Annexin A5 prevents amyloid-β-induced toxicity in choroid plexus: implication for Alzheimer’s disease

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In Alzheimer’s disease (AD) amyloid-β (Aβ) deposits may cause impairments in choroid plexus, a specialised brain structure which forms the blood–cerebrospinal fluid (CSF) barrier. We previously carried out a mass proteomic-based study in choroid plexus from AD patients and we found several differentially regulated proteins compared with healthy subjects. One of these proteins, annexin A5, was previously demonstrated implicated in blocking Aβ-induced cytotoxicity in neuronal cell cultures. Here, we investigated the effects of annexin A5 on Aβ toxicity in choroid plexus. We used choroid plexus tissue samples and CSF from mild cognitive impairment (MCI) and AD patients to analyse Aβ accumulation, cell death and annexin A5 levels compared with control subjects. Choroid plexus cell cultures from rats were used to analyse annexin A5 effects on Aβ-induced cytotoxicity. AD choroid plexus exhibited progressive reduction of annexin A5 levels along with progressive increased Aβ accumulation and cell death as disease stage was higher. On the other hand, annexin A5 levels in CSF from patients were found progressively increased as the disease stage increased in severity. In choroid plexus primary cultures, Aβ administration reduced endogenous annexin A5 levels in a time-course dependent manner and simultaneously increased extracellular annexin A5 levels. Annexin A5 addition to choroid plexus cell cultures restored the Aβ-induced impairments on autophagy flux and apoptosis in a calcium-dependent manner. We propose that annexin A5 would exert a protective role in choroid plexus and this protection is lost as Aβ accumulates with the disease progression. Then, brain protection against further toxic insults would be jeopardised.

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia in elderly1,2. The imbalance between amyloid-β (Aβ) peptide generation from amyloid precursor protein and clearance, induces its accumulation, aggregation, and deposition in the brain, which is thought to be an early and main pathogenic event in AD2,4. Besides accumulation and production in specific parenchyma areas, such as hippocampus and cortex, and blood vessels5, Aβ also accumulates in choroid plexus6,7. More specifically it was demonstrated that Aβ may be additionally produced and degraded in choroid plexus along with other brain areas6–10. Choroid plexus is a monolayer of specialised epithelial cells in the brain ventricles forming the blood – cerebrospinal fluid (CSF) barrier. The choroid plexus main function is to produce and secrete CSF (CSF turnover)

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protecting therefore CSF against external toxic insults. Works carried out by Jean Marie Serot et al., found choroid plexus functions of secretion, synthesis, and transport were deteriorated in AD as they observed morphological modifications including epithelial atrophy, fibrosis and calcifications of stroma, and thickened basement membrane. These impairments resulted in lower turnover and CSF stasis, reduced transthyretin levels, a sequestering protein, synthesised by choroid plexus, and oligomerisation and subsequent accumulation of Aβ (reviewed in[12]). Also, it was demonstrated that Aβ peptides accumulation in choroid plexus is largely responsible for an increased level of oxidative stress and cell death.[7,13] A decreased activity of enzymes involved in oxidative phosphorylation and mitochondrial activity, may also contribute to impair protein synthesis in choroid plexus. The observed decrease in the choroid plexus functional activity would be partially correlated with reduced protein secretion and mentioned CSF renewal, which may be involved in the initiation and progression of AD[15]. Although the detailed mechanism of Aβ-induced toxicity in choroid plexus is unclear, perturbation of Ca2+-homeostasis, and destabilisation of cellular metabolism by pronounced membrane permeabilisation might likely play an important role.

Annexin A5, a Ca2+-regulated, phospholipid-binding protein belongs to the annexins superfamily. This protein is abundantly expressed in a wide range of tissues[15] with intra- and extracellular locations[16]. Although annexin A5 is extensively used as an indicator of early apoptosis, it was demonstrated that annexin A5 exerts protective functions including inhibition of proinflammatory response and improvement of cardiac function and survival during endotoxemia in mice[17]. Also, annexin A5 was found interacting with amyloidogenic proteins reducing its toxicity in neurodegenerative diseases and type II diabetes mellitus[18]. It was also shown annexin A5 provided protection against Aβ cytotoxicity, and it was proposed that this effect occurs by competitive interaction with phosphatidylserine (PS) on the membrane surface[19–21]. Annexins are predominately located within the cell, where they mediate cellular processes such as exocytosis and endocytosis, membrane structure and generation of lipid rafts, but also extracellular roles such as inflammation[22]. Particularly, it was reported annexin A5 has antithrombotic properties, reducing vascular inflammation and improving endothelial function[24–26]. Annexin A5 was proposed as biomarker in heart injury[27] and also in nephrotic syndrome[28]. Moreover, annexin A5 was associated with various neuropathological conditions. Levels of annexin A5 were reported reduced in CSF from Parkinson's disease (PD) patients[29]. It was suggested that such reduction resulted as consequence of protein consumption during neuronal apoptosis[29]. Plasma levels of annexin A5 were found significantly higher in AD patients compared with healthy individuals[30]. These works suggested a defensive function for annexin A5 in these neurodegenerative diseases based on its interacting role with amyloidogenic proteins, islet amyloid polypeptides and α-synuclein inclusions by reducing the toxicity of these proteins and aggregates[31].

Using a mass proteomic based study, we previously found changes in annexin A5 protein levels in choroid plexus from AD patients[31]. The present study was focused on examining the potential effects of annexin A5 on Aβ toxicity in choroid plexus. For that purpose, we analysed choroid plexus tissue samples and CSF from MCI and AD patients, and choroid plexus primary cultures. Here, we show annexin A5 levels in CSF were found progressively increased as the disease stage increased in severity. Simultaneously, annexin A5 levels were found decreased with the disease severity in choroid plexus, in opposite way Aβ burden and cell death. We also show that annexin A5 may play a protective role in Aβ-induced cell toxicity in choroid plexus by a Ca2+-dependent mechanism.

