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Mitochondrial membrane permeabilisation by amyloid aggregates and protection by polyphenols

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ABSTRACT

Alzheimer's disease and Parkinson's disease are neurodegenerative disorders characterised by the misfolding of proteins into soluble prefibrillar aggregates. These aggregate complexes disrupt mitochondrial function, initiating a pathophysiological cascade leading to synaptic and neuronal degeneration. In order to explore the interaction of amyloid aggregates with mitochondrial membranes, we made use of two *in vitro* model systems, namely: (i) lipid vesicles with defined membrane compositions that mimic those of mitochondrial membranes, and (ii) respiring mitochondria isolated from neuronal SH-SY5Y cells. External application of soluble prefibrillar forms, but not monomers, of amyloid-beta ($A\beta_{42}$ peptide), wild-type α-synuclein (α-syn), mutant α-syn (A30P and A53T) and tau-441 proteins induced a robust permeabilisation of mitochondrial-like vesicles, and triggered cytochrome *c* release (CCR) from isolated mitochondrial organelles. Importantly, the effect on mitochondria was shown to be dependent upon cardiolipin, an anionic phospholipid unique to mitochondria and a well-known key player in mitochondrial apoptosis. Pharmacological modulators of mitochondrial ion channels failed to inhibit CCR. Thus, we propose a generic mechanism of thrilling mitochondria in which soluble amyloid aggregates have the intrinsic capacity to permeabilise mitochondrial membranes, without the need of any other protein. Finally, six small-molecule compounds and black tea extract were tested for their ability to inhibit permeation of mitochondrial membranes by $A\beta_{42}$, α-syn and tau aggregate complexes. We found that black tea extract and rosmarinic acid were the most potent mito-protectants, and may thus represent important drug leads to alleviate mitochondrial dysfunction in neurodegenerative diseases.

1. Introduction

Neurodegenerative diseases are a diverse group of disorders characterised by the progressive loss of select neurons in the brain. The two most frequent of these diseases are Alzheimer's disease (AD) and Parkinson's disease (PD). The hallmark in both pathologies is the accumulation and aggregation of misfolded proteins [1]. In AD, amyloid-beta (Aβ) peptides aggregate and classically have been described to deposit as extracellular amyloid plaques; however, the intracellular accumulation of Aβ is being increasingly recognised and is closely linked with neuronal loss [2,3]. The other critical protein in AD, the microtubular associated protein tau, deposits intracellularly as neurofibrillary tangles [4]. In PD, progressive intraneuronal aggregation of the protein α-synuclein (α-syn) has been proposed to play a central role, α-syn being the major component of pathognomonic cytoplasmic inclusions known as "Lewy Bodies" [5,6]. Point mutations of human α-syn (A30P, A53T) are related to familial autosomal-dominant forms of early-onset PD [7].

The aberrant assembly of such diverse proteins into mature amyloid fibrils proceeds through the formation of intermediate oligomeric assemblies. These soluble prefibrillar species, which are typically transient and structurally heterogeneous, are now widely recognised as the primary toxic determinants in both AD and PD [8–14]. A landmark study using transgenic mice has shown that α-syn variants that specifically form oligomers were significantly more neurotoxic than α-syn variants that form fibrils. In particular, *in vivo* toxicity was strongly associated with the higher ability of oligomers to interact

Abbreviations: AD, Alzheimer's disease; $A\beta_{42}$, amyloid-beta (1–42); α-syn, α-synuclein; CL, cardiolipin; Cyto *c*, cytochrome *c*; CCR, cytochrome *c* release; IM and IMM, inner mitochondrial membrane; OM and OMM, outer mitochondrial membrane; OGB-1, Oregon Green® 488 BAPTA-1; PD, Parkinson's disease; TX-100, Triton X-100; WT, wild-type

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with and potentially disrupt membranes [15]. Indeed, oligomeric complexes of Aβ or α-syn may penetrate cell membranes, stimulating Ca^{2+} influx and leading to cell death [16–18]. The mechanism of lipid bilayer disruption by aggregate species may involve insertion of distinct pore-like structures, formation of large "defects" in the membrane, or a combination of both [19–24]. Moreover, the first study has been recently published demonstrating increased phospholipid vesicle leakage, in association with decreased cell viability, induced by tau aggregation intermediates [25].

Membranes of organelles are potential targets of oligomeric complexes; this applies particularly to mitochondria which are abundant in synapses and neurons. Mitochondria are critical regulators of neuronal survival and death, and there is strong evidence that mitochondrial dysfunction might represent the fundamental initiator of the pathophysiological cascade in both AD and PD [26]. Mitochondrial processes of great importance to brain cells include energy metabolism [27,28], generation of free radicals [26,29], calcium homeostasis [30,31] and initiation of apoptosis *via* release of the respiratory protein, cytochrome *c* (Cyto *c*) [32–34].

It is now clear that mitochondria are important targets of the Aβ peptide, and that perturbation of membranes and bioenergetics may be key triggers of neuritic damage leading to dementia. Recognised mitochondrial targets of Aβ include the translocase import machinery, enzymes involved in Krebs cycle, respiratory chain enzymes, the mitochondrial permeability transition pore (mPTP), the mitochondrial matrix protein Aβ-binding alcohol dehydrogenase (ABAD), and mitochondrial DNA [35–41]. There is mounting evidence in favour of a direct relationship between mitochondria-specific Aβ accumulation, AD-related mitochondrial dysfunction and apoptotic cell death [42]. Not the least, one of the key enzymes involved in Aβ release, γ-secretase, has been located in mitochondrial membranes. This has been associated with the generation of cleavage fragments of the amyloid precursor protein, including Aβ peptides, locally in these organelles thus contributing to mitochondrial dysfunction [43]. The deleterious effects of Aβ peptides on brain mitochondria have been confirmed in several studies using transgenic mice, too [44–48]. For instance, the progressive accumulation of Aβ in mitochondria of amyloid precursor protein (APP) mutant mice is associated with decreased activity of complexes III and IV of the respiratory chain, and reduced consumption of oxygen [46]. Importantly, it has been possible using such models to attest that impairment of mitochondrial function by Aβ often occurs at the onset of cognitive loss, before amyloid plaque deposition [49,50].

The role of tau protein in AD-related mitochondrial dysfunction is still unclear, mainly because attention has hitherto been focused on the Aβ peptide. Interestingly, tau appears to impair mitochondrial function synergistically with Aβ: for example, tau causes mainly a deregulation of complex I activity, whilst Aβ impairs the function of complex IV [51]. Further, truncated tau disrupted mitochondrial function and transport when combined with Aβ in cortical neurons [52]. *In vivo* studies also attend to a pathogenic link between tau and mitochondria — a recent report identified that tau oligomers are acutely toxic and induce loss of mitochondrial and synaptic function in wild-type mice [53]. Nevertheless, the molecular mechanism was not explored.

Similar to Aβ and tau, multiple observations are providing evidence implicating an association between α-syn and mitochondria in the pathogenesis of PD [54,55]. Direct localisation of wild-type and mutant α-syn to mitochondria in dopaminergic neurons has been reported [56,57]. In fact, the intra-mitochondrial localisation of α-syn was more abundant in dopaminergic neurons than in other brain regions in rat models [58]. Since human α-syn has a mitochondrial targeting sequence at its N-terminal domain, the protein may gain direct entry into mitochondria *via* mitochondrial import channels [59]. An interaction between α-syn and mitochondria may lead to oxidative stress by decreasing complex I activity [59,60]; indeed, alpha-synuclein knock-out mice resisted toxicity induced by the neurotoxin and mitochondrial complex I inhibitor 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [61]. On the other hand, mice overexpressing the A53T mutant exhibit mitochondrial and neuronal degeneration [62]. Degeneration of mitochondria has been linked to mitochondrial fragmentation as a result of a direct interaction of α-syn with mitochondrial membranes, eventually resulting in neuronal death [63].

Given this background, we wanted to probe further the direct effects of the interaction between protein aggregates and mitochondrial membranes, also in relationship to a possible therapeutic treatment of AD and PD. In particular, we have previously shown that polyphenolic compounds, including black tea extract, are potent inhibitors of lipid membrane destabilisation by Aβ and α-syn aggregates [64,65]. Polyphenols are plant metabolites that are highly abundant in human diet, and several experimental and epidemiological evidences point to beneficial effects on AD and PD risks [66–68]. We first established the membrane permeabilising abilities of aggregate complexes of Aβ, WT α-syn, A30P α-syn, A53T α-syn and tau-441 in liposome systems and isolated mitochondria. These cell-free systems allow for a better understanding of the mechanisms in permeabilisation since they do away with many other factors which might otherwise be involved [69]. Next, the panel of *in vitro* assays was used to ascertain which of twelve small-molecule compounds and black tea extract can best inhibit permeation of mitochondrial membranes by the aggregates. Hence, knowledge was gained on the effectiveness of compound inhibitor activity in the presence of a mitochondrial phospholipid membrane interface.

