Polyphenolic compounds are novel protective agents against lipid membrane damage by α-synuclein aggregates *in vitro*

Mario Caruana [a,1], Johanna Neuner [a,b,1], Tobias Högen [c], Felix Schmidt [b], Frits Kamp [d], Charles Scerri [e], Armin Giese [b], Neville Vassallo [a,*]

[a] *Department of Physiology and Biochemistry, University of Malta, Msida, Malta*
[b] *Zentrum für Neuropathologie und Prionforschung, Ludwig-Maximilians-Universität, München, Germany*
[c] *Neurologische Klinik, Klinikum Großhadern, Ludwigs-Maximilians-Universität, München, Germany*
[d] *Adolf-Butenandt-Institut, Ludwig-Maximilians-Universität, München, Germany*
[e] *Department of Pathology, University of Malta, Msida, Malta*

ARTICLE INFO

ABSTRACT

Cumulative evidence now suggests that the abnormal aggregation of the protein α-synuclein (αS) is a critical factor in triggering neurodegeneration in Parkinson's disease (PD). In particular, a fundamental pathogenetic mechanism appears to involve targeting of neuronal membranes by soluble oligomeric intermediates of αS, leading to their disruption or permeabilisation. Therefore, a model assay was developed in which fluorophore-loaded unilamellar vesicles were permeabilised by soluble oligomers, the latter formed by aggregation of human recombinant αS protein. The αS oligomers induced an impairment of membrane integrity similar to that of the pore-forming bacterial peptide gramicidin. The lipid vesicle permeabilisation assay was then utilised to screen 11 natural polyphenolic compounds, 8 synthetic N′-benzylidene-benzohydrazide compounds and black tea extract for protection against membrane damage by wild-type and mutant (A30P, A53T) synuclein aggregates. A select group of potent inhibitory compounds included apigenin, baicalein, morin, nordihydroguaiaretic acid, and black tea extract. Structure–activity analysis further suggests that a 5,7-dihydroxy-chromen-4-one moiety appears to be favourable for the inhibition reaction. In conclusion, we have identified a group of polyphenols that can effectively hinder membrane damage by αS aggregates. These may serve as a viable source of lead compounds for the development and design of novel therapeutic agents in PD.

1. Introduction

Alpha-synuclein (αS) is a 14.5 kDa, highly-conserved, neuronal protein that is widely distributed throughout the brain and expressed predominantly in pre-synaptic terminals [1–4]. Increased expression of αS and pathologically altered forms of this protein have been implicated in the pathophysiology of both familial and sporadic Parkinson's disease (PD), culminating in a loss of nigrostriatal dopaminergic neurons [5].

PD is the most common movement disorder and affects approximately 1% of the population over the age of 50 with classical clinical manifestations of bradykinesia, muscle rigidity, resting tremor and postural instability [6].

The role of the various physical forms (i.e. monomers, soluble oligomers, protofibrils or fibrils) of αS in PD pathogenesis remains controversial. Deposits of high-molecular-weight, fibrillar αS aggregates in neurons, termed Lewy bodies, are a ubiquitous pathological feature of PD [7]. Substantial data from *in vitro* and *in vivo* studies, however, supports the hypothesis that soluble αS oligomeric intermediates represent the principal pathogenic species [8–10]. It has been shown that αS oligomers share a common structure with other amyloidogenic proteins, such as amyloid-beta, amylin, insulin, and the cellular prion protein, implying a common mechanism of pathogenesis [11]. Particularly, there is increasing evidence that these oligomers target biological membranes, possibly forming structures with pore-like morphologies that contribute to cytotoxicity in neurodegenerative diseases *via* the disruption of cellular and organelle membranes [12–14]. Lipid-bound oligomers of αS were isolated from brains of transgenic mice as well as from PD patients [15].

The nature of the interaction of αS with biological and artificial membranes is complex [16,17] and dependent on the phospholipid composition of the membranes, the size of the vesicles, as well as the ratio of membrane lipids to protein [18,19]. Thus, αS binds preferentially to small unilamellar vesicles (SUVs) containing acidic phospholipids such as phosphatidic acid, phosphatidylserine and phosphatidylglycerol but less strongly to vesicles with a net neutral charge such as phosphotidylcholine and phosphatidylethanolamine [20,21]. Only the positively-charged N-terminal and hydrophobic central parts of αS (residues 9–90) partake in lipid binding [22] whilst the negatively charged C-terminal region has been proposed to act as a scaffold to recruit additional proteins to the membranes [23].

Oligomeric αS has been observed to induce bilayer disruption upon tight binding to membranes containing anionic lipids [24,25]. The leakage of vesicular contents caused by αS oligomers showed a strong preference for low-molecular-mass molecules, implying a pore-like mechanism for permeabilisation; monomeric αS was determined to be less pore forming [24]. In support of these observations, soluble αS oligomers, but not monomers or fibrils, were able to form pores in planar lipid bilayers [26]. In cellular models, increased calcium influx has been reported using preformed aggregates of αS incubated with Fe^{3+} and organic solvent [27]. Expression of mutant αS (Ala30Pro [A30P] and Ala53Thr [A53T]) is associated with a non-selective ion permeability of the cellular membrane, presumably as a consequence of relatively large pores [28]. Moreover, both A30P and A53T mutants have been shown to form pore-like annular and tubular protofibrillar structures, whilst wild-type (WT) αS formed annular protofibrils only after extended incubation [29]. Pore formation may therefore play a role in the pathophysiology of the aggregated protein by permitting uncontrolled flux of ions into and out of cells. Aside from pore formation, another mechanistic possibility involves thinning of the plasma membrane [30,31]. Membrane thinning was directly observed during aggregation of WT and mutant αS on lipid bilayers; lipid molecules were extracted from the bilayer by the growing aggregates, ultimately resulting in extensive bilayer disruption [32]. A recent landmark study reported a direct link between membrane-bound αS oligomers and severe dopaminergic neuronal loss in a murine model [33]. The most severe neuronal toxicity correlated with those αS variants that formed oligomers; in turn, the more toxic oligomerising mutants exhibited a higher affinity to bind liposomes, implying a stronger reactivity to membranes [33]. Hence, membrane damage by oligomeric αS is considered a likely mechanism of cytotoxicity in PD, which in turn implies that compounds which interfere with disruption and permeabilisation of membranes by αS have a potential role in PD.

Currently, no preventive therapy is available for PD [34]. Over the past decade, intensive research has gone into identifying small organic molecules that can inhibit and/or disaggregate αS oligomer formation *in vitro* [27,35]. Among the most studied are the natural polyphenolic compounds, characterised by the presence of multiple hydroxyl groups on aromatic rings [36–40]. Polyphenols either form soluble, non-toxic, oligomeric complexes with the αS protein or disaggregate mature multimeric structures into smaller, non-toxic aggregates [41–44]. Polyphenols can also interact with and permeate phospholipid membranes [45]. Both polar and nonpolar forces were shown to have a significant impact on flavonoid–biomembrane interactions [46]. Results of a study in which alterations in membrane fluidity in a phospholipid bilayer were monitored, suggest that flavonoids and isoflavonoids, similar to cholesterol and alpha-tocopherol, partition into the hydrophobic core of the membrane and cause a dramatic decrease in lipid fluidity [47].

In summary, it is highly desirable to develop compounds that can interfere with membrane damage by oligomeric αS since they could offer powerful therapeutical potential. A liposome permeabilisation assay, in which synthetic lipid vesicles were exposed to pre-aggregated αS, was used as a robust screening method to identify such compounds. We thereby tested a select group of natural polyphenols and N′-benzylidene-benzohydrazide compound derivatives, that we had previously identified as potent inhibitors of αS oligomer formation [27,40].