Results

Annexin A5 levels are increased in CSF from AD patients. By doing a mass proteomic based study, we previously found annexin A5 protein levels were differentially expressed in choroid plexus from AD patients compared with healthy controls[31]. Here, we show annexin A5 levels were significantly increased in CSF from MCI and moderate AD patients determined by ELISA (Fig. 1a). We also checked by Western Blot annexin A5 levels in choroid plexus from brain donors with AD pathology and healthy subjects. Annexin A5 protein levels were significantly lower in Braak stages III/IV and stages V/VI compared with control cases (Fig. 1b). In contrast with our previous work[31], by increasing the number of samples here we increased the variability losing the significance significantly lower in Braak stages III/IV and V/VI compared with control samples (Fig. 1d). The effects of Aβ accumulation determined in choroid plexus using a Cell Death Detection ELISA kit resulted in progressive increased cell death in AD subjects that was significantly higher at Braak stages V/VI compared with healthy donors (Fig. 2c). These results were consistent with the previously reported findings showing deleterious effects of Aβ in choroid plexus from AD subjects and the AD transgenic APP/PS1 mouse model[17]. It is important to note that the observed progressive Aβ accumulation and cell death in choroid plexus match with the reduced annexin A5 levels (Fig. 1b), more evident at late AD stages (III-VI) (Fig. 2d).

Aβ-related choroid plexus cell death. AD-related pathology was confirmed analysing the Aβ load in choroid plexus by immunohistochemistry using an anti-Aβ antibody (Fig. 2a). The progressive pattern of Aβ accumulation in choroid plexus was estimated using an Aβ1–42 human specific ELISA kit (Fig. 2b). Aβ1–42 burden was significantly higher at Braak stages III/IV and stages V/VI when compared with control cases (Fig. 2b). The effects of Aβ accumulation determined in choroid plexus using a Cell Death Detection ELISA kit resulted in progressive increased cell death in AD subjects that was significantly higher at Braak stages V/VI compared with healthy donors (Fig. 2c). These results were consistent with the previously reported findings showing deleterious effects of Aβ in choroid plexus from AD subjects and the AD transgenic APP/PS1 mouse model[17]. It is important to note that the observed progressive Aβ accumulation and cell death in choroid plexus match with the reduced annexin A5 levels (Fig. 1b), more evident at late AD stages (III-VI) (Fig. 2d).

Aβ induces Annexin A5 release outside choroid plexus cells. Annexin A5 may be secreted extracellularly and increased annexin A5 levels were previously found in the supernatant of Aβ1–42-treated neuronal cells[32]. Hence, we asked if changes in annexin A5 levels between choroid plexus and CSF could be related to Aβ overload in choroid plexus. We used rat primary cultures of epithelial choroid plexus cells treated with oligomerised Aβ1–42 (10µM) at 6, 12, 24 and 48 hours. Aβ exposure in choroid plexus cells resulted in reduced annexin A5 protein

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levels in a time course-dependent manner (Fig. 3a). This reduction was significant compared with untreated cells 24 and 48 hours after Aβ treatment (Fig. 3a). Conversely, Aβ induced a time course-dependent increase of annexin A5 in the extracellular medium (Fig. 3b). Such annexin A5 increase in supernatant was clearly significant 24 and 48 hours after Aβ treatment (Fig. 3b).

Annexin A5 restores the Aβ-induced impairments on autophagy. A number of studies demonstrate autophagy is disrupted at one point in the progression of AD. This pathway may degrade accumulated aberrant proteins and peptides such as Aβ. Results above could suggest that annexin A5 may be beneficial in choroid plexus from AD patients; therefore we investigated the effect of annexin A5 on autophagy. Analysis of autophagy markers in postmortem tissue from AD and healthy donors by immunoblotting, showed significantly increased LC3-II levels in choroid plexus from all AD stages including early AD (Fig. 4a) and significantly increased p62 levels at Braak stages I-IV (Fig. 4b). These results may suggest both, increased autophagy induction and disrupted autophagosome–lysosome fusion. Then we investigated annexin A5 effects on Aβ-disrupted autophagy flux in choroid plexus cultures from rats. Cells were treated with and without annexin A5 for 1 hour and then exposed

Figure 1. CSF and choroid plexus annexin A5 protein levels were differentially expressed in AD patients. (a) Scatter plot showing CSF levels of annexin A5 protein in healthy donors and clinically diagnosed MCI and AD patients determined by ELISA. Annexin A5 levels in CSF samples were found higher in MCI and AD patients compared with controls (*p < 0.05, **p < 0.01; control n = 11; MCI n = 6; Mild AD n = 8; Moderate AD n = 6). In all cases, each individual value is shown along with the mean ± SEM per group. (b) Scatter plot showing annexin A5 protein levels in choroid plexus from AD and healthy donors. Annexin A5 levels were significantly reduced in choroid plexus from Braak stages III/IV and V/VI compared with control samples (*p < 0.05; control n = 22; AD I-II n = 21; AD III-IV n = 12; AD V-VI n = 13). In all cases, each individual value is shown along with the mean ± SEM per group. Bottom images show representative western blots. These images were cropped from full-length blots and they are shown in the Supplementary Information section.
to oligomerised Aβ_{42} to avoid extracellular Aβ sequestering by annexin A5, thereby preventing the toxicity. Immunoblotting revealed that Aβ_{42} exposure resulted in significantly elevated LC3-II and p62 levels compared with untreated cells suggesting autophagosome accumulation (Fig. 4c,d, respectively). Annexin A5 treatment in cells restored the LC3-II and p62 levels being similar to the obtained values in untreated or cells treated with annexin A5 only (Fig. 4c,d, respectively). In summary, the above results could indicate annexin A5 abolished the Aβ-induced impairments on autophagy in choroid plexus cells.