2. Materials and methods

2.1. Reagents

Chemical compounds and polyphenols obtained from Sigma-Aldrich (Munich, Germany) include: apigenin (Api), baicalein (Baic), black tea extract (BTE; >80% theaflavins), chrysin (Chr), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), (−)-epigallocatechin gallate (EGCG), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), cyclosporin A (CsA), fluorescein isothiocyanate conjugated to dextran (FITC-dextran 4 kDa, FITC-dextran 40 kDa, FITC-dextran 70 kDa), genistein (Gen), morin (Mor), myricetin (Myr), nordihydroguaiaretic acid (NDGA), purpurogallin trimethyl ether (Purp) and rosmarinic acid (RA). N′-benzylidene-benzohydrazide (NBB) compounds 301C09 and 293G02 were obtained from Chembridge Corp. (San Diego, CA, USA). Bongkrecic acid (BKA) and anti-VDAC antibody were from Calbiochem (Darmstadt, Germany). Stock solutions of 10 mM polyphenols and NBB compounds in 100% DMSO were stored at −20 °C. Gramicidin was purchased from Fluka Chemie AG (Buchs, Switzerland).

2.2. Preparation of amyloid aggregates

$Aβ_{42}$ and human recombinant tau-441 (2N4R) were purchased in lyophilised form (rPeptide, UK). Wild-type (WT) or mutant (A30P, A53T) human recombinant α-syn was expressed in *Eschericia coli* BL21 (DE3) cells and purified as described previously [70]. Seeding into soluble prefibrillar aggregates was achieved based upon established methods. Thus, 45 μM $Aβ_{42}$ in sterile PBS (pH 7.4) was aggregated for 2 h at 37 °C [64,71]; 7 μM WT, A30P or A53T α-syn was incubated in sterile PBS (pH 7.4) at 37 °C for 4 h with continuous shaking at 500 rpm [72]; 7 μM tau-441 was aggregated in the presence of 70 μM Al^{3+} at 37 °C for 4 h with continuous shaking at 500 rpm [73]. Shaking was performed in an Eppendorf Thermomixer® and microfuge tubes were sealed with Whatman® adhesive film to prevent evaporation. The oligomeric nature of soluble $Aβ_{42}$, synuclein and tau aggregates generated using these protocols was confirmed by electrophoresis on 4–12% Tris–Bis NuPAGE® gels, followed by immunoblotting or staining of proteins (Suppl. Fig. S1).

2.3. Preparation of lipid vesicles from defined lipid mixtures

For preparation of small unilamellar lipid vesicles (SUVs), egg phosphatidylcholine (PC), egg phosphatidylethanoloamine (PE), brain phosphatidylserine (PS), soybean phosphatidylinositol (PI), and heart cardiolipin (CL) in chloroform (Avanti Polar Lipids, Alabaster, AL, USA) were mixed in a glass tube at the following molar ratios: for OM-type vesicles having membranes with a lipid mixture corresponding to the lipid composition of outer mitochondrial membranes, PC, 53%, PE 27%, PI 9%, PS 7%, CL 4% [74]; for IM-type vesicles having membranes with a lipid mixture corresponding to the lipid composition of inner mitochondrial membranes, PC, 45%, PE 25%, PI 10%, PS 5%, CL 15% [75]; for L-type vesicles lacking cardiolipin, PC, 53%, PE 29%, PI 12%, PS 6%; and for C-type liposomes having membranes with a lipid mixture representative of the neuronal plasma membranes, a synthetic phospholipid blend consisting of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)/1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPS)/1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in a molar ratio of 5:3:2 (Avanti Polar Lipids). Lipid vesicles encapsulating Oregon Green® 488 BAPTA-1 (OGB-1; Invitrogen, Darmstadt, Germany) were prepared using a detergent-dialysis method as described previously [65]. The diameter of lipid vesicles was measured by the dynamic light scattering technique using Zetasizer Nano S (Malvern, Worcestershire, UK). Vesicles had an average diameter of 87 ± 20 nm, and hence classified as SUVs.

A similar protocol was used to prepare FITC-dextran loaded liposomes, but in this case the lipid film was resuspended in 2 mg/ml FITC-dextran (4 kDA; 40 kDA; 70 kDa) whilst the untrapped FITC-dextran molecules were removed by 3–4 cycles of centrifugation (22,000 *g* for 30 min).

2.4. Liposome permeabilisation assays

Liposome permeabilisation assays were carried out as described [65] using OGB-1 as the encapsulated fluorophore. Briefly, disruption of the vesicle membrane results in complexing of buffer Ca^{2+} with OGB-1 and thus an increase in fluorescence (exc. 485 nm, em. 528 nm). Aggregates ($A\beta_{42}$, α-syn or tau) were added to 25–50 μM liposomes in assay buffer (1 mM $CaCl_2$, 100 mM KCl, 10 mM MOPS/Tris, 1 mM EDTA, pH 7.0) and kinetic measurements taken for 60 min using FLx800-TBID microplate reader (BioTek, Germany). The maximum fold-increase in fluorescence of a sample was determined and normalised to the baseline fluorescence of control liposomes. Assays with IM-type liposomes were carried out using 0.1 mM (not 1 mM) Ca^{2+} in buffer to ensure stability of vesicles. Moreover, 10 μM Al^{3+} present in tau aggregation buffer does not significantly affect liposome integrity (<1.1-fold change).

Disruption of lipid vesicles by aggregates in the presence of compound was calculated as a percentage of permeabilisation caused by aggregates alone (theoretical maximum, 100%). Autofluorescence was measured for each compound and subtracted from the sample well values. Concentrations of small-molecule compounds and black tea extract in the assay were derived from previous studies [64,65].

2.5. Cell culture and isolation of mitochondria

SH-SY5Y human neuroblastoma cells (obtained from ATCC) were grown in flasks to ~90% confluency at 37 °C with 5% CO_2 in a humidified atmosphere using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Mitochondria were isolated from 5×10^7 cells using the MITOISO2 kit (Sigma-Aldrich) according to the manufacturer's recommendations. The mitochondrial pellet was finally resuspended in 1× storage buffer (50 mM HEPES, 25 mM succinate, 1.25 M sucrose, 5 mM ATP, 0.4 mM ADP, 10 mM K_2HPO_4, pH 7.5) at 1–1.5 mg/ml (final mitochondrial protein concentration determined using the BCA kit, Pierce). Mitochondria were kept on ice during the entire isolation procedure and prepared fresh for each experiment.

The purity of the mitochondrial fraction was confirmed by immunoblot analysis of cytosolic and mitochondrial isolates with anti-Hsp90 (Abcam, ab3931) and anti-Cyto *c* (Invitrogen) primary antibodies (Suppl. Fig. S2). Inner membrane integrity was assessed by measuring the uptake of the fluorescent dye JC-1 into mitochondria (Isolated Mitochondria Staining Kit, Sigma-Aldrich) (Suppl. Fig. S3).

2.6. Immunoassay for detection of Cyto c release from isolated mitochondria

Isolated mitochondria (12 μg) were incubated at 37 °C for 30–60 min: alone, with 2% Triton X-100 (v/v), with fresh or pre-aggregated amyloid aggregates, or with compounds (the latter were left for 10 min in presence of aggregates prior to addition of mitochondria). Final volume was 100 μl in 1× storage buffer. Supernatant fractions were obtained by pelleting the mitochondria (16,000 ×*g*, 10 min, 4 °C) and the amount of Cyto *c* present in supernatant was quantitated using a colourimetric enzyme-linked immunosorbent assay (Quantikine; R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Preliminary testing of small-molecule compounds and black tea extract with isolated mitochondria was carried out, and only those compounds that did not induce efflux of Cyto *c* at the tested concentration were selected (Suppl. Fig. S4).

