25 | 1.20± 0.23 |

Pericytes were exposed to 400 pg/mL IL-1$\beta$ for 48 hours and LYSO-ID® dual color reagent was added for1 hour at room temperature before being read by fluorescence using a Varioskan Flash microplate reader. Verapamil (10 $\mu$M), a lysosome-perturbation agent, is provided as a positivecontrol. Ratios of fluorescent intensity for LYSO-ID® red dye signal /fluorescent intensity for the blue nuclear counterstain were calculated and results were normalized to control. For Verapamil, ratio was 1.32 ± 0.12.

ISSN: 2330-2178

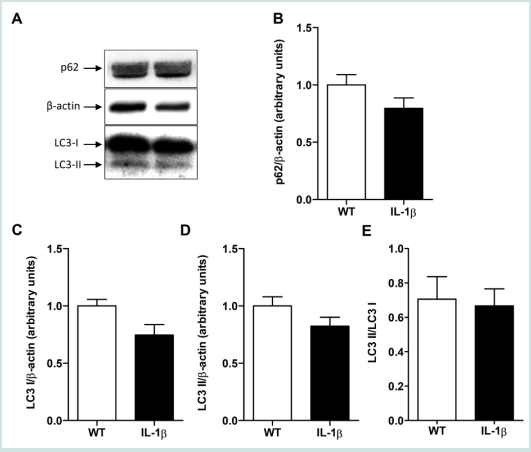


**Figure 3: Monitoring of p62 and LC3 levels in a murine pericyte cell line.** This cell line was prepared from B6C3F1 WT mice as indicated in method section. Pericytes were incubated either with 400 pg/mL of IL-1*β* or vehicle (sterile water) during 48 hr. Western blots were performed with cell lysates of pericytes to detect p62, LC3 I and LC3 II at 62, 16 and 14kDa, respectively and *β*-actin at 42 kDa as a loading control in Western blot analysis **(A)**. Ratios p62 or LC3 I or LC3 II/*β*-actin were calculated to determine the level of p62 **(B)**, LC3 I **(C)** and LC3 II **(D)** expression in our experimental conditions, respectively. Furthermore, ratios LC3 II/LC3 I were calculated to determine the translocation of LC3 I to LC3 II **(E)**. Results were expressed as mean ± SEM with arbitrary units of 13-15 independent experiments. Mann-Whitney test revealed no statistical difference (p value = 0.1178 and 0.7896 for p62 and LC3II/LC3I, respectively).

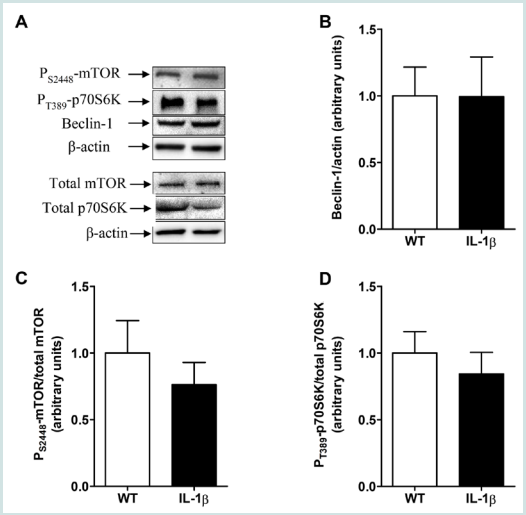


**Figure 4: Monitoring of mTOR signaling pathway and Beclin-1 in murine pericyte cell line.** This cell line was prepared from B6C3F1 WT mice as indicated in method section. Pericytes were incubated either with 400 pg/mL of IL-1*β* or vehicle (sterile water) during 48 hr. Western blots were performed with cell lysates of pericytes to detect mTOR, p70S6K and Beclin-1 at 289, 70 and 60kDa, respectively and *β*-actin at 42 kDa as a loading control in Western blot analysis **(A)**. Ratios mTOR, p70S6K and respective phosphorylated forms and Beclin-1/*β*-actin were calculated to determine the level of $P_{S2448}$-mTOR, total mTOR, $P_{T389}$-p70S6K and total p70S6K and Beclin-1 **(B)** expression in our experimental conditions. Furthermore, ratios $P_{S2448}$-mTOR/total mTOR and $P_{T389}$-p70S6K/total p70S6K were calculated to determine the activation of mTOR and p70S6K kinases, respectively **(C and D)**. Results were expressed as mean ± SEM with arbitrary units of 6 for mTOR and 15 independent experiments for Beclin-1 and p70S6K. Mann Whitney test revealed no statistical difference (p value = 0.5887, 0.4987, 0.8588 for P-mTOR/mTOR, P-p70S6K/p70S6k and Beclin-1, respectively).