**Annexin A5 protects against Aβ-induced apoptosis.** It was previously shown annexin A5 inhibited Aβ toxicity in neuronal cell cultures\cite{16,17,19,20,34,37}. We further investigated annexin A5 effect on Aβ-induced toxicity in choroid plexus cells. As Aβ-induced autophagy may precede Aβ-induced toxicity we tested the biological role of annexin A5 on cell viability carrying out bioactivity assays with choroid plexus cell cultures. Cells were previously treated with and without annexin A5 for 1 hour and then exposed to oligomerised Aβ_{42} (10 μM) for 48 hours (Fig. 5). Aβ_{42} induced 25% decrease in cell viability in choroid plexus cultures, as assessed by cell counting kit (CCK) assay, compared with control (Fig. 5a). Annexin A5 completely prevented cell loss when was added prior to Aβ_{42} (Fig. 5a). The reduction in...
pared with either untreated or cells treated with annexin A5 (Fig. 6b). Treatment of Aβ cells with annexin A5 prevented the reduction in the ΔΨ and the amount of Ca2+ levels of the untreated cell or cells treated with annexin A5 (Fig. 6d, right panel). Together, these results confirmed ΔΨ linked to mitochondria and mitochondrial health and function is reflected in the SEM.

Annexin A5 attenuates Aβ-induced impairments on mitochondrial function and [Ca2+] homeostasis in choroid plexus epithelial cells. Both, apoptosis and autophagy are two cellular processes closely linked to mitochondria and mitochondrial health and function is reflected in the ΔΨm. By using the fluorescent indicator TMRM, we analysed the ΔΨm (Fig. 6a). This dye accumulates in active mitochondria with intact membrane potentials, therefore the more fluorescence, the more mitochondrial health and quality. A significant decrease in ΔΨm was observed in Aβ-exposed choroid plexus cells reducing the TMRM signal to 74 ± 3% compared with either untreated or cells treated with annexin A5 (Fig. 6b). Treatment of Aβ-exposed choroid plexus cells with annexin A5 prevented the reduction in the ΔΨm, as TMRM signal showed similar values to untreated cells or cells with annexin A5 (Fig. 6b). Mitochondria are master regulators of Ca2+ homeostasis that simultaneously is linked to autophagy and apoptotic cell death regulation. Because annexin A5 is a Ca2+ binding protein we hypothesised that the protective role of annexin A5 could be related to its Ca2+ binding capacity. This could be supported by findings in which was demonstrated Aβ-induced toxicity in neurons involved perturbation of Ca2+ homeostasis. We analysed Ca2+ flux upon physiological Ca2+ stimuli in choroid plexus cells incubated for 24 hours with oligomerised Aβ1-42 in absence or presence of annexin A5 (Fig. 6c). Ca2+ signal was evaluated using fura-2. Purinergic receptors in epithelial choroid plexus cells were stimulated with ATP allowing the massive release of Ca2+ from the endoplasmic reticulum (ER) to the cytosol which was ratiometrically quantified (Fig. 6c, top panels). After recovery, mitochondrial Ca2+ uptake was analysed by adding the uncoupler FCCP and the amount of Ca2+ was quantified (Fig. 6c, top panels). Upon physiological Ca2+ stimuli, changes in ΔΨm were simultaneously analysed using Rh123 and the fluorescence was also quantified (dequench mode, Fig. 6c, bottom panels). ATP application to Aβ-treated choroid plexus cells was associated to loss of ΔΨm as Rh123 signal increased by 23% before FCCP addition. Annexin A5 treatment to Aβ-incubated choroid plexus cells prevented the mitochondrial depolarisation as Rh123 fluorescence did not increase after ATP addition. We found that Aβ1-42 incubation resulted in a significantly higher Ca2+ release from the ER in response to ATP in choroid plexus cells (Fig. 6d, left panel). Simultaneous incubation with Aβ and annexin A5 restored the ATP-induced Ca2+ release from the ER in choroid plexus cells resulting in a smaller Ca2+ signal similar to the obtained in untreated or only annexin A5 treated cells (Fig. 6d, left panel). Mitochondrial Ca2+ uptake after FCCP addition was also higher in Aβ treated cells compared with untreated or only annexin A5 treated cells (Fig. 6d, right panel). Treatment of choroid plexus cells with annexin A5 under Aβ presence restored the mitochondrial Ca2+ uptake reaching similar levels of the untreated cell or cells treated with annexin A5 (Fig. 6d, right panel). Together, these results confirmed cell viability on choroid plexus cells and the protective role of annexin A5 was studied analysing apoptotic cell death using the LIVE/DEAD Viability/Cytotoxicity Kit (Fig. 5b,c). 48 hours after incubation with Aβ, an increase of apoptotic cell death in choroid plexus cultures was found (Fig. 5b,c). The presence of annexin A5 attenuated the Aβ-induced apoptosis, recovering the amount of living cells and reducing the cell loss (Fig. 5b,c).
annexin A5 exerts its protective role on Aβ-induced autophagy and apoptotic cell death in choroid plexus in a Ca2+-dependent manner controlled by mitochondria.
Figure 5. Annexin A5 restores the Aβ-induced cell viability reduction and apoptosis. (a) Epithelial choroid plexus cell viability after incubation with and without oligomerised Aβ42 (10 µM) for 48 hours in absence or presence of annexin A5. A significant decrease in cellular viability was found in Aβ42 incubated cells compared with untreated cells or cells treated with annexin A5 only. Co-administration of annexin A5 (15 µl/ml) and Aβ42 (10 µM) restored cell viability in choroid plexus epithelial cell cultures (*p < 0.05 versus untreated cells; ####p < 0.0001 versus Aβ-treated cells; n = 7). In all cases, data represents mean ± SEM. (b) Apoptotic cell death was analysed using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes). Fluorescent images of epithelial choroid plexus cell culture show living cells (green) and apoptotic cells (red). Cells were incubated with and without oligomerised Aβ42 (10 µM), in absence and presence of annexin A5 (15 µl/ml). Scale bar = 44 μm. (c) Counting alive (green) and apoptotic (red) cells shows that Aβ42 incubation induced a significant increase of apoptotic cells compared with untreated or annexin A5 treated cells. Co-administration annexin A5 and Aβ42 reduced the number of apoptotic cells (**p < 0.01 versus Aβ42-treated cells; ***p < 0.001 versus untreated cells; n = 4). In all cases, data represents mean ± SEM.
Figure 6. Annexin A5 restores mitochondrial depolarisation in Aβ-treated choroid plexus cultures in a Ca²⁺-dependent manner. (a) Representative images of tetramethyl-rhodamine methylester (TMRM) fluorescence used in redistribution mode (40 nM) in choroid plexus cultures incubated with and without oligomerised Aβ42 (10 µM), in absence and presence of annexin A5 (15 µl/ml). (b) Annexin A5 restored the Aβ-induced mitochondrial depolarisation. Data were normalised to untreated cells and are represented as mean ± SEM from at least three independent experiments. (*p < 0.05 versus untreated cells; **p < 0.01 versus Aβ-treated cells; n = 4). (c) Traces showing changes-over time in fura-2 (upper panels) and simultaneous rhodamine 123 (Rh123) (bottom panels) fluorescence in choroid plexus epithelial cells upon physiological Ca²⁺ stimuli. Physiological Ca²⁺ was induced by addition of ATP (100 µM). Following Ca²⁺ release from ER, FCCP (1 µM) was added to obtain the maximal mitochondrial depolarisation allowing mitochondrial Ca²⁺ release. Upon stimulation of choroid plexus cells with ATP, Ca²⁺ stored in the ER was released and profound mitochondrial depolarisation was found in Aβ-incubated cells as shown by the increase in the Rh123 signal (iii). This effect was...
not observed in untreated cells (i) or annexin A5-treated cells only (ii). Annexin A5 treatment prevented the mitochondrial depolarisation when Ca\(^{2+}\) stimulus was added (iv). (d) Histograms showing ER Ca\(^{2+}\) (left panel) and mitochondrial Ca\(^{2+}\) levels (right panel) after addition of ATP and FCCP respectively as explained above. ER Ca\(^{2+}\) in choroid plexus cells incubated with Aβ were higher compared with untreated cells or annexin A5-treated cells only. Annexin A5 co-treatment restored the Aβ-induced increase of ER Ca\(^{2+}\) levels. Mitochondrial Ca\(^{2+}\) in Aβ-incubated choroid plexus cells was significantly higher compared with untreated cells or annexin A5-treated cells only. These levels were restored upon co-incubation with annexin A5. Data were normalised to untreated cells and are represented as mean ± SEM from at least three independent experiments. (***p < 0.0001 versus untreated cells; **p < 0.0001 versus Aβ-treated cells; n = 4).