2.7. Western blot analysis

For detection of Cyto *c* release by immunoblotting, mitochondria were incubated with permeabilisation agents or protein aggregates, for 30 min at 37 °C in 1× storage buffer, then pelleted at 16,000 ×*g* for 10 min at 4 °C. The supernatant was collected and subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes and then sequentially incubated with 5% nonfat dried milk blocking reagent in TBS-T overnight at 4 °C, primary monoclonal antibody to Cyto *c* (1:4000) and finally with anti-mouse IgG horseradish peroxidase conjugate (1:5000) for 2 h at room temperature. The membranes were processed for Cyto *c* detection using the enhanced chemiluminescent light (ECL Plus) kit according to specifications of the manufacturer (GE Healthcare, Munich).

In Western blot analysis to visualise α-syn aggregate species treated with polyphenolic compounds, each compound was incubated 10 min at room temperature with 0.7 μg α-syn oligomers. Samples were run on 15% SDS-PAGE gels and subsequently transferred onto nitrocellulose. After blocking overnight at 4 °C with 5% milk, membranes were probed for 2 h at room temperature with anti-synuclein antibody 15G7 (1:7000). Immunoreactivity was detected with peroxidase-conjugated anti-rat IgG (1:10,000; Sigma-Aldrich, Munich, Germany). For signal detection, ECL Plus was used.

2.8. Statistical analysis

Mean ± SEM are shown in all graphs, with *n* as the number of experiments. Statistical analyses were performed using GraphPad™ Prism 5 and one-way ANOVA was used for statistical testing. When ANOVA was significant ($p < 0.05$) Bonferroni's multiple comparison test was carried out to determine differences between experiments. Ranking of compounds in the various assays were correlated by the non-parametric Spearman rank correlation coefficient.

3. Results

3.1. Permeabilisation by amyloid aggregates of vesicles that mimic mitochondrial membranes

A unilamellar liposome model was first used to investigate whether recombinant $A\beta_{42}$ and α-syn oligomers can permeabilise mitochondrial-like phospholipid membranes. Lipid vesicles were formed from defined lipid mixtures similar to the observed composition

of outer mitochondrial membranes (OM-type) and loaded with the calcium-binding fluorophore OGB-1. Membrane disruption increases fluorescence as a result of Ca^{2+} ions in the buffer complexing with OGB-1. Over a time period of 60 min, 2-h aggregated $A\beta_{42}$ induced a robust increase in OM-type liposome permeabilisation over baseline fluorescence (0.5 μM: 1.79-fold; 1 μM: 2.28-fold). In comparison, 0.5 μM $A\beta_{42}$ monomeric (fresh) peptide had no observed effect on the OM-type liposomes (Fig. 1A). Wild-type α-syn behaved similarly: synuclein aggregates showed a dose-dependent effect on membrane leakage from 0.5 to 1 μM (0.5 μM: 1.3-fold; 0.75 μM: 1.82-fold; 1 μM: 2.32-fold) whilst non-aggregated (fresh) α-syn did not significantly differ from liposome control (Fig. 1B). An even more remarkable permeabilisation of OM-type vesicles was observed using mutant α-syn (Fig. 1C, D). For instance, 0.25 μM A30P or A53T α-syn aggregates were equally damaging to OM-type liposomes as 1 μM WT (>2-fold permeabilisation). Permeabilisation by A30P and A53T at the lowest tested concentration of 0.125 μM was approximately half compared to 0.25 μM, thereby exhibiting dose-dependence. Our data therefore suggest that mitochondrial membranes are indeed targeted by aggregate complexes of amyloidogenic peptides, with familial PD mutations in the α-syn gene evoking the strongest disturbance of membrane integrity.

In order to characterise better the mechanism of membrane destabilisation, a size-dependent fluorescence leakage assay was performed by preparing OM-type liposomes containing FITC-dextrans of three different molecular sizes (4 kDa, 40 kDa, and 70 kDa) and exposing them to $A\beta_{42}$ and WT α-syn oligomeric aggregates. In the case of Aβ aggregates, leakage of the differentially-sized markers occurred as follows: 79% ± 2 (4 kDa), 72% ± 4 (40 kDa), and 65% ± 2 (70 kDa). Permeabilisation by α-syn aggregates was observed as follows: 65% ± 3 (4 kDa), 61% ± 1 (40 kDa), and 63% ± 2 (70 kDa). Thus, there was essentially no major change in the leakage of larger-sized dextrans with respect to the smaller dextran molecules.

3.2. Preferential targeting of IMM-like vesicles by Aβ42, α-syn and tau assemblies

The liposomal model allows comparison of aggregate toxicity between vesicles having different phospholipid compositions. In view of the significant permeabilisation observed using OM-type liposomes, another three types of OGB-1 filled lipid vesicles were tested for permeabilisation, namely IM-type, L-type and C-type liposomes. IM-type liposomes are specifically characteristic for the inner mitochondrial membrane and therefore contain a higher proportion (15%) of the mitochondrial-specific phospholipid, CL. L-type liposomes maintain the same phospholipid composition as OM-type vesicles except for CL. Finally, 'C-type' liposomes were prepared using a synthetic phospholipid blend which models biological plasmalemma membranes. Thus, we were able to compare mitochondrial-like with cellular-like membranes *vis-a-vis* susceptibility to attack by aggregate samples, as well as to address the question regarding the specific role of CL.

Starting with $A\beta_{42}$, a significantly higher degree of permeabilisation of IM- and OM-type liposomes was observed when compared to L- and C-type liposomes: IM-type (2.01-fold) > OM-type (1.79-fold) > C-type (1.5-fold) > L-type (1.29-fold) (Fig. 2A). In other words, an increase in CL content correlated with enhanced $A\beta_{42}$ membrane destabilisation. Inner membrane-like liposomes were also the most damaged type of vesicle upon exposure to WT α-syn aggregates (2.34-fold); other

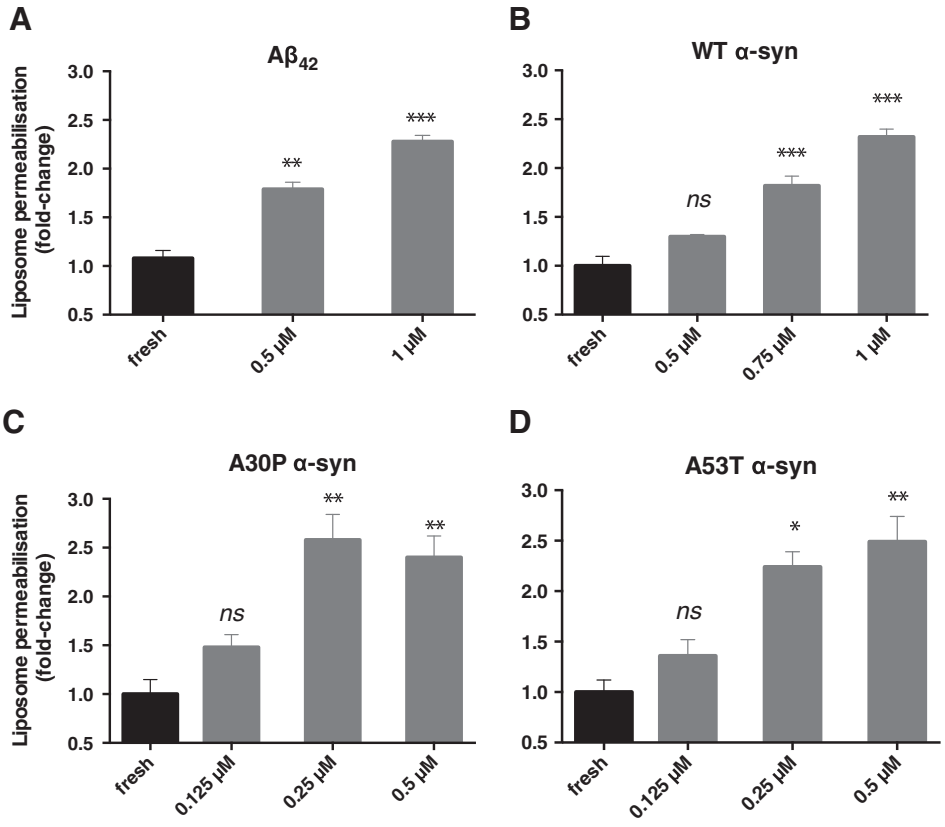

Fig. 1. Permeabilisation of lipid vesicles having a composition similar to the outer mitochondrial membrane. OM-type liposomes were incubated for 60 min with (A) 1 μM fresh and 0.5–1 μM aggregated $A\beta_{42}$, (B) 1 μM fresh and 0.5–1 μM aggregated WT α-syn, and (C, D) 0.5 μM fresh and 0.125–0.5 μM aggregated A30P or A53T α-syn. Concentrations are given in moles of monomeric peptide or protein. Results are expressed as the maximal fold increase over baseline fluorescence normalised to 1. Values are representative of means ± SEM ($n = 3$–4); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; *ns*, not significant.