described [16,19,48]. Studies showed that autophagy was enhanced in middle cerebral artery occlusion/reperfusion and oxygen-glucose deprivation/reperfusion experiments. In these contexts, autophagy would have either protective or detrimental roles in BBB function and ECs [49]. Autophagy could decrease BBB permeability and ischemic damage, but also induce apoptosis and cell death [49].

For the first time, we observed that primary pericytes purified from brain of APPswePS1dE9 mice showed an increase of Beclin-1, p62 and LC3 II expression levels at 3 months compared to age-matched WT mice. Then, levels of these autophagy markers were like those of WT mice at 6 and 12 months. These autophagy changes in primary pericytes at 3 months revealed an activation of autophagy with no accumulation of autophagosomes or lysosomes, indicating no impairment of autophagic flow. At 3 months, APPswePS1dE9 mice displayed no amyloid deposits but produced intracellular A*β*. At 6 months, extracellular A*β* was mainly depicted in cerebral parenchyma [50]. It is known that A*β*34, a marker for amyloid clearance in AD, was predominantly detectable in a subset of brain capillaries associated with pericytes, while in later clinically diagnosed AD stages, this pericyte-associated A*β*34 immuno reactivity was largely lost [51].BBB-associated pericytes clear A*β* aggregates *via* an LRP1-dependentApoE isoform-specific mechanism with apoE4 disrupting A*β* clearance compared to apoE3 [52,53]. One may propose that enhanced autophagy at 3 months in these transgenic AD mice could be explained by the proteolytic degradation of A*β*40 and A*β*42.

Contrary to primary microglia [1,35], IL-1*β*-induced inflammation did not modify expression of Beclin-1, p62 and LC3 II in primary WT or AD pericytes. Furthermore, no modification of autophagy markers and of the mTOR signaling pathway was observed in pericyte cell line. The concentration of IL-1*β* (400 pg/mL) may be questioned. It was chosen based on our previous work on microglia where higher concentrations led to cell death [1]. Recently, authors showed that inhibition of the NLRP3-contained inflammasome reducedpericyte cell coverage and decreased protein level of PDGFR *β* contrary to IL-1*β* which is the major product of NLRP3 [54]. Concentrations of IL-1*β* were 12.5 to 125 times higher than those used in our study and authors concluded that NLRP3 activation maintained healthy pericytes in the brain and warned of therapeutic strategies to inhibit inflammasome [54]. Besides the relationship between NLRP3 and pericyte survival, we can ask the question of the resistance of the pericytes to this inflammatory stress at the autophagic level because inflammation generally stimulates autophagy, or even blocks the flow and leads to cell death.. However, no data were available in the literature about the autophagic status of pericytes in AD. In stroke and cocaine exposure, authors showed that in pericytes autophagy was regulated by the sigma-1 receptor signaling pathway [55,56].

For the first time, we show that autophagy is activated in primary pericytes at 3 months. Proteolytic degradation of amyloid peptides may explain this activation. In addition, inflammation has no impact on autophagic flow under our experimental conditions, but data in the literature highlighted that NLRP3 activation might be essential to maintain pericytes in the healthy brain. Further experiments will be needed to understand the relationship between autophagy and inflammation in pericytes.

ISSN: 2330-2178

## Acknowledgement

This work has benefited from the facilities and expertise of PREBIOS platform (University of Poitiers, France). The authors thank Damien Chassaing (EA3808 Neurovascular Unit and Cognitive Disorders, University of Poitiers) for his technical skills and the Fondation Claude Pompidou for financial.