2. Materials and methods

2.1. Materials

Compounds tested in this work were obtained as follows: apigenin (Api), baicalein (Baic), black tea extract (BTE; >80% theaflavins), (−)-epigallocatechin gallate (EGCG), genistein (Gen), ginkgolide B (Gink), morin (Mor), nordihydroguaiaretic acid (NDGA), propyl gallate (PropylG), purpurogallin trimethyl ether (Purp) and resveratrol (Resv) were all purchased from Sigma-Aldrich (Munich, Germany); scutellarein (Scut) was obtained from Pharmasciences Laboratories (Cour-bevoie, France); N′-benzylidene-benzohydrazide (NBB) compounds were obtained from Chembridge Corp. (San Diego, CA, USA). In all cases, the purity of the compounds was >98%. Polyphenols, NBB compounds and BTE were prepared as stock solutions in DMSO and stored at −20 °C. During the experiments, compounds were protected from light and used immediately after thawing. Unless otherwise stated, all other chemicals indicated in the protocols below were purchased from Sigma.

2.2. Expression and purification of α-synuclein

Wild-type (WT) or mutant (A30P, A53T) human recombinant αS-containing pET-5a plasmid was transfected into *Eschericia coli* BL21 (DE3) cells (Novagen, Madison, WI, USA). Expression was induced with isopropyl-D-thiogalactopyranose (Promega, Mannheim, Germany) and the proteins purified as described previously [48]. Protein concentration was determined using a bicinchoninic acid protein-quantification kit (Pierce, Rockford, IL, USA). Aliquots of purified recombinant synucleins (0.5–1.0 mg/l) were stored at −80 °C.

2.3. Fluorescent labelling of α-synuclein

Fluorescent labelling of αS with Alexa Fluor-488-O-succinimidylester (green) and Alexa Fluor-647-O-succinimidylester (red) (Invitrogen, Eugene, OR, USA) was performed as previously described [27,48]. Quality control of labelled αS was done by fluorescence correlation spectroscopy measurements on an Insight Reader (Evotec-Technologies, Hamburg, Germany). Aliquots of purified recombinant fluorescently-labelled monomeric synuclein were stored at −80 °C.

2.4. Preparation of wild-type and mutant α-synuclein aggregates

The aggregation assay was performed as described [48]. Briefly, 7 μM monomeric αS with 1% (v/v) DMSO and 10 μM $FeCl_3$ in sterile phosphate-buffered saline (PBS) buffer (pH 7.4) was incubated for 4 h at 25 °C. Concentrations given for aggregated αS refer to the respective equivalent monomer concentration.

2.5. Confocal single particle analysis

Fluorescence correlation spectroscopy (FCS), fluorescence intensity distribution assay (FIDA) and scanning for intensely fluorescent target (SIFT) measurements were carried out on an Insight Reader (Evotec-Technologies, Hamburg, Germany) according to established protocols [48]. In general, fluorescence emitted from dual colour excitation at 488 and 633 nm was recorded simultaneously with two single-photon detectors. Photons were summed over time intervals of constant length (bins) using a bin length of 40 μs. The frequency of specific combinations of "green" and "red" photon counts, derived from monomeric control or aggregation measurements respectively,

was recorded and presented in two-dimensional intensity distribution histograms, as shown in Fig. 1A.

2.6. Thioflavin T assay

Thioflavin T (ThT) is a dye widely used for the detection of cross-β-sheet structures in proteins, characteristic of the amyloid fibrillar form. The ThT assay was modified from an established protocol [49]. Aggregation of αS was monitored over 3 weeks; at each sampling time, an aliquot of the αS solution was diluted in 12 μM ThT (in PBS, pH 7.5). Fluorescence was measured in black 96-well plates using an FLx800-TBID microplate reader (BioTek, Germany), with excitation at 440 nm and emission at 490 nm (slits of 10 nm). To allow for background fluorescence, the fluorescence intensity of a blank PBS solution was subtracted from all readings. Data were measured in triplicates and averaged for evaluations.

2.7. Preparation of SUVs

Vesicles were prepared from a synthetic phospholipid blend (50 mg/ml, Avanti Polar Lipids, Alabaster, AL, USA) consisting of 1,2-dioleoyl phosphatidylethanolamine (DOPE), 1,2-dioleoyl phosphatidylserine (DOPS), 1,2-dioleoyl phosphatidylcholine (DOPC) in a molar ratio of 5:3:2 (w/w) in chloroform. These vesicles have a net negative charge and lipid moieties native to the neuronal environment [50]. Typically, 50 μg of lipids was thoroughly dried from a chloroform solution under vacuum, to yield a thin lipid film at the bottom of a glass vial. The thin film was hydrated by adding 500 μl of 100 mM KCl, 10 mM MOPS/Tris, 1 mM EDTA pH 7.0, containing 50 μM Oregon Green® 488 BAPTA-1 (OGB-1; Invitrogen, Darmstadt, Germany). The suspension was left to hydrate, with vortexing approximately every 15 min until it turned very cloudy. Subsequently, 60 μl of 0.8 M Mega-9 detergent was added to the suspension, which was then

Fig. 1. Aggregation of purified recombinant wild-type αS. (A) Two-dimensional intensity distribution histograms obtained in an independent experiment showing the induction of large oligomers using 1% DMSO and 10 μM Fe^{3+} from monomeric αS. The αS aggregates are detected as high-intensity signals by SIFT analysis. (B) Fibrillisation kinetics of WT aggregated αS monitored by ThT fluorescence (in arbitrary units, a.u.) compared to control aggregation buffer only (1% DMSO and 10 μM Fe^{3+} in sterile PBS, pH 7.4). Sampling of the αS mix (WT αS) and the aggregation buffer (CTR) was carried out over 21 days. Data shown as the mean of duplicate measurements (S.E.M. bars < 10%).

vortexed gently until it appeared clear again. The lipid-solution was then carefully loaded into Spectra/Por® membrane tubing (pore size, 3.5 kDa; Spectrum Labs, Breda, The Netherlands) and subjected to dialysis for 6 h at 4 °C against a buffer (5 l) containing 100 mM KCl, 10 mM MOPS/Tris, pH 7.0. This was followed by two successive overnight dialyses at 4 °C in the dark, with renewal of fresh buffer each time. Liposomes were retrieved from the tubing and kept at 4 °C covered in aluminium foil. The size and uniformity of the vesicle population were measured using a Zetasizer Nano S dynamic light scattering device (Malvern, Worcestershire, UK). The vesicles were relatively uniformly-sized with an average diameter of 65 ± 10 nm (data not shown), and hence categorised as SUVs.

A similar protocol was used to prepare calcein-loaded liposomes, with the following changes: (i) the lipid film was resuspended in 500 μl of 50 mM calcein, and (ii) untrapped calcein molecules were removed using G-25 Sephadex columns (GE Healthcare, Munich, Germany) after detergent-dialysis.