vesicles representing outer mitochondrial and cellular membranes were significantly less efficiently permeabilised (<1.5-fold) by WT α-syn (Fig. 2B). Both α-syn A30P and A53T aggregates, however, induced a marked 2.5 to 3-fold increase in permeabilisation across all lipid compositions (Fig. 2C & D). This further suggests that familial PD mutants are associated with more severe membrane perturbations than WT α-syn. We also extended the liposome permeabilisation assay to the other major misfolded protein implicated in AD, the neuronal microtubule-associated protein tau. Soluble tau aggregates were generated *in vitro* from recombinant monomeric tau and incubated with the four types of liposomes. Strikingly, the permeabilisation effects of tau aggregates mirrored those of $A\beta_{42}$, with IM-type and OM-type vesicles being significantly more damaged than either L-type or C-type vesicles (IM-type, 2.15-fold; L-type, 1.35-fold; C-type, 1.29-fold increase in permeabilisation; Fig. 2E). Summing up, CL-rich IM-type liposomes appeared to be the only vesicles that were strongly permeabilised (>2-fold) irrespective of the nature of amyloid aggregate to which they had been exposed ($A\beta_{42}$, WT or mutant α-syn, tau-441). A similar pattern of differential permeabilisation of IM-vesicles was seen when the well-known pore-forming antibiotic gramicidin was incubated with liposomes. Here again, the integrity of IM-type lipid vesicles was disrupted most (2.24-fold) in comparison with the other vesicle types (circa 1.5-fold) (Fig. 2F).

The accelerated loss of membrane integrity by IM-type liposomes is particularly evident from the kinetics of vesicle permeabilisation (Fig. 3). $A\beta_{42}$ and tau aggregates demonstrate a similar kinetic profile

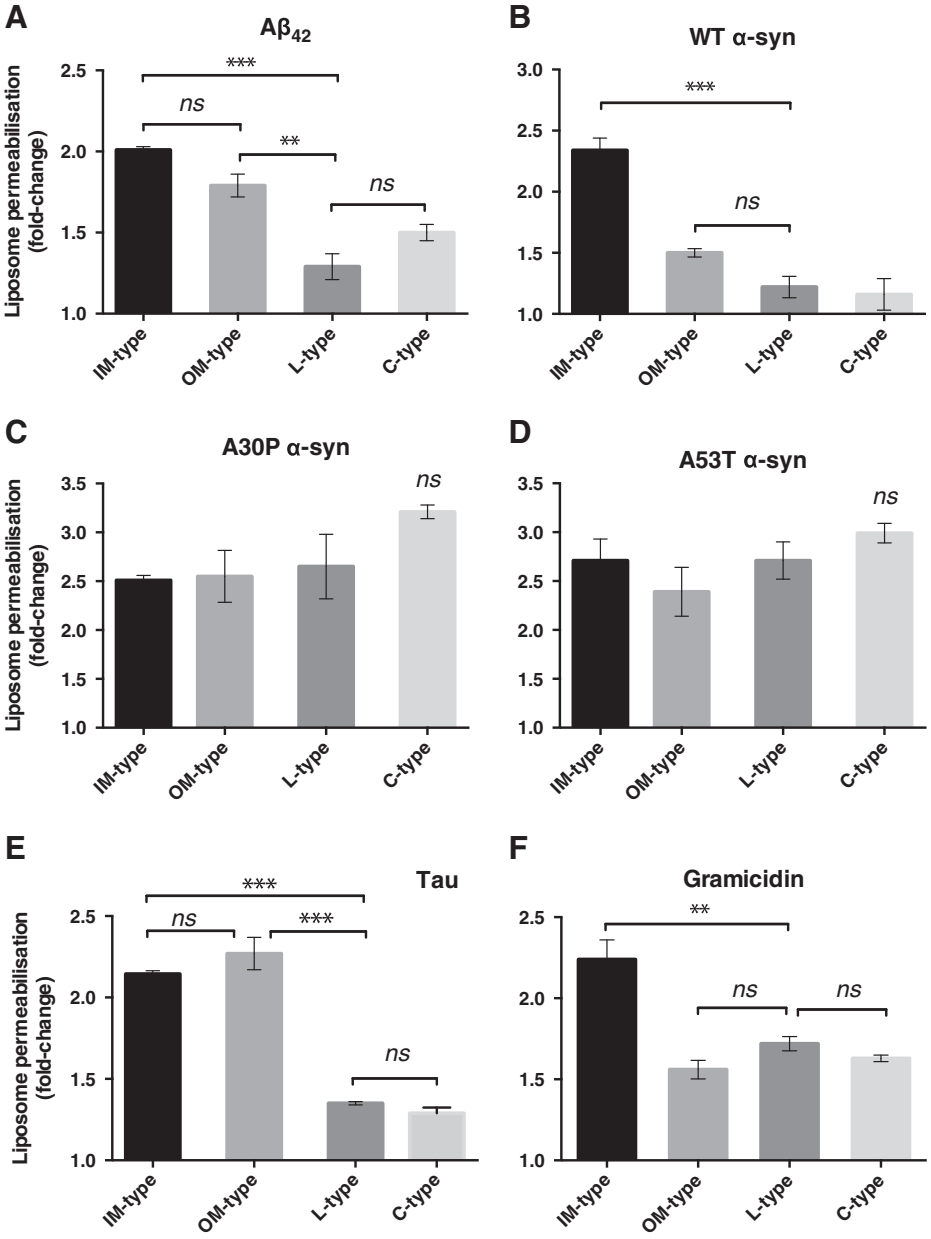

Fig. 2. Differential permeabilisation of synthetic lipid vesicles mimicking mitochondrial and cellular membranes. OGB-1 loaded liposomes consisting of either the same lipids as mitochondrial inner membrane (IM-type), mitochondrial outer membrane (OM-type), cardiolipin-free outer membrane (L-type) or cellular plasma membrane (C-type), were treated with pre-aggregated (A) 0.5 μM $A\beta_{42}$, (B) 0.5 μM WT α-syn, (C) 0.5 μM A30P α-syn, (D) 0.5 μM A53T α-syn, (E) 0.1 μM tau and (F) 0.025 mg/ml gramicidin for 60 min. When compared to L-type and C-type membranes, the phospholipid composition of IM-type vesicles appears to be preferentially targeted by soluble aggregates of $A\beta_{42}$, WT α-syn and tau proteins, as well as by the channel-forming ionophore gramicidin. Permeabilisation is indicated as the maximal fold increase in fluorescence over liposome control (incubated with buffer only). The phospholipid compositions of IM-, OM-, L-, and C-type liposomes are specified in 'Materials and methods'. Values represent means ± SEM ($n = 3$); $^{**}p < 0.01$; $^{***}p < 0.001$; *ns*, not significant.

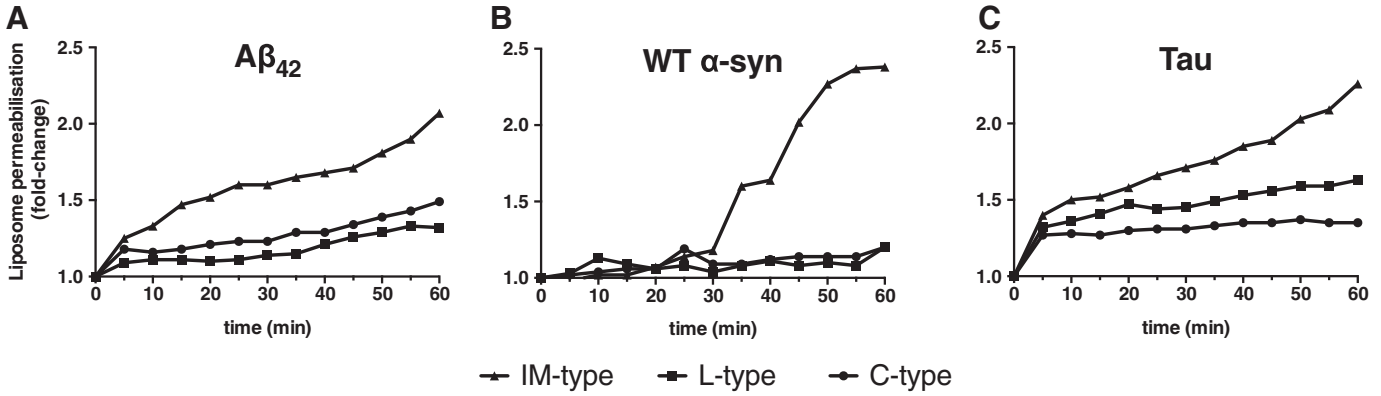

Fig. 3. Kinetics of lipid vesicle permeabilisation. The time course of the fold-change in fluorescence is shown for IM-type, L-type and C-type liposomes incubated with soluble preformed aggregates of (A) 0.5 μM $A\beta_{42}$, (B) 0.5 μM WT α-syn and (C) 0.1 μM tau-441, for 60 min. Kinetic fluorescence readings were recorded at 5-min intervals and data presented as fold increase over baseline at time = 0. Data are representative of at least 3 experiments.