## References

1. François A, Terro F, Janet T, Bilan AR, Paccalin M, et al. (2013) Involvement of interleukin-1$\beta$ in the autophagic process of microglia: relevance to Alzheimer's disease. J Neuroinflammation 10: 915.
2. François A, Terro F, Quellard N, Fernandez B, Chassaing D, et al. (2014) Impairment of autophagy in the central nervous system during lipopolysaccharide-induced inflammatory stress in mice. Mol Brain 7: 56.
3. François A, Rioux Bilan A, Quellard N, Fernandez B, Janet T, et al. (2014) Longitudinal follow-up of autophagy and inflammation in brain of APPswePS1dE9 transgenic mice. J Neuroinflammation 11: 139.
4. Anwar S, Rivest S (2020) Alzheimer's disease: microglia targets and their modulation to promote amyloid phagocytosis and mitigate neuroinflammation. Expert Opin Ther Targets 24: 331-344.
5. Sengillo JD, Winkler EA, Walker CT, Sullivan JS, Johnson M, et al. (2013) Deficiency in Mural Vascular Cells Coincides with Blood-Brain Barrier Disruption in Alzheimer's Disease: Pericytes in Alzheimer's Disease. Brain Pathol 23: 303-310.
6. Sweeney MD, Zhao Z, Montagne A, Nelson AR, Zlokovic BV (2019) Blood-Brain Barrier: From Physiology to Disease and Back. Physiol Rev 99: 21-78.
7. Montagne A, Nation DA, Sagare AP, Barisano G, Sweeney MD, et al. (2020) APOE4 leads to blood-brain barrier dysfunction predicting cognitive decline. Nature. 581: 71-76.
8. Sweeney MD, Sagare AP, Pachicano M, Harrington MG, Joe E, et al. (2020) A novel sensitive assay for detection of a biomarker of pericyte injury in cerebrospinal fluid. Alzheimers Dement 16: 821-830.
9. Sá-Pereira I, Brites D, Brito MA (2012) Neurovascular Unit: a Focus on Pericytes. Mol Neurobiol 45: 327-347.
10. Langen UH, Ayloo S, Gu C (2019) Development and Cell Biology of the Blood-Brain Barrier. Annu Rev Cell Dev Biol 35: 591-613.
11. Bennett HC, Kim Y (2021) Pericytes Across the Lifetime in the Central Nervous System. Front Cell Neurosci 15: 627291.
12. Daneman R, Zhou L, Kebede AA, Barres BA (2010) Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature 468: 562-566.
13. Winkler EA, Bell RD, Zlokovic BV (2011) Central nervous system pericytes in health and disease. Nat Neurosci 14: 1398-1405.
14. Sweeney MD, Ayyadurai S, Zlokovic BV (2016) Pericytes of the neurovascular unit: key functions and signaling pathways. Nat Neurosci 19: 771-783.
15. Hellström M, Gerhardt H, Kalén M, Li X, Eriksson U, et al. (2001) Lack of Pericytes Leads to Endothelial Hyperplasia and Abnormal Vascular Morphogenesis. J Cell Biol 153: 543-554.
16. Sagare AP, Bell RD, Zlokovic BV (2012) Neurovascular Dysfunction and Faulty Amyloid -Peptide Clearance in Alzheimer Disease. Cold Spring Harb Perspect Med 2: a011452-a011452.
17. Sagare AP, Bell RD, Zhao Z, Ma Q, Winkler EA, et al. (2013) Pericyte loss influences Alzheimer-like neurodegeneration in mice. Nat Commun 4: 2932.
18. Procter TV, Williams A, Montagne A (2021) Interplay between brain pericytes and endothelial cells in dementia. Am J Pathol 191: 1917-1931.
19. Winkler EA, Sagare AP, Zlokovic BV (2014) The Pericyte: A Forgotten Cell Type with Important Implications for Alzheimer's Disease?: Pericytes in Alzheimer's Disease. Brain Pathol 24: 371-386.
20. Nortley R, Korte N, Izquierdo P, Hirunpattarasilp C, Mishra A, et al. (2019) Amyloid $\beta$ oligomers constrict human capillaries in Alzheimer's disease via signaling to pericytes. Science 365: eaav9518.