**Discussion**

Annexins are widely distributed among tissues and their ubiquitous distribution suggests they have important roles in cell biology. In particular, annexin A5 has been found exerting anti-inflammatory, antiocoagulant and anti-apoptotic functions\(^{30-40}\). In endothelial cells, annexin A5 was able to protect against vascular inflammation contributing to protect barrier integrity\(^{30}\). Expression of other annexins, annexin A1, A4 and A6, was described in choroid plexus\(^{47,48}\), but little is known about expression and role of annexin A5 in this brain structure. Here we report increased annexin A5 levels in CSF from clinically diagnosed MCI and AD patients and simultaneous reduced annexin A5 levels in choroid plexus from AD brains. These findings are accompanied by increased Aβ overload in choroid plexus related to the disease severity and significantly altered autophagic flux and increased cell death at late AD stages. In vitro, we also demonstrate that annexin A5 extracellular release in choroid plexus cells could be Aβ-overload dependent. We propose that Aβ in choroid plexus may induce increased autophago-some accumulation. Such effects result finally in increased cell death and annexin A5 down-regulation. On the other hand, annexin A5 protects against Aβ toxicity restoring the Aβ-induced Ca\(^{2+}\) homeostasis dysregulation linked to the mitochondrial health and function. Together, our findings suggest that annexin A5 plays a crucial role in maintaining physiological protection of blood-CSF barrier that is impaired in AD.

The higher levels of annexin A5 found in CSF from MCI and AD subjects compared with healthy donors suggests that secreted annexin A5 may indicate early changes in choroid plexus from these patients. Increased annexin A5 levels were also found in plasma from AD patients and the AD transgenic mouse model Tg2576\(^{32}\). We suggest that choroid plexus epithelial cells contribute to increase the annexin A5 levels in CSF along with other sources as neurons. Our results indicate CSF levels of annexin A5 become increased even at early stages of disease, including MCI, when Aβ accumulation in the brain becomes evident. This is consistent with other findings observed in neuronal cell cultures, describing increased annexin A5 levels in the supernatant of Aβ\(^{34}\)-treated cells\(^{32}\).

Aβ deposition in brains of AD patients agrees with the hypothesis suggesting Aβ accumulation as the main cause for neuronal cell death\(^{1,5,49}\). Exposure of cells to Aβ peptides cause typical apoptotic cell death\(^{30}\), although lower Aβ concentrations appear to only affect expression of neuronal apoptotic genes, without directly causing apoptosis\(^{50,51}\). Here we show that Aβ accumulation in AD is also present in choroid plexus. This observation was found gradually increasing as the disease progresses and is consistent with the increased cell death observed in choroid plexus from AD subjects. We demonstrate Aβ overload in choroid plexus cultures induced annexin A5 reduction in cells and subsequently increased annexin A5 in extracellular medium. Annexin A5 release outside cells occurs from the cellular apical compartment as has been demonstrated in epithelial cell cultures\(^{34,35}\). Upon Ca\(^{2+}\) stimulation, cytoplasmic annexin A5 is translocated to the plasma membrane while nuclear annexin A5 is moved to the nuclear membrane, in all cases binding to phosphatidylserine (PS)\(^{35}\). As Aβ also binds PS, both annexin A5 and Aβ could exert competitive interaction to each other. Reduced levels of intracellular annexin A5 result in lower competitive inhibition with Aβ at PS interaction site leading to increasing Aβ-induced cytotoxicity\(^{19}\). We may suggest Aβ overload in patient choroid plexus could induce annexin A5 drain from these cells, hence contributing to the observed increased levels in CSF.