with respect to IM-lipid vesicle damage, with a relatively constant permeabilisation rate of 0.15-fold increase in fluorescence every 10 min, starting from 10 min after addition of the protein (Fig. 3A, C). WT α-syn has a more distinctive profile of permeabilisation, which only commences 30 min after incubation with the aggregates, and then proceeds at a fast rate of around 0.55-fold increase in fluorescence every 10 min (Fig. 3B). Permeabilisation of mitochondrial-like lipid vesicles lacking CL (L-type) or plasma membrane-like vesicles (C-type) occurs at rates not exceeding 0.06-fold increase every 10 min, excluding a rapid initial increase by the tau aggregates. Furthermore, even after 60 min, leakage from the CL-containing membranes was still occurring rapidly whilst that from other types of liposomal membranes had stabilised (Fig. 3).

Taken together, these data support the concept that soluble assemblies derived from diverse amyloidogenic proteins preferentially target the defined phospholipid composition of the inner mitochondrial membrane, and in particular cardiolipin.

3.3. Aggregates induced Cyto c release from isolated mitochondria

Next we investigated the effects of amyloid aggregates on isolated mitochondria — and hence on biological, rather than synthetic, mitochondrial membranes. Cyto *c* is a protein normally present in the intermembrane mitochondrial space, with loosely and tightly bound pools attached to the CL-rich IMM [76]. Liberation of Cyto *c* into the cytosol, along with that of other pro-apoptotic proteins (*e.g.* apoptosis-inducing factor, Omi and Smac/Diablo) occurs as a result of mitochondrial membrane permeabilisation and is a critical event in cell death [33]. Respiring mitochondria isolated from SH-SY5Y cells were exposed to soluble aggregate complexes, samples were centrifuged and Cyto *c* efflux determined using a quantitative immunoassay technique. Cyto *c* release (CCR) induced by Triton X-100 was taken as reference and set to 100%. Comparing CCR induced by 2 μM (in moles of monomeric peptide) of the various aggregate complexes, the most damaging were mutant A30P (74%) and A53T (67%) α-syn aggregates, followed by tau (62%) and WT α-syn (52%). In the case of $A\beta_{42}$, 10 μM aggregates were needed to induce 68% CCR (Fig. 4). Notably, when mitochondria were incubated with amyloid-β, α-syn or tau in the monomeric form, CCR was minimal. Data obtained in the immunoassay experiments was qualitatively confirmed by detection of CCR using immunoblot analysis. Efflux of Cyto *c* from mitochondria occurred upon exposure to all aggregate types and is comparable to that induced by 2% Triton X-100 (Suppl. Fig. S5).

Collectively, these results corroborate those obtained by the liposome assays and suggest a direct role for soluble prefibrillar aggregates in mitochondrial permeabilisation.

3.4. Aggregate-induced CCR is independent of the mitochondrial permeability transition pore

One proposed mechanism of CCR assumes the formation of the mitochondrial permeability transition (mPT) pore, which results in subsequent swelling of mitochondria and physical breakage of the mitochondrial membrane barrier [77]. The mPT pore is a supramolecular complex thought to be composed of the voltage-dependent anion channel (VDAC) in the outer membrane, adenylate translocator (ANT) in the inner membrane and cyclophilin D in the mitochondrial matrix [78]. We wanted to determine whether Cyto *c* redistribution from mitochondria upon exposure to soluble prefibrillar aggregates involves opening of the mPT pore. To explore this mechanistically, we treated isolated respiring mitochondria with inhibitors of the various components of the mPT pore complex: BKA, an inhibitor of ANT; CsA, which targets cyclophilin D; DIDS, a VDAC inhibitor; and anti-VDAC antibodies to block VDAC activity and hence Cyto *c* release. As can be seen in Fig. 5, neither the pharmacological blockers of mPT nor the VDAC antibodies provided any protection against CCR triggered by $A\beta_{42}$ and WT α-syn aggregates. Moreover, FCCP, a proton ionophore that causes rapid and complete dissipation of the mitochondrial membrane potential, increased CCR additionally by 40–50% (Fig. 5). Taken together, these results suggest that aggregate-induced CCR occurs without formation of the mPT channel, and hence support a model in which $A\beta_{42}$ and α-syn interact directly with mitochondrial membranes in the induction of membrane permeabilisation.

3.5. Select compounds protect against targeting of mitochondrial membranes by aggregates

In previous studies we have shown that polyphenolic compounds can potently interfere with damage to phospholipid membranes induced by amyloid aggregates. Hence, we wanted to evaluate the efficacy of ten selected polyphenols (Api, Baic, Chr, EGCG, Gen, Mor, Myr, NDGA, Purp, RA) together with BTE and two NBB compounds (301C09, 293G02) on maintaining the integrity of mitochondrial membranes. However, the following compounds were found to evoke a significant disturbance of mitochondrial integrity by themselves and were thus eliminated from further testing: Api, Gen, Myr, 301C09, Purp, and Chr (Suppl. Fig. S4). An exception was made for NDGA in view of its previously demonstrated high efficacy in protecting neuronal-like membranes against damage by amyloid-β and α-syn oligomers [64,65].

Thus, Baic, EGCG, Mor, NDGA, RA, BTE and 293G02 were extensively tested using the liposome permeabilisation assay and the mitochondrial CCR assay, in the presence of $A\beta_{42}$ and WT α-syn aggregates. In each case, compounds were allowed to interact with preformed aggregate complexes for 10 min prior to incubation with

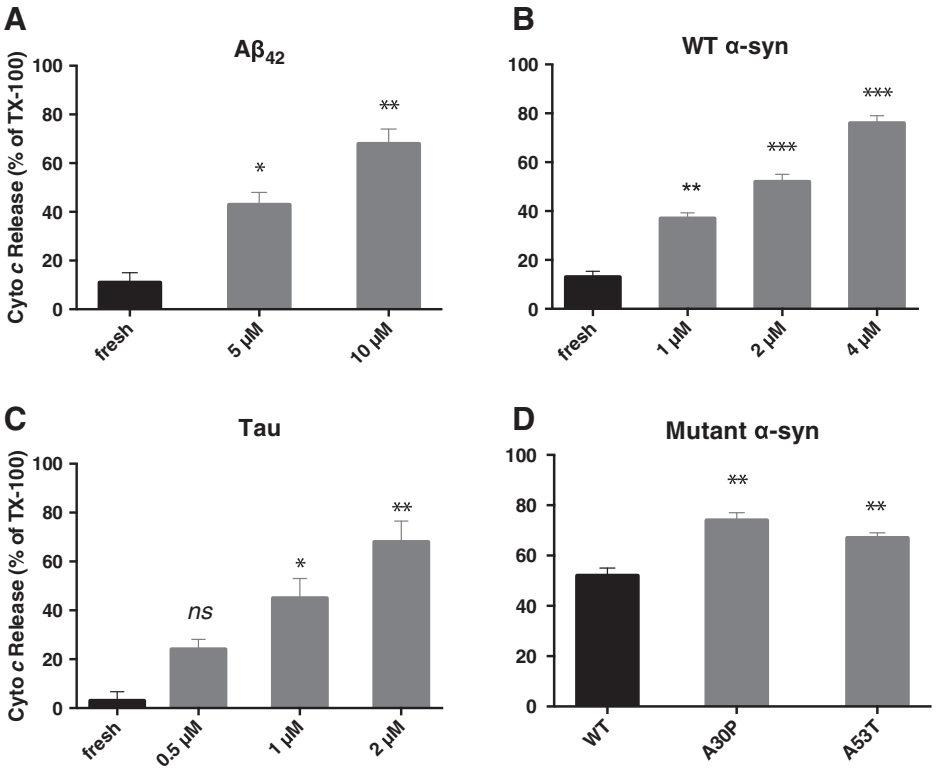

Fig. 4. Quantification of Cyto *c* release from isolated mitochondria. Mitochondria, isolated from SH-SY5Y cells, were incubated for 1 h at 30 °C with soluble prefibrillar aggregates: (A) 5 μM fresh (monomeric) and 5, 10 μM pre-aggregated $A\beta_{42}$; (B) 2 μM fresh and 1, 2, 4 μM pre-aggregated WT α-syn; and (C) 2 μM fresh and 0.5, 1, 2 μM pre-aggregated tau. A direct comparison was also made between 2 μM WT and 2 μM mutant (A30P and A53T) α-syn aggregates (D). Mitochondria were pelleted by centrifugation and the amount of Cyto *c* liberated in the resultant supernatant quantified using an immunoassay technique, as described in 'Materials and methods'. CCR by aggregates is expressed as a percentage of detergent Triton X-100 (theoretical maximium, 100%). Values represent means ± SEM ($n = 3$); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; *ns*, not significant.