2.8. Liposome permeabilisation assays

Liposome permeabilisation assays were carried out as described previously [51], using OGB-1 or calcein as encapsulated fluorophores. Disruption of liposomes containing OGB-1 causes an increased fluorescence (exc. 485 nm, em. 528 nm) as a result of Ca^{2+} influx and/or OGB-1 efflux. Release of calcein molecules from inside the lipid vesicles increases fluorescence (exc. 495 nm, em. 520 nm) due to relief of self-quenching by the dye. Fluorescence measurements were carried out using an FLx800-TBID microplate reader (BioTek, Germany) whilst fluorescence intensity data was acquired using KC Junior™ software (BioTek, Germany). Aggregated αS was added to 50 μM liposomes in assay buffer (1 mM $CaCl_2$, 100 mM KCl, 10 mM MOPS/Tris, 1 mM EDTA, pH 7.0). Typically, the 5-min values were selected and the percentage permeabilisation effect was calculated according to the equation:

$$\text{Permeabilisation } (\%) = 100 \times (F-F_0)/(F_{max}-F_0),$$

where F denotes the fluorescence intensity of the measured sample; F_{max} denotes the maximum fluorescence intensity after the addition of 2 μM ionomycin (a chelating calcium ionophore) in the case of OGB-1 liposomes (ca. 6-fold increase), or after the addition of 5% M-PER® membrane detergent (Pierce, Rockford, IL, USA) in the case of calcein liposomes (ca. 10-fold increase); and F_0 represents the fluorescence of intact vesicles. Fluorescence intensity values are expressed as relative fluorescence units (RFUs).

2.9. Assays for inhibition of αS-induced liposome permeabilisation

Small-molecule compounds and BTE (typically 50 μM polyphenols, 10 μM NBB compounds and 3 μg/ml BTE) were tested for inhibition of liposome permeabilisation by two protocols. In protocol *A*, the compounds were incubated for 10 min with 50 μM fluorophore-loaded liposomes, before adding 0.5 μM aggregated αS. In protocol *B*, compounds were first incubated with aggregated αS for 10 min, prior to the addition of liposomes. Disruption of lipid vesicles by αS in the presence of compound was calculated as a percentage of permeabilisation caused by αS alone (theoretical maximum, 100%). Autofluorescence was measured for each compound and subtracted from the sample well values. Fluorescence intensity values are given in RFU.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad™ Prism 5 software (GraphPad Software, Inc.). Unless expressly indicated in the figure legend, results were expressed as the means and the
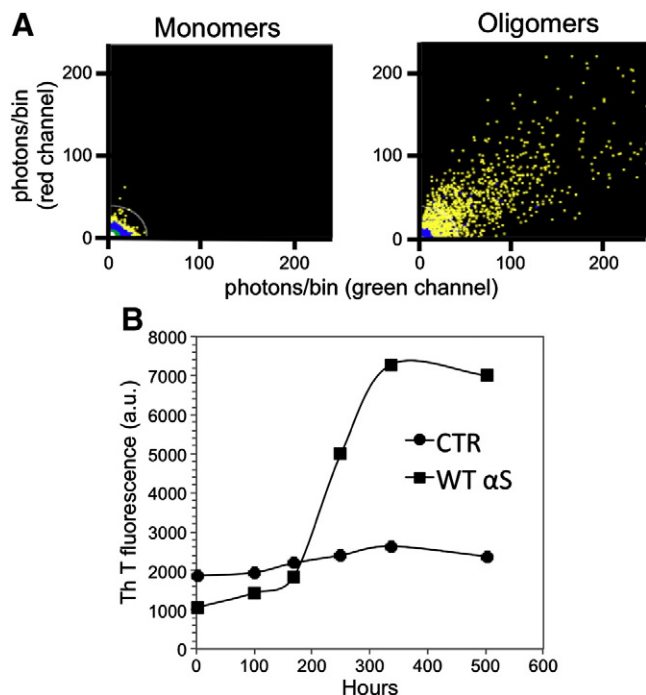
standard error of the mean (SEM) values, with n as the number of experiments. Differences between means were determined by unpaired Student's t test. In all analyses, the null hypothesis was rejected at the 0.05 level.

3. Results and discussion

3.1. Generation of wild-type αS oligomers

In several previous studies we have robustly shown that organic solvent (DMSO) and low micromolar Fe^{3+} ions trigger *in vitro* aggregation of monomeric αS into oligomeric species [27,40,48,52]. Oligomers were detected by FIDA and SIFT analysis, indicating formation of large aggregates of >100meres (Fig. 1A) [27,48]. AFM measurements confirmed these results and revealed an approximately 100-fold increase in particle size compared to αS monomers [27]. Characterisation of the aggregate species by immunoblotting additionally showed dimers, trimers, tetramers, pentamers and hexamers [48]. The latter is not unexpected, given the relatively low concentration of αS monomer (7 μM), the short duration of incubation (4 h) and the temperature (22 °C). In the present study, we additionally carried out ThT assays to demonstrate that there was no increase in fibrillar structure of the αS sample during the first 4 h of incubation. Rather, a gradual aggregation of WT αS into amyloid fibrils occurred over 2–3 weeks, consistent with a slow nucleation-dependent polymerisation model (Fig. 1B) [53].

3.2. Kinetics of lipid vesicle permeabilisation

The next step involved testing the αS aggregates for their effects on lipid vesicle integrity. SUVs having a net negative charge and lipid moieties native to the neuronal environment were prepared and loaded with OGB-1, a Ca^{2+}-sensitive fluorescent dye, and exposed to various concentrations of preformed WT αS oligomers. Enhanced fluorescence from Ca^{2+}-bound OGB-1 was monitored for approximately 30 min; thus, the kinetic measurements reflect impairment of vesicle membrane integrity over time. A typical kinetic recording of the permeabilisation process is shown in Fig. 2A. The permeabilisation reaction was characterised by a rapid initial phase (~5 min) followed by a steady, but more gradual, increase in signal (0.1 and 0.2 μM αS) or saturation (0.5 μM αS). Indeed, aggregated wild-type αS manifested a clear dose-dependent increase in vesicle permeabilisation over 0.1–0.8 μM, comparable to that reported in previous studies (Fig. 2B) [24,54]. It is likely that the increase in permeabilisation with higher concentrations of αS oligomers reflects a larger fraction of affected liposomes [54]. Due to a robust (ca. 30%) and immediate (5 min) liposome disruptive effect with 0.5 μM αS, this concentration was chosen for most of the subsequent experiments. Of note, 0.5 μM 'fresh' (i.e. non-aggregated) αS had a 3-fold lower permeabilising effect on the vesicles (Fig. 2B). This confirms the greater membrane affinity of oligomers compared to monomers [54]. Since Ca^{2+} ions might artificially influence the OGB-1 liposome permeabilisation assay, calcein-filled liposomes were also prepared and exposed to aggregated WT αS. Accordingly, 0.5 μM oligomeric αS induced a significant increase in calcein fluorescence at 5 min when compared to liposomes alone. In terms of kinetics, the results obtained were similar to the OGB-1 assays (Fig. 2A and Suppl. Fig. S1). Therefore, the presence of Ca^{2+} ions in the OGB-1 assays was not having any influence on the ability of αS oligomers to disrupt vesicular membranes.