Our experiments show that annexin A5 restores the Aβ-induced autophagic flux impairments in choroid plexus cells reducing the Aβ\(^{34}\)-increased levels of LC3-II and p62. Previous works showed elevated LC3-II and p62 levels in brains from AD patients at different stages of disease\(^{36,37}\). Here we found an increase in these autophagy markers in human choroid plexus suggesting that such increase is due to Aβ overload. In support of this observation several works showed similar effects increasing autophagy markers after Aβ incubation in human neuroblastoma H4 cells\(^{48}\) and in cells overexpressing mutant forms of PSEN2 and APP, both linked to Aβ overproduction in AD familial cases\(^{38,39}\). Therefore, we may suggest an induction of autophagy is triggered in choroid plexus due to Aβ overload. Then autophagosomes containing Aβ cannot be fused with lysosomes resulting in their accumulation and subsequent increased levels of autophagic markers LC3-II and p62. Apart of the mentioned cellular models\(^{38,39}\) this dual effect has been also observed in AD brains showing high autophagy flux. Such increased flux would try to maintain low Aβ\(^{34}\) levels through autophagic clearance but this is followed by impaired autolysosomal proteolysis\(^{33,34,37,61-63}\). We found that annexin A5 treatment restored the levels of autophagy markers to similar values that those obtained in untreated cells. We suggest the annexin A5 effect on autophagy may work enhancing autophagosome – lysosomes fusion allowing lysosomal cargo degradation. This agrees with other observations where annexin A5 was proposed stimulating autophagy\(^{44}\). In this work, authors showed that overexpression of annexin A5 in HEK293T cells decreased LC3-II levels. They proposed such decrease was produced by the annexin A5 effect of increasing the autophagosomal conversion into autolysosomes and enhancing lysosomal proteolysis\(^{44}\).

Linked to the protective role on autophagy could be the observation that annexin A5 reduces the number of Aβ\(^{34}\)-induced apoptotic cells. The idea of Aβ\(^{34}\)-induced cell death is preceded by impaired autophagy was also showed by other authors\(^{55,66}\). Further analysis about this connection was discussed by Nixon and Yang where they
Mitochondria play an important role in cells as they control bioenergetics, Ca\(^{2+}\) homeostasis, and cell death could be related to its affinity binding preferentially acidic phospholipids in a Ca\(^{2+}\) homeostasis regulation. Indeed, our work show A\(^{3}\) impaired Ca\(^{2+}\) homeostasis, and annexin A5 prevented this effects. A\(^{3}\) exposure made the choroid plexus cells to increase the ER Ca\(^{2+}\) uptake was higher. Both effects were restored by annexin A5 incubation. It has been shown A\(^{3}\) affects intracellular Ca\(^{2+}\) concentration by generating Ca\(^{2+}\)-permeable channels in plasma membrane of neurons and astrocytes\(^{70,71}\). Works carried out in neuronal cells showed A\(^{3}\) restored the A\(^{3}\)-induced mitochondrial depolarisation reflected by a reduction in the mitochondrial membrane potential which mirrors the mitochondrial health and function. Therefore, mitochondrial depolarisation may indicate altered Ca\(^{2+}\) homeostasis regulation. In summary, we show that levels of annexin A5 in choroid plexus from AD are reduced in late stages of disease\(^{67}\). This is consistent with our results as we observed a reduction in autophagy markers and apoptotic cell death when rat primary cultures of epithelial choroid plexus cells were incubated with A\(^{3}\) in presence of annexin A5. Both, stimulation of autophagy and apoptotic cell death are tightly regulated by intracellular Ca\(^{2+}\) level\(^{68,69}\). On this regard, mitochondria play an important role in cells as they control bioenergetics, Ca\(^{2+}\) homeostasis and apoptotic cell death. Failures in this regulation have been recognised in neurodegenerative disorders. Here, we show that annexin A5 restored the A\(^{3}\)-induced mitochondrial depolarisation reflected by a reduction in the mitochondrial membrane potential which mirrors the mitochondrial health and function. Therefore, mitochondrial depolarisation may indicate altered Ca\(^{2+}\) homeostasis regulation. Indeed, our work show A\(^{3}\) impaired Ca\(^{2+}\) homeostasis, and annexin A5 prevented this effects. A\(^{3}\) exposure made the choroid plexus cells to increase the ER Ca\(^{2+}\) stores. Then, by inducing Ca\(^{2+}\)-dependent mechanism under mitochondrial control.