liposomes or mitochondria. In this manner, compounds could potentially antagonise membrane perturbation by disruption of aggregate assemblies and/or by interfering with their membrane interaction. Indeed, the above-mentioned 8 compounds were almost all significantly effective in protecting synthetic and biological mitochondrial membranes from $A\beta_{42}$ and α-syn insult ($p < 0.001$, Fig. 6). Permeabilisation of OM-type lipid vesicles and mitochondrial CCR induced by $A\beta_{42}$ aggregates were decreased by at least 50% in the presence of compounds. The top protective compounds against $A\beta_{42}$ insult in both the liposome permeabilisation and the mitochondrial CCR assays were Baic, BTE, Mor and RA (Fig. 6A, B). The latter three compounds (BTE, Mor and RA) additionally proved to be highly effective against α-syn oligomers, by inhibiting permeabilisation of mito-like vesicles as well as efflux of Cyto *c* from mitochondria (Fig. 6C, D). We extended testing of RA against aggregates derived from mutant α-syn (A30P, A53T) and tau-441 peptide. RA remarkably decreased CCR induced by A30P α-syn aggregates to 8.7% (±4.3%, $n = 2$), by A53T α-syn aggregates to 17.3% (±1.7%, $n = 2$), and by tau aggregates to 11.7% (±1.1%, $n = 2$).

Statistical analysis by non-parametric Spearman's *rho* correlations (Table 1) revealed a striking correlation between rank order of compounds inhibiting mitochondrial membrane damage induced by $A\beta_{42}$ and WT α-syn aggregates (95% CI, $r = 0.857$, $p = 0.012$ for CCR; 95% CI, $r = 0.750$, $p = 0.033$ for OM-vesicles). Significant correlations were additionally found when comparing the two $A\beta_{42}$ (95% CI, $r = 0.786$, $p = 0.024$) and the two α-syn assays (95% CI, $r = 0.714$, $p = 0.044$) separately. The inference is that compounds are protecting membranes

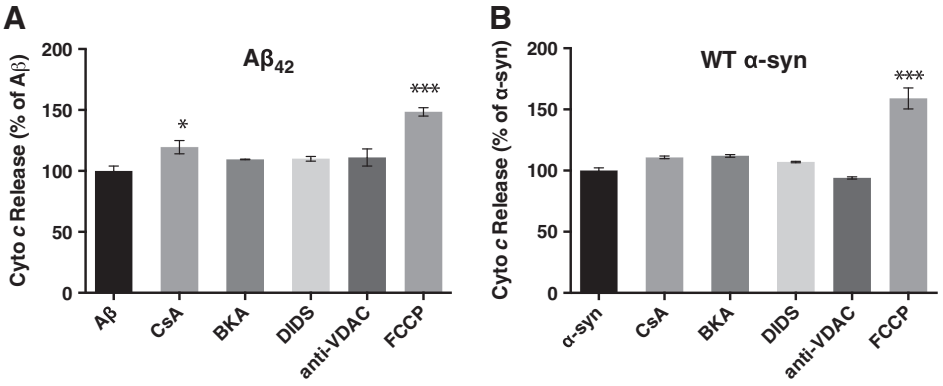

Fig. 5. Effect of modulators of the mPT pore on aggregate-induced CCR. Isolated mitochondria were pretreated with inhibitors of the PT pore (1 μM CsA and 10 μM BKA), inhibitors of VDAC (50 μM DIDS and 0.1 μg/μl anti-VDAC antibody) and the uncoupler FCCP (5 μM) for 10 min, before exposure to pre-aggregated 10 μM $A\beta_{42}$ (A) and 2 μM WT α-syn (B). After 30 min at 37 °C, mitochondria were centrifuged and the amount of Cyto *c* released into the supernatant was quantified as specified under 'Materials and methods'. None of the mPT inhibitors and VDAC blockers decreased CCR by either $A\beta_{42}$ or WT α-syn aggregates. Values represent means ± SEM ($n = 3$); $^{*}p < 0.05$; $^{***}p < 0.001$.

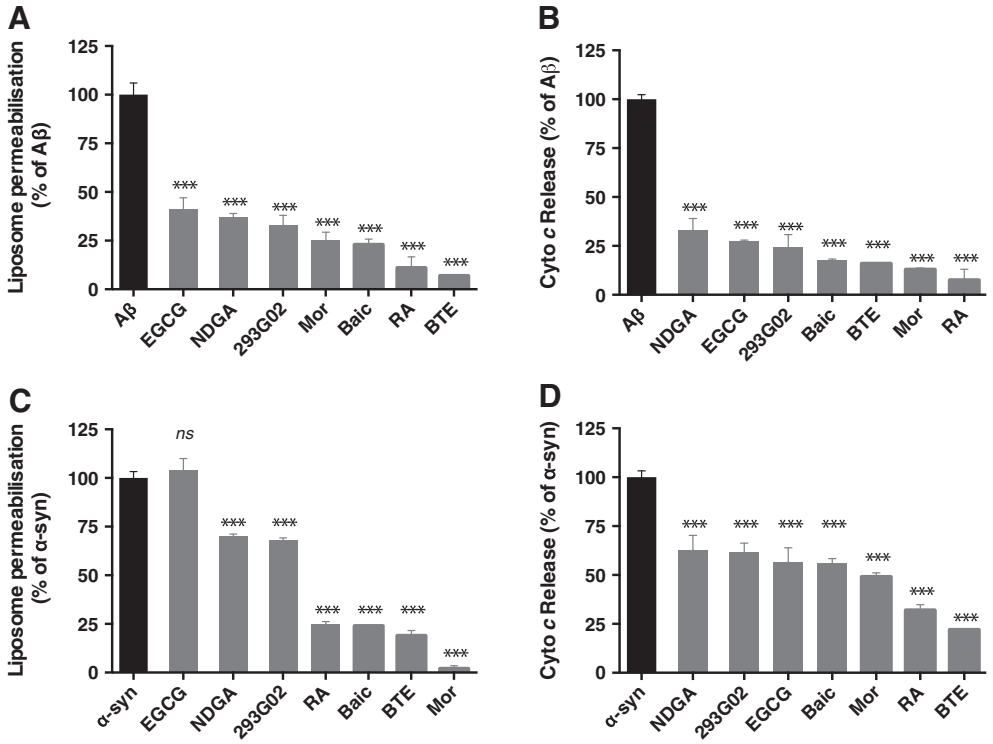

Fig. 6. Inhibitory effects of small-molecule compounds and black tea extract on the loss of mitochondrial membrane integrity induced by $A\beta_{42}$ and α-syn aggregates. 50 μM polyphenols, 10 μM NBB compound 293G02 and 3 μg/ml BTE were incubated with 1 μM $A\beta_{42}$ (A) or 0.1 μM α-syn (C) aggregates for 10 min. Subsequently, OM-vesicles were added and disruption of liposomal membranes monitored over 60 min. Values are calculated as a percentage of maximal liposome permeabilisation by Aβ or α-syn (100%; *y*-axis). For experiments using isolated mitochondria, 10 μM polyphenols, 5 μM NDGA, 10 μM 293G02 and 0.5 μg/ml BTE were incubated with 10 μM $A\beta_{42}$ (B) or 2 μM α-syn (D) aggregates for 10 min before adding respiring mitochondria. CCR was determined as a percentage of that induced by Aβ or α-syn alone (100%; *y*-axis). Values represent means ± SEM ($n \geq 3$) and were compared statistically using one-way ANOVA followed by Bonferroni's post-hoc test; $^{***}p < 0.001$; *ns*, not significant, when compared to Aβ or α-syn alone.

by mechanisms independent of the nature of the peptide. To gain more insight, we selected the top 3 compounds (BTE, Mor and RA) and looked at their effects on pre-formed oligomers of WT α-syn. Immunoblots were performed after incubating oligomers for 10 min with each of the 3 polyphenolic compounds (Fig. 7). Interestingly, the immunoblots illustrate varied effects of the polyphenols: BTE decreased low-molecular-weight oligomers whilst accelerating the formation of nontoxic fibrillar species; Mor decreased low-molecular-weight oligomers only; RA had no effect on the distribution of α-syn aggregate species.