3.3. Membrane perturbation is suggestive of a pore-like mechanism

Two results from the permeabilisation assays point to the likelihood that aggregated WT αS is relatively efficient at destabilising vesicles through a pore-like mechanism, linked to small oligomeric complexes (trimers to octamers). First, Zn^{2+} (but not Mg^{2+}) ions
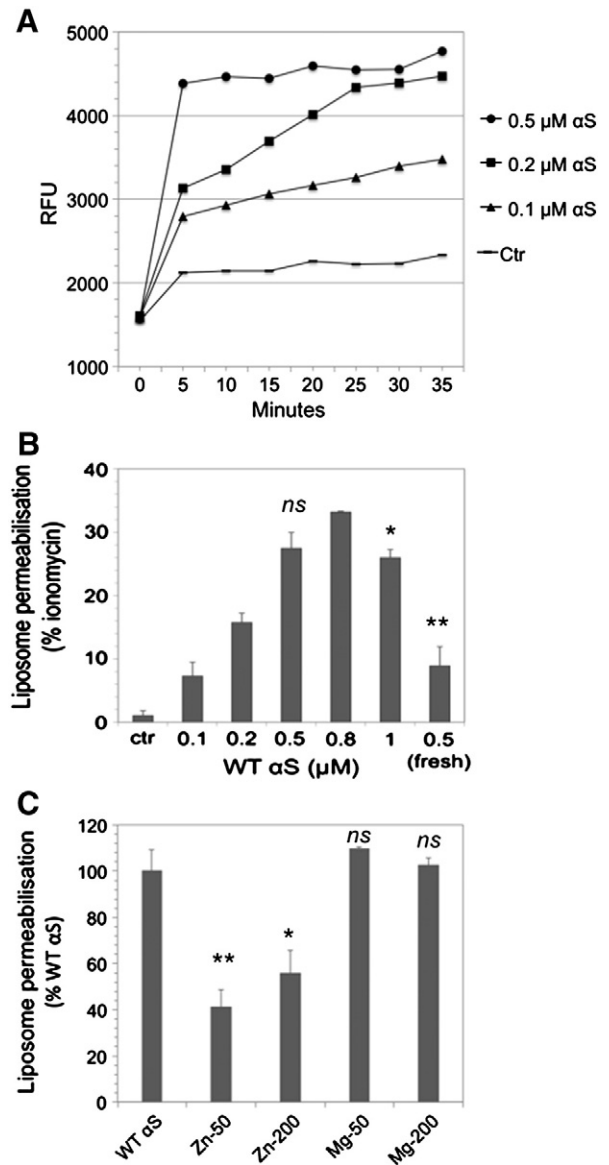

Fig. 2. Lipid vesicle permeabilisation by wild-type αS. The graph in (A) shows permeabilisation kinetics upon incubating liposomes with 1% DMSO and 10 μM Fe^{3+} alone (Ctr) and with 0.1 μM–0.5 μM pre-aggregated WT αS. Data are representative of at least 3 independent experiments. (B) Concentration-dependence of vesicle permeabilisation by WT αS oligomers (0.1–1.0 μM), calculated as a percentage of the effect induced by 2 μM ionomycin; 0.5 μM non-aggregated WT αS, representing monomeric αS, is also included. Values represent means ± S.E.M. ($n \geq 2$); ns, not significant, compared to 0.8 μM αS; *$p < 0.05$, compared to 1.0 μM αS; **$p = 0.001$, compared to 0.5 μM oligomeric αS. (C) Zn^{2+} and Mg^{2+} ions (50 and 200 μM) were tested for their effect on liposome permeabilisation, after 10 min incubation with pre-aggregated 0.2 μM αS (WT αS). Results are expressed as a percentage of the leakage induced by WT αS alone (100%; y-axis). Values represent means ± S.D. ($n = 3$); *$p < 0.05$; **$p < 0.005$.

blocked lipid vesicle disruption by aggregated αS by ~60% (Fig. 2C). Zinc (II) ions have been reported to block non-selective cation channels formed by αS aggregates in lipid bilayers [55]. Secondly, kinetic profiles of permeabilisation by αS display a striking resemblance to those of the bacterial pore-forming peptide gramicidin (Fig. 2A and Suppl. Fig. S2A). In contrast, a detergent-like mechanism would have resulted in a non-gradual, and immediately complete, permeabilisation even at low (<0.5 μM) concentrations of αS (Suppl. Fig. S2B).

The suggested pore-like mechanism is consistent with a number of reported studies: Danzer and co-workers similarly used Fe^{3+} to induce αS oligomers that were cytotoxic and showed pore-like single

channel recordings [27,56]. In another investigation, cold denaturation of αS fibrils was used to prepare oligomeric αS species, which also exhibited pore-like characteristics [26].

3.4. Polyphenolic compounds and BTE attenuate lipid vesicle permeabilisation by α-synuclein oligomers

The liposome permeabilisation assay was next used to identify compounds having an inhibitory effect on membrane perturbation by aggregated WT αS. A total of 11 polyphenols and BTE were screened for any potential impairment of vesicle membrane integrity by the compounds themselves. None of the compounds significantly compromised membrane integrity (Suppl. Fig. S3). The inhibitory effects of polyphenols and BTE on lipid vesicle permeabilisation by aggregated WT αS were then assayed using two protocols A and B, as outlined in Section 2.9. Briefly, protocol A allows polyphenols and BTE to first interact with the phospholipid membranes of the vesicles, before exposure to αS. Conversely, protocol B allows for the primary interaction of the inhibitory compound with the aggregated αS species, before including the liposomes and monitoring permeabilisation. With regard to protocol B, increasing the incubation time of the compounds with αS aggregates from 10 to 60 min did not alter the inhibitory potency of the compounds (data not shown). Results and comparisons between 11 polyphenols and BTE used in protocols A and B are given in Table 1. Furthermore, by combining data from the two protocols (A and B), an overall 'score' value was assigned to each of the 11 polyphenolic compounds and BTE (Table 1). In summary, these data establish that the compounds with the highest scores, namely Mor (6/6), BTE (5/6), Baic (4/6) and NDGA (4/6), can be regarded as the best overall in protecting lipid vesicle membranes from WT αS oligomer-mediated permeabilisation. The polyphenols Mor, NDGA and BTE were additionally effective at lower concentrations (Fig. 3A). In order to demonstrate that the observed protective effects were independent of the permeabilisation assay itself, Baic, Mor and BTE were also tested on calcein-entrapped liposomes. Indeed, experiments with the calcein-filled liposomes gave similar results to the ones obtained using OGB-1-filled liposomes: Mor, 31% ± 3 ($p < 0.005$); BTE, 7% ± 4 ($p < 0.005$); Baic, 33% ± 7 ($p < 0.005$).

We have recently published a study in which confocal fluorescence spectroscopy techniques were used to monitor WT αS aggregation at the single-molecule level in the presence of naturally-occurring polyphenolic compounds and BTE [40]. Correlating the results of these anti-aggregation assays with those of the liposome permeabilisation assay above, it is striking to note that BTE, Baic, Mor and NDGA were all very effective in both assay types. Similarly, compounds having the lowest scores in the permeabilisation assay, namely Resv, Gink and Purp, also exhibited the weakest effects in the anti-aggregation assays. The implication is that the dominant, although not necessarily exclusive, mechanism involved in protection of lipid vesicles from αS-induced permeabilisation is in fact destabilisation of the aggregates by the polyphenolic compounds. Exceptions to these observations include Api and Gen, both of which potently attenuated permeabilisation in protocol B whilst being overall weak in disaggregating activity. A possible mechanism here might presuppose direct binding of the compounds to soluble αS oligomers, without causing disaggregation of the latter, but still preventing their interaction with lipid vesicle membranes.

3.5. Polyphenols do not inhibit permeabilisation of liposomes by gramicidin or detergents

To determine whether the ability of polyphenols to counter membrane destabilisation by αS was a specific effect against oligomer permeabilisation, OGB-1-filled liposomes were exposed to the toxin gramicidin and Triton X-100 detergent, in the presence of Mor and BTE. Both polyphenolic compounds did not inhibit vesicle damage by these two permeabilising agents; by contrast, Mor actually enhanced

Table 1
Inhibitory effect of polyphenols and BTE on WT α-synuclein-induced lipid vesicle permeabilisation using protocols A and B, and ranking by overall score.