### Table 1. Demographic and clinical characteristics of CSF donors. MCI: mild cognitive impairment; AD: Alzheimer's disease; Mod: moderate-severe; SD: standard deviation; MMSE: Minimental Status Examination; ns: non-significant. # p-value indicates statistical differences between all groups; *p < 0.05, **p < 0.01, ***p < 0.001, versus control group.

|                | Control (n = 26) | MCI (n = 26) | Mild AD (n = 20) | Mod AD (n = 20) | P-value |
|----------------|-----------------|--------------|-----------------|----------------|---------|
| Age, mean (SD) | 70.9 (9.2)      | 70.7 (7.3)   | 76.6 (6.5)      | 78.3 (3.6)     | 0.01    |
| Sex female, n (%) | 14 (53.8%)    | 15 (57.7%)  | 11 (55.0%)     | 10 (50.0%)     | ns      |
| MMSE, mean (SD) | 29.0 (1.2)     | 24.5 (3.4)   | 19.3 (4.4)     | 12.3 (5.2)     | <0.001  |
| CDR             | 0               | 0.5          | 1               | 2.3            | <0.001  |
| ApoE ε4, %      | 0%              | 41.6%        | 46.1%           | 40.2%          | 0.01    |
| CSF A\(^{3}\)\(_{42}\), mean (sem) | 885.2 (105.2) | 457.3 (53.3)*** | 513.8 (95.9)*** | 339.1 (46.2)*** | <0.001 |
| CSF t-tau, mean (sem) | 394.5 (54.6)  | 534.8 (123.5) | 690.4 (73.4)**  | 982.8 (104.5)** | 0.01   |

### Material and Methods

All methods were performed following the relevant guidelines and regulations approved by the local ethical review committee from the Hospital 12 de Octubre Research Institute. Other review committees partially involved in the present project were the following: 12 de Octubre Hospital, (Madrid, Spain) and Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) review committees for research related to CSF samples; The Institute of Neuropathology Brain Bank IDIBELL-Hospital Universitari de Bellvitge (Hospital de Llobregat, Spain) review committee, The Netherlands Brain Bank (NBB) (Amsterdam, The Netherlands) review committee and Banco de Tejidos, Fundación CIEN (Centro de Investigación de Enfermedades Neurológicas, Madrid, Spain) review committee for research involving the use of choroid plexus samples from human donors. Informed consent was obtained from all participants and/or their legal guardians.

### CSF collection.

CSF samples (10 ml per subject) from 26 mild cognitive impairment (MCI), 20 mild AD, 20 moderate AD, and 26 healthy control subjects were collected by lumbar puncture upon informed patient consent. All samples were centrifuged at 3000 rpm at 4°C for 10 min to remove any cell and debris, aliquoted in small volumes and stored in low bind polypropylene tubes at –80°C. Samples were obtained from the Hospital 12 de Octubre Neurology Service (Madrid, Spain), and Hospital de la Santa Creu i Sant Pau Neurology Service (Barcelona, Spain). Subjects were clinically diagnosed as probably having AD according to the National Institute on Aging and Alzheimer’s Association (NIA-AA) guidelines\(^{77}\). MCI was diagnosed in patients with cognitive impairment that did not fulfil the criteria for dementia\(^{68,79}\). All subjects had mini-mental state examination (MMSE) scores available. Inclusion criteria for cognitively normal older individual subjects were MMSE scores 24–30, no history or clinical signs of neurological or psychiatric disease or cognitive symptoms\(^{80}\). Subjects’
Table 2. Demographic and clinical characteristics of choroid plexus donors. AD I–VI: Alzheimer disease-related changes, stages of Braak and Braak; SD: standard deviation; ns: non-significant.

|               | Control (n = 22) | AD I–II (n = 21) | AD III–IV (n = 14) | AD V–VI (n = 13) | p-value |
|---------------|-----------------|-----------------|-------------------|-----------------|---------|
| Age, mean (SD)| 71.2 (10.0)     | 69.4 (9.9)      | 78.9 (7.7)        | 78.3 (6.6)      | <0.001  |
| Sex female, n (%) | 9 (40.9%)       | 7 (33.3%)       | 6 (42.9%)         | 6 (46.2%)       | ns      |

*Consent was obtained according to Declaration of Helsinki, and approval came from the research ethics committee of each responsible institution. Written informed consent was given from all participants or representatives. Subject demographic and clinical characteristics are listed in Table 1.*

**Tissue samples.** Post-mortem choroid plexus tissue was obtained from brain donors diagnosed with AD and control individuals. Frozen samples were supplied by the Institute of Neuropathology Brain Bank IDIBELL–Hospital Bellvitge (Hospitalita de Llobregat, Spain), The Netherlands Brain Bank (NBB) (Amsterdam, The Nederlands), and Banco de Tejidos, Fundación CIEN (Centro de Investigación de Enfermedades Neurológicas, Madrid, Spain). In all cases, post-mortem delays before sampling was between 3–8 hours. Subjects’ consent was obtained according to the Declaration of Helsinki, and approval came from the research ethics committee of each responsible institution. For all cases, written informed consent is available. Subjects were selected on the basis of post-mortem diagnosis of AD according to neurofibrillar pathology and Aβ plaques. Control cases were considered those without neurological symptoms and lesions in the neuropathological examination. A total of 70 samples were categorised into four groups, as presented in Table 2.

**Choroid plexus cultures.** Choroid plexus epithelial cell cultures were prepared as described previously. Choroid plexus were dissected from 3–5-day-old Wistar rats (Charles River, MA, USA). ARRIVA guidelines for the care and use of animals were followed. Once disaggregated, choroid plexus epithelial cells were seeded at a 4 × 10^4 cells/cm² density on laminin-coated plates and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA), 2 mM L-glutamine and 1% penicillin/streptomycin (Thermo Fisher Scientific, MA, USA) at 37 °C and 5% CO2. After 5–7 days on culture cells reached confluence and they were serum-deprived for 2 hours and oligomerised Aβ42 (10 μM; AnaSpec, Inc., San Jose, CA) was added. In the experiments with annexin A5 (15 μl/ml; Sigma-Aldrich, St. Louis, USA), this drug was added 1 hour before oligomerised Aβ42, to allow annexin A5 enter into the cells and avoiding extracellular sequestering of oligomerised Aβ42 stock was previously dissolved in acetic acid 0.1 M. Oligomeric Aβ42 was prepared by incubating a volume of stock solution in DMEM at 4°C for 24 hours prior addition to the cell cultures as previously described.