In conclusion, the best compounds overall were black tea extract (high content of theaflavins), the small-molecule phenolic acid rosmarinic acid, and the flavonoids morin and baicalein. These compounds enhanced the resilience of the mitochondrial membrane barrier against perturbation induced by $A\beta_{42}$ and WT α-syn aggregates in all assays, albeit by different mechanisms.

4. Discussion

The progressive intraneuronal accumulation of amyloidogenic proteins and peptides like $A\beta_{42}$ [3], α-syn [5] and tau [53], exposes membranes of organelles including mitochondria to potential damage by the amyloid aggregates. Herein, we employed two simplified model systems to explore the generic molecular mechanisms operating upon interaction of amyloid aggregates with mitochondrial membranes. These *in vitro* models allowed us to avoid the complexity of the cellular milieu and consist of: (i) a liposomal model, an artificial membrane system in which defined liposomes were formed that mimic lipid compositions of outer and inner mitochondrial membranes; and (ii) an isolated mitochondria model, consisting of whole organelles isolated from SH-SY5Y human neuroblastoma cells.

Previous studies have reported Cyto *c* release upon the *in vitro* incubation of isolated mitochondria with high (20 μM or more) concentrations of $A\beta_{42}$ [79–81], and wild-type or mutant forms of α-syn [60,82]. In the present study, we compared monomeric and aggregated species of five different amyloidogenic polypeptides and applied them in physiological ranges compatible with those typically observed in the brain (1 μM or less). We first show that external application of soluble oligomeric forms of $A\beta_{42}$, tau, WT and mutant (A30P, A53T) forms of α-syn, efficiently permeabilised liposomes mimicking mitochondrial membranes by more than two-fold. Permeabilisation by Aβ and WT α-syn aggregates did not show any size selection, allowing FITC-dextran molecules (4–70 kDa) to pass through the membrane. This may argue against a pore-forming mechanism, although one cannot exclude the formation of pores that gradually coalesce leading to complete destruction of the membrane of a small unilamellar vesicle. Indeed, the kinetic profile of IM-type lipid vesicle permeabilisation suggests a pore-like mechanism, with a steady increase in vesicle leakage over time rather than an abrupt detergent-like membrane rupture. Lastly, we also demonstrate an affinity for the IMM by the channel-forming antimicrobial peptide, gramicidin. Gramicidin has been shown to bind CL-rich domains on the inner membrane of Gram-negative bacteria, causing leakage of cell contents into the periplasmic space [83]. Mitochondria have an endosymbiotic origin and retain many of the vestiges of their bacterial ancestry, including a double membrane.

Apart from liposome permeabilisation, low concentrations of soluble amyloid aggregates also induced efflux of Cyto *c* from isolated mitochondria, detected primarily by immunoassay and confirmed qualitatively by immunoblot. In fact, the maximal CCR effect of the aggregate complexes was comparable to that of Triton X-100 detergent, which solubilises membranes (60–80% of Trx-100).

Since none of the monomeric peptides were significantly damaging to mitochondrial membranes, permeabilisation most likely represents a specific, toxic gain-of-function of the aggregate species. Comparing the membrane-disruptive effects of the various proteins, it is notable that

Table 1
Correlations between the inhibitory effects of the tested compounds on (a) lipid vesicle permeabilisation and (b) mitochondrial CCR, induced by pre-formed aggregates of $A\beta_{42}$ and WT α-syn. Correlations were determined from the ranking of compounds based upon the results shown in Fig. 6.[a]

	Inhibition of vesicle permeabilisation by $A\beta_{42}$	Inhibition of vesicle permeabilisation by α-syn	Inhibition of mitochondrial CCR by $A\beta_{42}$	Inhibition of mitochondrial CCR by α-syn
Inhibition of vesicle permeabilisation by $A\beta_{42}$	$r = 1$	$r = 0.75$ ($p = 0.033$)	$r = 0.786$ ($p = 0.024$)	–
Inhibition of vesicle permeabilisation by α-syn	$r = 0.75$ ($p = 0.033$)	$r = 1$	–	$r = 0.714$ ($p = 0.044$)
Inhibition of mitochondrial CCR by $A\beta_{42}$	$r = 0.786$ ($p = 0.024$)	–	$r = 1$	$r = 0.857$ ($p = 0.012$)
Inhibition of mitochondrial CCR by α-syn	–	$r = 0.714$ ($p = 0.044$)	$r = 0.857$ ($p = 0.012$)	$r = 1$

[a] The Spearman rank-order correlation coefficient, *rho*, ranges from −1 to +1; r = −1.0, perfect inverse correlation; r = 0, the two variables do not vary together at all; r = +1.0, perfect correlation.

mutant A30P and A53T were much more damaging to mitochondrial membranes than their wild-type counterpart. This effect was consistent on both biological and synthetic membranes. Hence, the accelerated neurotoxicity of familial PD mutants [84] may be mechanistically linked with a higher potential for mitochondrial interaction and disruption. Previous biophysical studies report similar lipid binding profiles for WT and A53T α-syn, but decreased lipid affinity for the A30P mutation; however, such studies were not conducted on mitochondrial-like membranes [85,86]. Another interesting aspect from our studies is the high toxicity of tau aggregates on mitochondrial membranes. For instance, liposome permeabilisation by 0.1 μM tau is equivalent to 1 μM $A\beta_{42}$ (circa 2-fold), whilst the extent of CCR from isolated mitochondria induced by 2 μM tau is similar to 10 μM $A\beta_{42}$ (circa 1.6-fold). These results indicate that tau may be the more potent mitochondrial membrane destabiliser of the two peptides.

The general observation that results obtained with isolated mitochondria mirror those obtained with artificial membrane systems (*i.e.* lipid vesicles), strengthens the hypothesis that amyloid species are able to directly perforate mitochondrial membranes. To substantiate further, we treated mitochondria with known pharmacological inhibitors of CCR from mitochondria, namely blockers of the inner-membrane mPT pore (CsA, BKA) or of the outer-membrane VDAC (DIDS, anti-VDAC antibody). However, none of these agents could mitigate aggregate-induced CCR, suggesting that indeed the mechanism most likely involves direct OMM and/or IMM destabilisation *via* an interaction of aggregate species with membrane phospholipids. That CCR by aggregates does not occur as a consequence of depolarisation

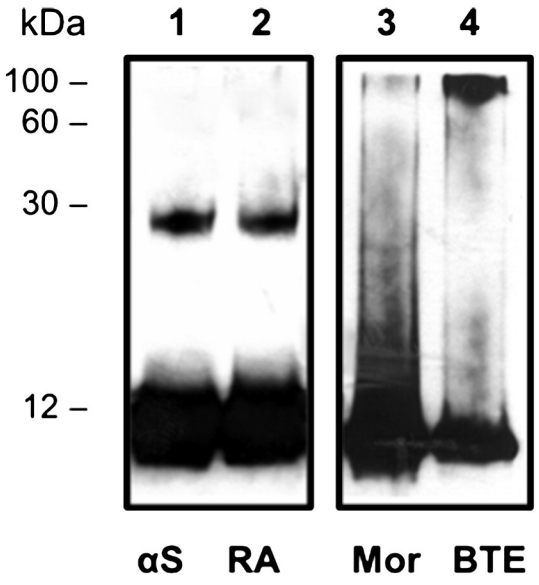

Fig. 7. Western blots of recombinant wild-type α-syn incubated with polyphenolic compounds. 0.7 μg aggregated WT α-syn (Lane 1) was incubated for 10 min with 100 μM RA (Lane 2), 100 μM RA (Lane 3) and 6 μg/ml BTE (Lane 4). After, the samples were separated using SDS-PAGE and probed with anti-synuclein (15G7) monoclonal antibody.

of the IMM is also indicated by the fact that pre-incubation of mitochondria with FCCP, which induces a complete collapse of the mitochondrial membrane potential, further enhances CCR. Albeit beyond the scope of the current study, it should be emphasised that, in the cellular context, release of Cyto *c* from the intermembrane mitochondrial space is highly regulated by actions of members of the Bcl-2 family. Pro-apototic members such as Bax, Bad and Bid, induce loss of OMM integrity with subsequence CCR, whereas anti-apoptotic members such as Bcl-2 and Bcl-X_L protect the integrity of the mitochondrion and prevent release of death-inducing factors [87]. Hence, amyloid aggregates could potentially induce CCR by altering the levels of these key regulatory molecules. In fact, apoptosis in hippocampal neurons incubated with soluble Aβ oligomers was associated with increased levels of Bid, Bax and Cyto *c* and lower levels of the anti-apoptotic Bcl-2 protein [88]. In PD, disruption of 14-3-3 signalling function by α-syn results in the release of pro-apoptotic factors such as Bax [89]. It would therefore be important to address such aspects in future work.