Polyphenols	Protocol A[a]	Protocol B[a]	Overall score[b]	Difference of A vs. B[c] ($p < 0.05$)
Mor	23 ± 4%*** (+++)	25 ± 2%*** (+++)	6	ns
BTE	32 ± 7%*** (++)	27 ± 2%*** (+++)	5	ns
Baic	30 ± 5%*** (++)	33 ± 7%*** (++)	4	ns
NDGA	58 ± 1%** (++)	50 ± 5%** (++)	4	ns
Api	77 ± 1%* (+)	31 ± 7%*** (++)	3	More effective in B
EGCG	75 ± 5%* (+)	55 ± 4%** (++)	3	More effective in B
Gen	113 ± 6%ns (−)	44 ± 6%** (++)	2	More effective in B
Scut	81 ± 3%ns (+)	80 ± 5%* (+)	2	ns
PropylG	101 ± 2%ns (−)	77 ± 2%* (+)	1	More effective in B
Resv	99 ± 4%ns (−)	88 ± 4%ns (+)	1	ns
Gink	95 ± 6%ns (−)	93 ± 7%ns (−)	0	ns
Purp	98 ± 4%ns (−)	94 ± 0.3%ns (−)	0	ns

[a] Eleven polyphenols (50 μM) and BTE (3 μg/ml) were either first incubated with 50 μM OGB-1 liposomes for 10 min, after which WT αS oligomers were included (protocol A), or were first incubated with WT αS oligomers for 10 min, after which 50 μM liposomes were added (protocol B). In both cases, permeabilisation was monitored for 30 min and calculated as a percentage of the liposome damage caused by αS alone. Potency of each compound was classified as: no effect (>90%; −); weak effect (60–90%; +); moderate effect (30–59%;++); strong effect (<30%; +++). Values represent means ± S.E.M. ($n ≥ 3$); *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$.
[b] The overall score was determined by assigning an arbitrary score of 0–3 points to each compound, based upon the percentage effectiveness obtained from the two protocols utilised in the study. The best score that can be obtained by a compound is therefore '6'.
[c] Differences between protocols A and B were denoted as not significant (ns) or significant ($p < 0.05$), if the compound was more effective in either strategy.

permeabilisation by gramicidin (Fig. 3B). Hence, specific structural features, rather than broad biochemical characteristics, likely determine the inhibitory effect upon synuclein-induced permeabilisation.

3.6. Additive inhibition by pairwise combinations of polyphenols

Taking into consideration the fact that in natural products there is a heterogeneous mix of polyphenols, we decided to test whether pairwise combinations of polyphenols would result in an overall enhanced, or diminished, biological activity. Thus, NDGA was associated with 4 other polyphenols in paired combinations: namely BTE (strong inhibitor), EGCG (moderate inhibitor), PropylG (weak inhibitor) and Purp (no effect). In each case, the paired combination resulted in an additive inhibitory effect on liposome permeabilisation by WT αS (Fig. 3C). In particular, BTE plus NDGA prevented damage to lipid membranes almost completely (1.6%, $p < 0.01$ when compared to NDGA alone). Accordingly, at least with these combinations, it appears that the mechanisms of inhibition by the compounds were independent and/or overlapping but not conflicting.

3.7. Inhibition of αS-induced lipid vesicle permeabilisation by N′-benzylidene-benzohydrazide derivates

In previous studies, we had identified several NBB derivates inhibiting iron-dependent αS oligomer species formation in vitro [27], as well as in cultured cells [52]. Accordingly, it was of interest to determine whether this class of synthetic compounds could also prevent membrane damage by WT αS oligomers. As with the polyphenols, NBB compounds were first tested for any possible direct membrane-damaging effects on the synthetic lipid vesicles. None of the compounds, including the DMSO control, caused any significant liposome disruption (Suppl. Fig. S3). Next, the NBB compounds were tested using the liposome permeabilisation assay (protocol B only). Compounds #5 (67%), #7 (84%), and #8 (75%) were found to be the three most effective compounds. Their chemical structures
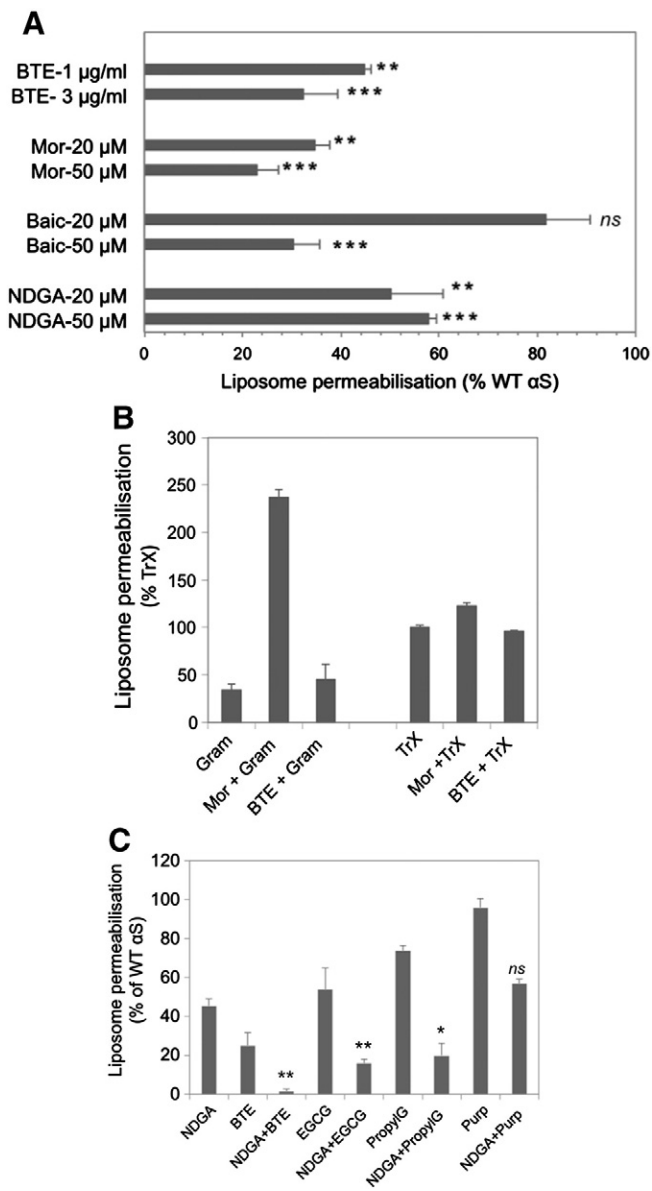
Fig. 4. Effect of NBB compounds on lipid vesicle permeabilisation by WT αS. 10 μM NBB compounds (#1–#8) and 10 μM NDGA, were incubated for 10 min with pre-aggregated WT αS, and 50 μM OGB-1 liposomes added to assess permeabilisation. Values represent means ± S.E.M. ($n = 2$); *$p < 0.5$; **$p < 0.05$; ***$p < 0.005$.

same time leaving the lipid vesicles intact [57]. This finding confirms the attractiveness of such compounds for drug development in PD.