21. Montagne A, Barnes SR, Sweeney MD, Halliday MR, Sagare AP, et al. (2015) Blood-Brain Barrier Breakdown in the Aging Human Hippocampus. Neuron 85: 296-302.
22. Zenaro E, Piacentino G, Constantin G (2017) The blood-brain barrier in Alzheimer's disease. Neurobiol Dis 107: 41-56.
23. Soto-Rojas LO, Pacheco-Herrero M, Martínez-Gómez PA, Campa-Córdoba BB, Apátiga-Pérez R, et al. (2021) The Neurovascular Unit Dysfunction in Alzheimer's Disease. Int J Mol Sci 22: 2022.
24. Qian X, Song X, Liu X, Chen S, Tang H-U (2021) Inflammatory pathways in Alzheimer's disease mediated by gut microbiota. Ageing Res Rev 68: 101317.
25. Heneka MT, Carson MJ, Khoury JE, Landreth GE, Brosseron F, et al. (2015) Neuroinflammation in Alzheimer's disease. Lancet Neurol 14: 388-405.
26. Webers A, Heneka MT, Gleeson PA (2020) The role of innate immune responses and neuroinflammation in amyloid accumulation and progression of Alzheimer's disease. Immunol Cell Biol 98: 28-41.
27. Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, et al. (2005) Extensive Involvement of Autophagy in Alzheimer Disease: An Immuno-Electron Microscopy Study. J Neuropathol Exp Neurol 64: 113-122.
28. Li Q, Liu Y, Sun M (2017) Autophagy and Alzheimer's Disease. Cell Mol Neurobiol 37: 377-388.
29. Zare-shahabadi A, Masliah E, Johnson GVW, Rezaei N (2015) Autophagy in Alzheimer's disease. Rev Neurosci 26: 385-395.
30. Onyango IG, Jauregui GV, Čarná M, Bennett JP, Stokin GB (2021) Neuroinflammation in Alzheimer's Disease. Biomedicines 9: 524.
31. Blamire AM, Anthony DC, Rajagopalan B, Sibson NR, Perry VH, et al. (2000) Interleukin-1β-Induced Changes in Blood-Brain Barrier Permeability, Apparent Diffusion Coefficient, and Cerebral Blood Volume in the Rat Brain: A Magnetic Resonance Study. J Neurosci 20: 8153-8159.
32. Rustenhoven J, Jansson D, Smyth LC, Dragunow M (2017) Brain Pericytes As Mediators of Neuroinflammation. Trends Pharmacol Sci 38: 291-304.
33. Kovac A, Erickson MA, Banks WA (2011) Brain microvascular pericytes are immunoactive in culture: cytokine, chemokine, nitric oxide, and LRP-1 expression in response to lipopolysaccharide. J Neuroinflammation 8: 139.
34. Navarro R, Compte M, Álvarez-Vallina L, Sanz L (2016) Immune Regulation by Pericytes: Modulating Innate and Adaptive Immunity. Front Immunol 7: 1-10.
35. Álvarez-Arellano L, Pedraza-Escalona M, Blanco-Ayala T, Camacho-Concha N, Cortés-Mendoza J, et al. (2018) Autophagy impairment by caspase-1-dependent inflammation mediates memory loss in response to $\beta$-Amyloid peptide accumulation. J Neurosci Res 96: 234-246.
36. Jaeger PA, Wyss-Coray T (2010) Beclin 1 Complex in Autophagy and Alzheimer Disease. Arch Neurol 67: 67: 1181-1184.
37. Bitto A, Lerner CA, Nacarelli T, Crowe E, Torres C, Sell C (2014) p62/SQSTM1 at the interface of aging, autophagy, and disease. AGE 36: 9626.
38. Tanida I, Ueno T, Kominami E (2008) LC3 and Autophagy. In (Ed: Deretic V). Autophagosome and Phagosome. Humana Press: Totowa, NJ. Pp: 77-88.
39. Vérité J, Janet T, Chassaing D, Fauconneau B, Rabeony H, et al. (2018) Longitudinal chemokine profile expression in a blood-brain barrier model from Alzheimer transgenic versus wild-type mice. J Neuroinflammation 15: 182.
40. Burek M, Salvador E, Förster CY (2012) Generation of an Immortalized Murine Brain Microvascular Endothelial Cell Line as an In Vitro Blood Brain Barrier Model. J Vis Exp (66): e4022.
41. Kang R, Zeh HJ, Lotze MT, Tang D (2011) The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ 18: 571-580.