**Immunohistochemistry.** Human choroid plexus tissue was fixed for 24 hours in 4% paraformaldehyde (PFA) by immersion. Then, choroid plexus samples were OCT embedded and stored at −80 °C for subsequent cryostat sectioning (Leica, Wetzlar, Germany). 20 μm thick sections were processed free-floating for immunohistochemistry. To carry out immunohistochemistry for Aβ deposits, brain slices were 20 min pre-incubated with 88% formic acid and immunolabeled with mouse anti-Aβ antibody (1:500, MBL, Nagoya, Japan). After overnight incubation, primary antibody staining was revealed using the avidin-biotin complex method (VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine chromogenic reaction (Vector Laboratories, Inc.). Images were captured using a light microscope (Zeiss microscope; Carl Zeiss Microimaging, GmbH, Oberkochen, Germany).

**ELISA.** Levels of human endogenous Aβ42 in choroid plexus fractions from human subjects were determined with human specific ELISA kit (Innoltest β-amyloid1–42, Innogenetics, Ghent, Belgium), according to the manufacturer’s instructions. Prior to tissue homogenisation, endothelial vessels were removed in order to have pure choroid plexus epithelium. Obtained data were normalised to protein content.

DNA fragmentation undergoing apoptosis was detected in choroid plexus tissue from human subjects with a Cell Death Detection ELISAPLUS kit (Roche, Basel, Switzerland). According to the manufacturer’s instructions, unfixed frozen tissue was incubated with incubation buffer (lysis buffer provided in the kit) and tissue lysates were transferred to a streptavidin-coated 96 well microplate and allowing nucleosomes to interact with monoclonal antibodies: antihistone (biotin-labeled) and anti-DNA (peroxidase-conjugated). Finally, the amount of coloured product was determined using a microplate reader.

Annexin A5 levels in CSF from human subjects were determined with specific human ELISA kit (Abcam, Cambridge, UK). Briefly, CSF samples were added to the pre-coated wells, followed by the antibody mix. Finally, the signal intensity was measured at 450 nm using a microplate reader.

**Western blot.** Conditional medium from choroid plexus cultures was collected and protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland) were added. Choroid plexus tissue and cultured choroid plexus epithelial cells were homogenised in lysis buffer (50 mM Tris/HCl buffer, pH 7.4 containing 2 mM EDTA, 0.2% Nonidet P-40, 1 mM PMSE, protease and phosphatase inhibitor cocktails – Roche, Basel, Switzerland), and centrifuged for 10 min at 14000 rpm. The supernatant was recovered and stored at −80°C. Protein content from cell lysates, and extracellular medium was determined with the BCA method (Thermo Fisher Scientific, MA, USA). Equal amount of protein (20 μg per lane) were separated by SDS-PAGE (4–12%) and transferred onto
polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). For human choroid plexus tissue homogenates 25 μg of protein were loaded. Non-specific bindings were blocked by incubation in 5% non-fat milk in Tris-buffered saline (100 mM NaCl, 10 mM Tris, pH 7.4) containing 0.1% Tween (TTBS) for 1 hour at room temperature. Afterward, membranes were 4°C incubated overnight with mouse anti-annexin A5 antibody (ab54577, 1:1000; Abcam, Cambridge, UK), rabbit monoclonal anti-p62 antibody (ab91526, 1:20000; Abcam, Cambridge, UK), and rabbit polyclonal anti-LC3 antibody (NB100–2220, 1:1000; Novus, CO, USA). Protein loading was monitored using a mouse monoclonal antibody against β-actin (A1978, 1:10000; Sigma-Aldrich, St. Louis, USA). Membranes were then incubated for 1 hour in the appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000; Dako, CA, USA), and immunocomplexes were revealed by an enhanced chemiluminescence reagent (ECL Clarity; Bio Rad, CA, USA). Densitometric quantification was carried out with Image Studio Lite 5.0 software (Li-COR Biosciences, NE, USA). Protein bands were normalised to β-actin levels and expressed as percentage of the control group.

**Bioactivity and cell death quantification.** In vitro, cell viability was assessed using Cell Counting Kit-8 (CCK-8 assay, Sigma, St. Louis, USA) according manufacturer’s instructions. Briefly, primary cultures of choroid plexus epithelial cells were incubated 48 hours with and without oligomerised Aβ(1-42) (10 μM) and treated with or without annexin A5 (15 μg/ml). Then, CCK-8 solution was added, and absorbance was measured 1 hour later at 450 nm using a microplate reader.

Cell death was assessed 48 hours after incubation with and without both, oligomerised Aβ(1-42) (10 μM) and annexin A5 (15 μg/ml) using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Thermo Fisher Scientific, MA, USA). Fixed cells were labelled with propidium iodide. Healthy cells were recognised by their morphology and green staining, whereas propidium iodide-positive red cells (with condensed choromatin) were scored as apoptotic. Resulting images were taken using a fluorescence microscopy (Zeiss microscope; Carl Zeiss Microimaging, GmbH, Oberkochen, Germany) at a magnification of 40×. Fluorescence was quantified using the Volocity software (PerkinElmer, Waltham, MA, USA).

**Measurement of mitochondrial membrane potential (ΔΨ<sub>m</sub>).** ΔΨ<sub>m</sub> was measured as was described previously<sup>39</sup>. Briefly, cells plated on 25 mm laminin-coated coverslips and loaded with 40 nM tetramethyl-rhodamine methyl ester (TMRM) in a HEPES-buffered salt solution (HBSS) (composed of 156 mM NaCl, 3 mM KCl, 2 mM MgSO<sub>4</sub>, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 10 mM glucose and 10 mM HEPES; pH adjusted to 7.35 with NaOH) for 40 min at room temperature and keeping the dye present in the chamber at the time of recording. TMRM is a cell-permeant fluorescent dye used in the redistribution mode to assess ΔΨ<sub>m</sub> and therefore, a reduction in TMRM fluorescence represents mitochondrial depolarisation. Confocal images were obtained using a Zeiss 510 microscope equipped with META detection system (Zeiss, Oberkochen, Germany) and ×40 oil immersion objective. Excitation wavelength for TMRM was 560 nm and emission was detected above 580 nm. Z-stack images were obtained and analysed using the Volocity software (PerkinElmer, Waltham, MA, USA) and TMRM values for untreated cells were set to 100%. TMRM values for Aβ(1-42) and/or annexin A5 treated cells were expressed relative to untreated cells.