3.8. Extension of compound testing to liposome permeabilisation by mutant αS aggregates

Having established the relative effectiveness of 11 natural polyphenols and 8 NBB compounds in maintaining integrity of membranes disrupted by WT αS oligomers, we proceeded with testing all the polyphenols (incl. BTE) and two NBB compounds (#5 and #8) against membrane damage induced by mutant αS aggregates, namely A30P and A53T αS. The point mutations represent amino acid substitutions in the α-synuclein gene associated with autosomal-dominant early onset familial forms of PD, known to accelerate formation of αS oligomers [58]. Indeed, monitoring of the fibrillisation kinetics of aggregated mutant αS showed that after 7 days both mutants had entered the log-phase of fibril formation, whilst WT αS reached the log-phase after a further week of incubation. Importantly, after only 4 h incubation (representing the aggregation protocol) mutant and WT αS had very low ThT fluorescence, implying no cross-β-sheet fibril formation until that stage (Fig. 5A). Lipid vesicle permeabilisation by 0.5 μM A30P and A53T aggregates was comparable to that of 0.5 μM WT αS (Fig. 5B).

Inhibition of liposome permeabilisation by the 11 polyphenols (at 50 μM, except for 20 μM NDGA) was investigated for A30P and A53T αS, using protocol B. The results are summarised in Table 2, shown together with the previous data pertaining to WT αS (also using protocol B). This enabled pooling of all the data for each compound and a ranking table was constructed. In essence, each compound was assigned an arbitrary score of 0–9 points, based upon its overall effectiveness in inhibiting permeabilisation by the three synucleins (A30P, A53T, WT). Table 2 shows that NDGA, Api, Gen, Mor, and BTE can be classified as the most potent inhibitors, obtaining a high score of 8 points. A moderate inhibitory effect was manifested by EGCG (6 points), Baic (5 points), PropylG and Scut (4 points). The least effective were Purp and Resv (3 points) and Gink (1 point). The same method was applied to NBB compounds #5 and #8 (at 10 μM), in direct comparison with NDGA (also at 10 μM). As shown in Table 2, the polyphenol NDGA compared very favourably to the synthetic compounds (7 points for NDGA and 3 points each for the NBB compounds).

3.9. Structure–activity relationships

An attempt was made to infer potential associations between chemical scaffolds and compound bioactivity from Table 2 (refer to Suppl. Fig. S4 for chemical structures of all compounds). Essentially, the 5,7-dihydroxy-chromen-4-one moiety (found in the flavones Api, Gen and Mor) appears to be the key pharmacophore favoured in the inhibition of vesicle permeabilisation (Fig. 6). The other

Fig. 3. Effect of polyphenols on liposome permeabilisation by WT αS, gramicidin and membrane detergent. (A) Polyphenols (20–50 μM) and BTE (1–3 μg/ml) were incubated with 50 μM OGB-1 liposomes for 10 min, prior to addition of aggregated WT αS. For each tested compound, permeabilisation is shown as a percentage of the effect induced by WT αS alone (100%; x-axis). Values represent means ± S.E.M. ($n ≥ 2$); **$p < 0.005$; ***$p < 0.001$. (B) Mor (50 μM) and BTE (3 μg/ml) were incubated with 50 μM OGB-1 liposomes for 10 min, after which they were exposed to either 0.05 mg/ml gramicidin (Gram) or 10% (v/v) Triton X-100 (TrX). Permeabilisation is shown as a percentage of the effect by detergent TrX (100%; y-axis). Values represent means ± S.E.M. ($n = 3$). (C) Paired combination of 20 μM NGDA with 50 μM BTE, EGCG, PropylG and Purp reveals an additive potentiation of their inhibitory effect against liposome permeabilisation by WT αS. Values shown here are means of triplicate readings ± S.E.M. ($n = 2$); *$p < 0.05$ and **$p < 0.01$ *versus* NDGA alone.

were used at a later stage when searching for meaningful structure–activity relationships (Section 3.8). With regard to the other NBB compounds, permeabilisation by WT αS still reached >90%. In direct comparison, 10 μM NDGA reduced permeabilisation to 49% (Fig. 4).

Recently, we directly evaluated the influence of the compounds Baic and NBB #8 on the interaction between αS and model lipid vesicles [57]. The synuclein and liposomes were labelled with a different fluorophore and confocal single-molecule FIDA was performed for qualitative and quantitative evaluation of the oligomer population and vesicle integrity. Notably, both these compounds potently dissolved membrane-bound, large αS oligomers into monomers, at the
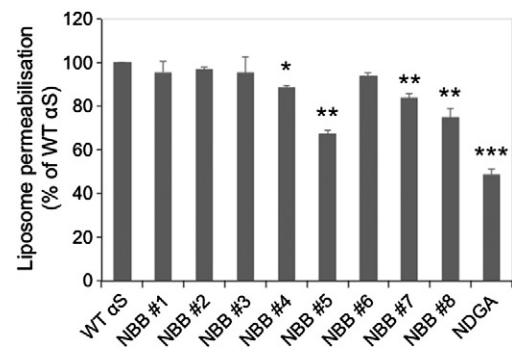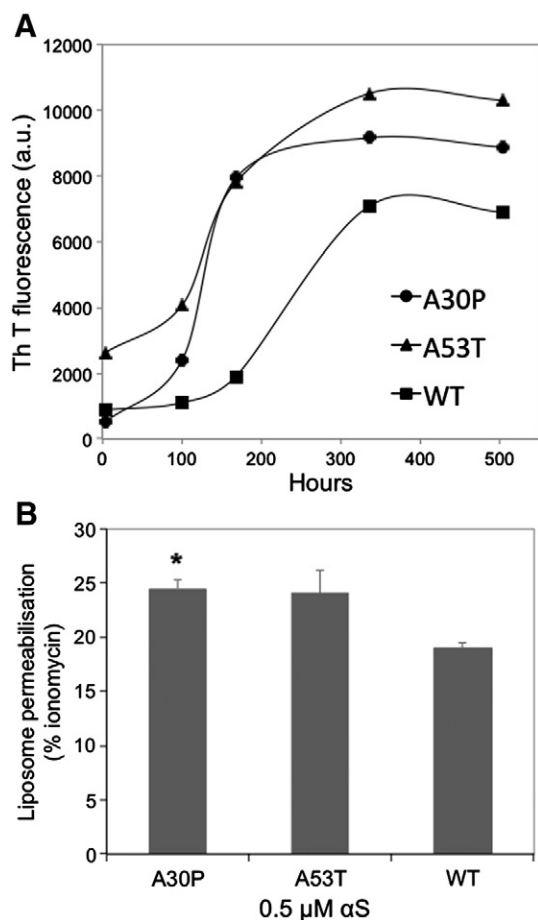
Fig. 5. (A) Fibrillisation kinetics of mutant αS (A30P, A53T). Aggregation of wild-type (WT), A30P and A53T α-synuclein was monitored for 3 weeks in a ThT assay (ThT fluorescence, arbitrary units). Shown is the mean of duplicate measurements. (B) Comparison of liposome permeabilisation by mutant (A30P, A53T) and WT aggregated αS. Permeabilisation of OGB-1 lipid vesicles is given as a percentage of the maximum ionomycin effect (% ionomycin, y-axis). Values were taken after 5 min permeabilisation and represent means ± S.E.M. ($n = 4$); $^*p < 0.05$ compared to WT.

flavones Baic and Scut possess an additional hydroxyl group and hence a trihydroxylated aromatic ring, in a similar fashion to EGCG and PropylG. Consequently, trihydroxylated rings appear to be less effective than dihydroxylated rings for inhibitory activity on permeabilisation. Besides, EGCG has a chroman ring structure, which lacks the carbonyl oxygen on the 'C' ring present in the chromene moiety of the polyphenols Api, Gen and Mor. Interestingly, the symmetrical dihydroxylation pattern found in Api, Gen and Mor is also a feature of NDGA, the only other potent small-molecule compound which is not a flavonoid. Its compact and symmetrical structure, with two dihydroxyphenyl rings connected by a rigid linker, may be particularly suitable for specific binding to, and destabilisation of, aggregated αS [36,40]. It is particularly revealing that among the NBB compounds having the best anti-permeabilisation activity, NBB #5, #7 and #8 all feature an analogous structure to NDGA, with a dihydroxyphenyl ring at one end of the molecule. Moreover, NBB #8 (293 G02) has been shown to interfere with αS oligomer formation in living cells [52].