ISSN: 2330-2178

42. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, et al. (2016) Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 12: 1-222.

43. Rocchi A, Yamamoto S, Ting T, Fan Y, Sadleir K, et al. (2017) A Becn1 mutation mediates hyperactive autophagic sequestration of amyloid oligomers and improved cognition in Alzheimer's disease. PLOS Genet 13: e1006962.

44. Lucin KM, O'Brien CE, Bieri G, Czirr E, Mosher KI, et al. (2013) Microglial Beclin 1 Regulates Retromer Trafficking and Phagocytosis and Is Impaired in Alzheimer's Disease. Neuron 79: 873-886.

45. Whyte LS, Lau AA, Hemsley KM, Hopwood JJ, Sargeant TJ (2017) Endo-lysosomal and autophagic dysfunction: a driving factor in Alzheimer's disease? J Neurochem 140: 703-717.

46. Papp D, Kovács T, Billes V, Varga M, Tarnóci A, et al. (2016) AUTEN-67, an autophagy-enhancing drug candidate with potent antiaging and neuroprotective effects. Autophagy 12: 273-286.

47. Wang W-Y, Tan M-S, Yu J-T, Tan L (2015) Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann Transl Med 3: 136.

48. Chakraborty A, de Wit NM, van der Flier WM, de Vries HE (2017) The blood brain barrier in Alzheimer's disease. Vascul Pharmacol 89: 12-18.

49. Kim K-A, Shin D, Kim J-H, Shin Y-J, Rajanikant GK, et al. (2018) Role of Autophagy in Endothelial Damage and Blood-Brain Barrier Disruption in Ischemic Stroke. Stroke 49: 1571-1579.

50. Garcia-Alloza M, Robbins EM, Zhang-Nunes SX, Purcell SM, Betensky RA, et al. (2006) Characterization of amyloid deposition in the APPswe/PS1dE9 mouse model of Alzheimer disease. Neurobiol Dis 24: 516-524.

51. Kirabali T, Rigotti S, Siccoli A, Liebsch F, Shobo A, et al. (2019) The amyloid-$\beta$ degradation intermediate A$\beta$34 is pericyte-associated and reduced in brain capillaries of patients with Alzheimer's disease. Acta Neuropathol Commun 7: 194.

52. Ma Q, Zhao Z, Sagare AP, Wu Y, Wang M, Owens NC, et al. (2018) Blood-brain barrier-associated pericytes internalize and clear aggregated amyloid-$\beta$42 by LRP1-dependent apolipoprotein E isoform-specific mechanism. Mol Neurodegener 13: 57.

53. Bell RD, Winkler EA, Singh I, Sagare AP, Deane R, et al. (2012) Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. Nature 485: 512-516.

54. Quan W, Luo Q, Tang Q, Furihata T, Li D, et al. (2020) NLRP3 Is Involved in the Maintenance of Cerebral Pericytes. Front Cell Neurosci 14: 276.

55. Zhang Y, Zhang X, Wei Q, Leng S, Li C, et al. (2021) Correction to: Activation of Sigma-1 Receptor Enhanced Pericyte Survival Via the Interplay between Apoptosis and Autophagy: Implications for Blood-Brain Barrier Integrity in Stroke. Transl Stroke Res 12: 689-690.

56. Sil S, Niu F, Tom E, Liao K, Periyasamy P, et al. (2019) Cocaine Mediated Neuroinflammation: Role of Dysregulated Autophagy in Pericytes. Mol Neurobiol 56: 3576-3590.

57. Couturier J, Paccalin M, Morel M, Terro F, Milin S, et al. (2011) Prevention of the $\beta$-amyloid peptide-induced inflammatory process by inhibition of double-stranded RNA-dependent protein kinase in primary murine mixed co-cultures. J Neuroinflammation 8: 72.