An additional key finding of this study is the preference shown by the aggregated forms of $A\beta_{42}$, WT α-syn and tau amyloids for compromising the integrity of membranes with a high content of CL, a unique anionic phospholipid specific to mitochondria.

In fact, membranes with 15% CL content (similar to inner mitochondrial membranes) sustained the highest degree of permeabilisation; on average, at least twice that sustained by liposomes lacking CL. Exceptionally, mutant A30P and A53T α-syn aggregates were not sensitive to CL content and strongly permeabilised all types of lipid vesicles. Regarding oligomeric α-syn, the targeted disruption of lipid vesicles having negatively charged membranes, including those containing CL, has been previously cited [90]. More recently, Zigoneanu et al. [91] also concluded that CL is essential for the interaction between WT and A30P α-syn with large unilamellar vesicles whose composition is similar to that of the inner mitochondrial membrane. Finally, affinity of α-syn to CL has been postulated to drive mitochondrial fission by α-syn oligomers, but not monomers [63].

The affinity of CL to amyloid aggregates likely provides a platform for rapid and enhanced membrane destabilisation, as demonstrated by the fact that CL-containing vesicles showed a more sustained increase in permeabilisation. In summary, it is highly plausible that mitochondria-specific CL is a novel *leitmotif* in the targeting of the inner mitochondrial membrane by aggregation-prone peptides. Cardiolipin would increase protein accumulation at the inner membrane, hence acting as an effective catalyst for generation of an amyloid peptide's most toxic form *in situ*. In relation to this, it was reported that CL was the phospholipid found to most strongly stimulate Aβ aggregation (CL > PI > PS > PC = PE) [92].

The significance of such a scenario lies in the fact that CL orchestrates the launching of mitochondrial apoptosis, with detachment of Cyto *c* from CL and its mobilisation into the cytosol representing key events in the apoptotic process [93]. Interestingly, it has been shown using similar artificial membrane systems to ours, that mitochondrial membrane targeting and permeabilisation by Bcl-2 family proteins also requires CL [74,94]. An intriguing possibility is therefore that amyloid aggregates seem to be mimicking the action of endogenous pro-apoptotic factors, such as tBid and Bax, on mitochondrial

membranes. The capacity of amyloid aggregates to specifically target and directly disrupt mitochondrial membranes is also strikingly reminiscent of the action of virulent toxins from bacterial pathogens; examples include PorB porin of *Neisseria gonorrhoeae* [75,95], toxin B from *Clostridium difficile* [96] and VacA toxin from *Helicobacter pylori* [97]. Hence, the idea that the molecular mechanisms by which misfolded protein aggregate complexes cause disease are shared by those of bacterial pore-forming toxins [98], can now also be extended to the involvement of mitochondrial membranes.

Finally, we decided to screen low-molecular-weight molecules (Baic, EGCG, Mor, NDGA, RA, 239G02 and black tea extract) for their ability to block, or attenuate, perturbation of mitochondrial membranes by amyloid peptides. The tight correlation ($r = 0.714–0.857$) between the ranking of the compounds in the liposome and CCR assays is remarkable. The implication is that compounds are acting on common structural elements in aggregate complexes, on the mitochondrial lipid membranes themselves, or both. The most effective compound overall is BTE, which accelerated the formation of nontoxic fibrillar species, at the expense of low-molecular-weight oligomers. Theaflavins, the main polyphenolic constituents present in black tea, in fact direct the formation of Aβ and α-syn into nontoxic assemblies [99]. We have also reported BTE as a highly effective compound in protecting against membrane permeabilisation by $A\beta_{42}$ and α-syn oligomers in our previous studies [64,65,100]. Besides, several epidemiological studies support the notion of a protective effect of tea drinking on the development of dementia or PD [101–103].

Rosmarinic acid was found to be the most potent of the small-molecule compounds, efficiently inhibiting permeabilisation of liposomes and also blocking CCR from isolated mitochondria. RA is a polyphenol naturally present in several herbs in the *Lamiaceae* family; dietary sources include rosemary, oregano, sage and thyme. RA can interact with the polar headgroups of lipids and insert spontaneously in the membrane, without causing any alteration of membrane structure [104]. Its mechanism of activity could also involve binding to the aggregate complexes, although we did not find any direct effect of RA on the size of WT α-syn oligomers by immunoblotting. This is conformant with our previous studies using confocal single-molecule fluorescence spectroscopy, in which RA was only found to be a weak disaggregator of α-syn oligomers [100]. Nonetheless, it is still possible that RA interferes directly with the interaction between existing amyloid aggregates and membranes. Interestingly, pre-treatment of dopaminergic cells with RA protected against mitochondrial insult by 1-methyl-4-phenylpyridinium (MPP^+) and restored complex I activity of the mitochondrial respiratory chain [50]. Taken together with our findings, such evidence suggests that RA can be regarded as a potential mito-protective agent in AD and PD.

As in our previous permeabilisation studies, Mor and Baic were also highly effective compounds [64,65]. Both are flavonoids having a common 5,7-dihydroxy-4-oxo-moiety; this structural characteristic has been reported to significantly enhance the affinity for lipid bilayers [105]. Morin is also a strong disaggregator of toxic Aβ [106] and α-syn [100] oligomers, in agreement with this study in which a decrease in low-molecular-weight oligomers was observed in Western blots. Baicalein and 293G02 dissolved α-syn oligomers bound to lipid vesicles, without rupturing the latter [107]. In comparison to Baic and Mor, EGCG was a much less effective inhibitor of mitochondrial membrane permeabilisation, ranking 5th–7th in both the liposome and organelle assays. In fact, although EGCG can disassemble preformed Aβ and α-syn amyloid fibrils [108], it is much less effective in the presence of lipid membranes [109].

A recognised obstacle to the therapeutic use of polyphenols is their poor bioavailability and inefficient delivery to the brain following oral administration [66]. Nevertheless, promising results have recently been obtained using improved delivery methods in which polyphenol-conjugated nanoliposomes, or nanolipidic particles, are administered. Oral bioavailability of EGCG in rats, for instance, was doubled [110] whilst a robust increase of resveratrol concentration in brain tissue of rats was achieved *in vivo* [111]. This provides important preliminary evidence that such delivery methods would allow translation of *in vitro* research on polyphenols into useful application for targeted drug delivery in clinical trials.

5. Conclusion

Our findings point to a common mechanism through which mitochondrial dysfunction in neurodegenerative diseases is induced by misfolded amyloid aggregates. Such a mechanism would involve destabilisation of mitochondrial membranes *via* a direct interaction of intracellular aggregate species with membrane phospholipids, without the requirement of any other protein. Rather, the specific targeting to mitochondria is mediated by an intrinsic affinity of aggregate complexes for cardiolipin, an acidic phospholipid unique to mitochondrial membranes and an important integrator of apoptosis. Mitochondria are abundant in synapses, hence damage by the amyloid aggregates would initiate apoptotic biochemical cascades locally in synapses and dendrites, resulting in the early synaptic degeneration characteristic of AD and PD [112]. We also identify black tea extract, rosmarinic acid, morin and baicalein as potential drug candidates for enhancing the resilience of the mitochondrial membrane barrier against insult by amyloid aggregates.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.06.026.

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