Black tea is one of the most widely consumed beverages in the world. Indeed, in a large epidemiological study conducted in Asia, black tea showed an inverse association with Parkinson's disease risk that was not confounded by other factors, like total caffeine intake or tobacco smoking [59]. It has also been reported that BTE provided neuroprotection and neurorescue in a neuronal cell model of PD [60]. These reports are therefore in line with our assay results

Table 2
Overall polyphenol and N′-benzylidene-benzohydrazide compound scores.

	No effect (>90%)	Mild (60–90%)	Moderate (30–59%)	Strong (<30%)	Overall score
NDGA (20 μM)			WT, 50%**	A30P, 28%** A53T, 21%**	8
Api			WT, 31%**	A30P, 25%** A53T, 20%**	8
Gen			WT, 44%**	A30P, 25%** A53T, 20%**	8
Mor			A53T, 37%**	A30P, 18%** WT, 26%**	8
BTE (3 μg/ml)			A53T, 35%**	A30P, 28%** WT, 27%**	8
EGCG			A30P, 49%** WT, 55%**	A53T, 10%**	7
Baic		A30P, 62%**	A53T, 59%** WT, 33.2%**		5
PropylG		WT, 76%* A30P, 75%**	A53T, 47%**		4
Scut		A30P, 90%** WT, 81%**	A53T, 48%**		4
Purp	WT, 95%ns	A30P, 81%**	A53T, 44%**		3
Resv		A30P, 71%** A53T, 68%* WT, 63%*			3
Gink	A30P, 104%ns WT, 93%ns	A53T, 86%*			1
NDGA (10 μM)			WT, 49%** A30P, 38%**	A53T, 24%**	7
NBB #5 (10 μM)		WT, 67%** A30P, 82%** A53T, 82%**			3
NBB #8 (10 μM)		WT, 75%** A30P, 62%** A53T, 67%**			3

Each compound was assigned an arbitrary score of 0–3 points, based upon its effectiveness in inhibiting permeabilisation by the three synucleins (A30P, A53T, WT). Values represent means ± S.E.M. ($n \geq 3$); ns = no significant difference; $^*p < 0.05$; $^{**}p < 0.005$. No effect = 0 points; Mild effect = 1 point; Moderate effect = 2 points; Strong effect = 3 points. The maximum score that can be obtained by a compound is '9' ($3 + 3 + 3$).

indicating a significant effect of BTE in limiting lipid membrane damage by aggregated synucleins.

4. Conclusion

In agreement with others, we confirmed the ability of α-synuclein oligomers to permeabilise phospholipid membranes, potentially *via* a pore-forming mechanism analogous to membrane disruption by bacterial toxins [24,55,56,61]. This process of membrane damage could resemble the mechanism of neuronal toxicity *in vivo* [62].

In this study, we identified a group of small-molecule natural polyphenols (NDGA, Mor, Baic and Api), as well as black tea extract, that were found to strongly protect against membrane perturbation induced by aggregated wild-type and mutant α-synuclein. Our structure–function comparisons, based upon the polyphenols and synthetic N′-benzylidene-benzohydrazide derivatives, suggest that the presence of dihydroxy-phenyl rings, especially within a 5,7-dihydroxy-chromen-4-one scaffold, appears to be favourable for the inhibition reaction. Indeed, it has been suggested that one way of improving screening libraries is to include molecules resembling biogenic scaffolds [63]. More detailed investigations into structure–activity relationships of compounds found in natural products may guide the design of natural product-inspired molecules with enhanced properties *in vivo* (*e.g.* ability to penetrate blood–brain barrier), but which retain the bioactivity characteristic of the natural product scaffold. In this respect, it is important to keep in mind that polyphenols found in human diet are highly metabolised by microbial enzymes in the colon [64]. These metabolites may have a different biological activity from the native
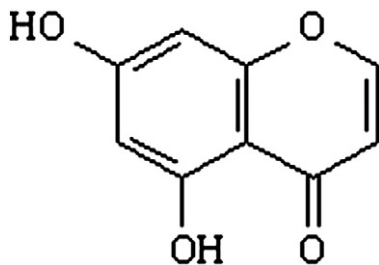
Fig. 6. The 5,7-dihydroxy-chromen-4-one moiety (found in the flavones apigenin, genistein and morin) appears to be the key pharmacophore favoured in the inhibition of vesicle permeabilisation by αS.

substances. Nevertheless, oral administration of polyphenolic compounds has been successful in modulating amyloid aggregation pathways and ameliorating neuropathology of various disease models *in vivo*. Among these are polyphenols which were found to be particularly effective in our assays, including NDGA [65], Gen [66], BTE [67], EGCG [68] and Baic [69]. To conclude, we highlight a promising strategy for the development of novel lead compounds in Parkinson's disease.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.05.019.

References

[1] S. Totterdell, G.E. Meredith, Localization of alpha-synuclein to identified fibers and synapses in the normal mouse brain, Neuroscience 135 (2005) 907–913.

[2] A. Adamczyk, J. Solecka, J.B. Strosznajder, Expression of alpha-synuclein in different brain parts of adult and aged rats, J. Physiol. Pharmacol. 56 (2005) 29–37.

[3] S.J. Lee, H. Jeon, K.V. Kandror, Alpha-synuclein is localized in a subpopulation of rat brain synaptic vesicles, Acta Neurobiol. Exp. (Wars) 68 (2008) 509–515.

[4] J. Burre, M. Sharma, T. Tsetsenis, V. Buchman, M.R. Etherton, T.C. Sudhof, Alpha-synuclein promotes SNARE-complex assembly *in vivo* and *in vitro*, Science 329 (2010) 1663–1667.

[5] V.M. Lee, J.Q. Trojanowski, Mechanisms of Parkinson's disease linked to pathological alpha-synuclein: new targets for drug discovery, Neuron 52 (2006) 33–38.

[6] A.J. Lees, J. Hardy, T. Revesz, Parkinson's disease, Lancet 373 (2009) 2055–2066.

[7] M.G. Spillantini, M.L. Schmidt, V.M. Lee, J.Q. Trojanowski, R. Jakes, M. Goedert, Alpha-synuclein in Lewy bodies, Nature 388 (1997) 839–840.

[8] B. Caughey, P.T. Lansbury, Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders, Annu. Rev. Neurosci. 26 (2003) 267–298.

[9] T.F. Outeiro, P. Putcha, J.E. Tetzlaff, R. Spoelgen, M. Koker, F. Carvalho, B.T. Hyman, P.J. McLean, Formation of toxic oligomeric alpha-synuclein species in living cells, PLoS One 3 (2008) e1867.