**Imaging of [Ca<sup>2+</sup>]<sub>i</sub>**. For cytosolic [Ca<sup>2+</sup>]<sub>i</sub> (<Ca<sup>2+</sup>><sub>i</sub>) measurements cells were plated on 25 mm laminin-coated coverslips and maintained in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1% penicillin/streptomycin at 37 °C and 5% CO<sub>2</sub>. After 5–7 days on culture, media was replaced with HBSS (156 mM NaCl, 3 mM KCl, 2 mM MgSO<sub>4</sub>, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 10 mM glucose and 10 mM HEPES; pH adjusted to 7.35) and cells were loaded with fura-2 AM (5 μM; Molecular Probes, Thermo Fisher Scientific, MA, USA) and 0.005% pluronic for 30 minutes. For simultaneous measurement of [Ca<sup>2+</sup>]<sub>i</sub> and ΔΨ<sub>m</sub>, rhodamine123 (Rh123, 10 μM; Molecular Probes, Thermo Fisher Scientific, MA, USA) was added into the cultures during the last 15 min of the [Ca<sup>2+</sup>]<sub>i</sub> loading period, and cells were then washed. We stimulated P2Y receptors from choroid plexus cells with 100 μM ATP and we added 1 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine (FCCP) to depolarise mitochondria. Fluorescence measurements were obtained on an epifluorescence inverted microscope equipped with a ×100 fluorecein inverted objective. Simultaneous [Ca<sup>2+</sup>]<sub>i</sub> and ΔΨ<sub>m</sub> were monitored in single cells using excitation light provided by a Xenon arc lamp, the beam passing sequentially through 10 nm pass filters centred at 340, 380 and 490 nm housed in computer-controlled filter wheel (Cairn Research, Kent, UK). Emitted fluorescence light was reflected through a 515 nm long-pass filter to a cooled CCD camera (Retiga, QImaging, Canada). All imaging data were collected and analysed using the Andor software (Belfast, UK). The fura-2 data were not calibrated in terms of [Ca<sup>2+</sup>]<sub>i</sub>, because of the uncertainty arising from the use of different calibration techniques. The fluorescent signal is quenched by Rh123 accumulation in polarised mitochondria; in response to depolarisation the fluorescence signal is dequenched; an increase in Rh123 signal therefore indicates mitochondrial depolarisation. We normalised the signals between resting level (set to 0) and a maximal signal generated in response to the uncoupler FCCP (1 μM; set to 100%).

**Data and statistical analysis.** In vivo and in vitro results are shown related to healthy donors and untreated cells, respectively. All of them are expressed as mean ± standard error of the mean (SEM) in percentage. In vitro data were generated from a minimum of three independent replicates per experiment (n = 3) performed in different days. For all imaging, each replicate consisted of at least 1 coverslip per condition where a number of 15–30 cells per coverslip were analysed. Statistical analysis and exponential curve fitting were performed using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA) software. Grubbs outlier filter was used for all data. Statistical significance for multiple comparisons was calculated by one-way ANOVA followed by Fisher’s LSD correction. In all cases, statistical significance was set at p < 0.05 (**p < 0.05; ***p < 0.01; ****p < 0.001; ####p < 0.005, ###p < 0.01, ##p < 0.05, ##p < 0.01, ####p < 0.001, ######p < 0.0001).
Ethical approval and informed consent. All participants gave written informed consent for participation. ARRIVA guidelines for the care and use of animals were followed. The project in full was approved by the 12 de Octubre Research Institute ethical review committee. Additionally, research related to CSF samples was also approved by the 12 de Octubre Hospital, (Madrid, Spain) and Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) review committees; research involving the use of choroid plexus samples from human donors was also approved by the Institute of Neuropathology Brain Bank IDIBELL-Hospital Universitari de Bellvitge (Hospitalet de Llobregat, Spain) review committee, The Netherlands Brain Bank (NBB) (Amsterdam, The Nederlands) review committee and Banco de Tejidos, Fundación CIEN (Centro de Investigación de Enfermedades Neurológicas, Madrid, Spain) review committee.

Data availability
The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 10 February 2020; Accepted: 9 May 2020;
Published online: 10 June 2020

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Acknowledgements

We are grateful to the patients and donors without which this work would not have been possible. Also, we thank Dr. Marife Cano who kindly helped us to obtain and perform choroid plexus cell cultures. This study was supported by grants from the Instituto de Salud Carlos III (PI2016/01), the Instituto de Salud Carlos III, supported by grants from the Comunidad de Madrid (S2017/BMD-3700; NEUROMETAB-CM), Fundación Ramón Areces (P12016/01), and the Alzheimer’s Association (CIVP16A1825). Part of the work was supported by the NIHR Queen Square Dementia Biomedical Research Unit. This work was undertaken at UCLH/UCL which receives a proportion of funding from the Department of Health’s NIHR Biomedical Research Centres funding scheme.

Author contributions

F.B. and E.C. were responsible for experimental designs, data interpretation and writing of the paper. F.B., A.K., M.C., C.P. and D.A. performed and analysed the experiments. C.S., A.V.G., A.R., J.F., D.A., A.L.L. and I.F. provided human samples. J.H. and A.Y.A. contributed feedback the manuscript. J.H., A.Y.A. and E.C. obtained the funding. All authors reviewed and corrected the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-66177-5.

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