[10] D.P. Karpinar, M.B. Balija, S. Kugler, F. Opazo, N. Rezaei-Ghaleh, N. Wender, H.Y. Kim, G. Taschenberger, B.H. Falkenburger, H. Heise, A. Kumar, D. Riedel, L. Fichtner, A. Voigt, G.H. Braus, K. Giller, S. Becker, A. Herzig, M. Baldus, H. Jackle, S. Eimer, J.B. Schulz, C. Griesinger, M. Zweckstetter, Pre-fibrillar alpha-synuclein variants with impaired beta-structure increase neurotoxicity in Parkinson's disease models, EMBO J. 28 (2009) 3256–3268.

[11] R. Kayed, E. Head, J.L. Thompson, T.M. McIntire, S.C. Milton, C.W. Cotman, C.G. Glabe, Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis, Science 300 (2003) 486–489.

[12] M.J. Volles, S.J. Lee, J.C. Rochet, M.D. Shtilerman, T.T. Ding, J.C. Kessler, P.T. Lansbury Jr., Vesicle permeabilization by protofibrillar alpha-synuclein:

[13] R. Kayed, Y. Sokolov, B. Edmonds, T.M. McIntire, S.C. Milton, J.E. Hall, C.G. Glabe, Permeabilization of lipid bilayers is a common conformation-dependent activity of soluble amyloid oligomers in protein misfolding diseases, J. Biol. Chem. 279 (2004) 46363–46366.

[14] Y. Yoshiike, R. Kayed, S.C. Milton, A. Takashima, C.G. Glabe, Pore-forming proteins share structural and functional homology with amyloid oligomers, Neuromolecular Med. 9 (2007) 270–275.

[15] R. Sharon, I. Bar-Joseph, M.P. Frosch, D.M. Walsh, J.A. Hamilton, D.J. Selkoe, The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease, Neuron 37 (2003) 583–595.

[16] I. Dikiy, D. Eliezer, Folding and misfolding of alpha-synuclein on membranes, Biochim. Biophys. Acta 1818 (2011) 1013–1018.

[17] C.M. Pfefferkorn, Z. Jiang, J.C. Lee, Biophysics of alpha-synuclein membrane interactions, Biochim. Biophys. Acta 1818 (2011) 162–171.

[18] H.J. Lee, C. Choi, S.J. Lee, Membrane-bound alpha-synuclein has a high aggregation propensity and the ability to seed the aggregation of the cytosolic form, J. Biol. Chem. 277 (2002) 671–678.

[19] V.V. Shvadchak, D.A. Yushchenko, R. Pievo, T.M. Jovin, The mode of alpha-synuclein binding to membranes depends on lipid composition and lipid to protein ratio, FEBS Lett. 585 (2011) 3513–3519.

[20] E. Rhoades, T.F. Ramlall, W.W. Webb, D. Eliezer, Quantification of alpha-synuclein binding to lipid vesicles using fluorescence correlation spectroscopy, Biophys. J. 90 (2006) 4692–4700.

[21] V.V. Shvadchak, L.J. Falomir-Lockhart, D.A. Yushchenko, T.M. Jovin, Specificity and kinetics of alpha-synuclein binding to model membranes determined with fluorescent excited state intramolecular proton transfer (ESIPT) probe, J. Biol. Chem. 286 (2011) 13023–13032.

[22] T. Bartels, L.S. Ahlstrom, A. Leftin, F. Kamp, C. Haass, M.F. Brown, K. Beyer, The N-terminus of the intrinsically disordered protein alpha-synuclein triggers membrane binding and helix folding, Biophys. J. 99 (2010) 2116–2124.

[23] M. Drescher, F. Godschalk, G. Veldhuis, B.D. van Rooijen, V. Subramaniam, M. Huber, Spin-label EPR on alpha-synuclein reveals differences in the membrane binding affinity of the two antiparallel helices, Chembiochem 9 (2008) 2411–2416.

[24] M.J. Volles, P.T. Lansbury Jr., Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism, Biochemistry 41 (2002) 4595–4602.

[25] B.D. van Rooijen, M.M. Claessens, V. Subramaniam, Membrane binding of oligomeric alpha-synuclein depends on bilayer charge and packing, FEBS Lett. 582 (2008) 3788–3792.

[26] H.Y. Kim, M.K. Cho, A. Kumar, E. Maier, C. Siebenhaar, S. Becker, C.O. Fernandez, H.A. Lashuel, R. Benz, A. Lange, M. Zweckstetter, Structural properties of pore-forming oligomers of alpha-synuclein, J. Am. Chem. Soc. 131 (2009) 17482–17489.

[27] M. Kostka, T. Hogen, K.M. Danzer, J. Levin, M. Habeck, A. Wirth, R. Wagner, C.G. Glabe, S. Finger, U. Heinzelmann, P. Garidel, W. Duan, C.A. Ross, H. Kretzschmar, A. Giese, Single particle characterization of iron-induced pore-forming alpha-synuclein oligomers, J. Biol. Chem. 283 (2008) 10992–11003.

[28] K. Furukawa, M. Matsuzaki-Kobayashi, T. Hasegawa, A. Kikuchi, N. Sugeno, Y. Itoyama, Y. Wang, P.J. Yao, I. Bushlin, A. Takeda, Plasma membrane ion permeability induced by mutant alpha-synuclein contributes to the degeneration of neural cells, J. Neurochem. 97 (2006) 1071–1077.

[29] H.A. Lashuel, B.M. Petre, J. Wall, M. Simon, R.J. Nowak, T. Walz, P.T. Lansbury Jr., Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils, J. Mol. Biol. 322 (2002) 1089–1102.

[30] A. Demuro, E. Mina, R. Kayed, S.C. Milton, I. Parker, C.G. Glabe, Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers, J. Biol. Chem. 280 (2005) 17294–17300.

[31] G. Valincius, F. Heinrich, R. Budvytyte, D.J. Vanderah, D.J. McGillivray, Y. Sokolov, J.E. Hall, M. Losche, Soluble amyloid beta-oligomers affect dielectric membrane properties by bilayer insertion and domain formation: implications for cell toxicity, Biophys. J. 95 (2008) 4845–4861.

[32] N.P. Reynolds, A. Soragni, M. Rabe, D. Verdes, E. Liverani, S. Handschin, R. Riek, S. Seeger, The mechanism of membrane interaction and disruption by alpha-synuclein, J. Am. Chem. Soc. 133 (2011) 19366–19375.

[33] B. Winner, R. Jappelli, S.K. Maji, P.A. Desplats, L. Boyer, S. Aigner, C. Hetzer, T. Loher, M. Vilar, S. Campioni, C. Tzitzilonis, A. Soragni, S. Jessberger, H. Mira, A. Consiglio, E. Pham, E. Masliah, F.H. Gage, R. Riek, *In vivo* demonstration that alpha-synuclein oligomers are toxic, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 4194–4199.

[34] W.G. Meissner, M. Frasier, T. Gasser, C.G. Goetz, A. Lozano, P. Piccini, J.A. Obeso, O. Rascol, A. Schapira, V. Voon, D.M. Weiner, F. Tison, E. Bezard, Priorities in Parkinson's disease research, Nat. Rev. Drug Discov. 10 (2011) 377–393.

[35] D.A. Amer, G.B. Irvine, O.M. El-Agnaf, Inhibitors of alpha-synuclein oligomerization and toxicity: a future therapeutic strategy for Parkinson's disease and related disorders, Exp. Brain Res. 173 (2006) 223–233.

[36] K. Ono, M. Yamada, Antioxidant compounds have potent anti-fibrillogenic and fibril-destabilizing effects for alpha-synuclein fibrils in vitro, J. Neurochem. 97 (2006) 105–115.

[37] M. Masuda, N. Suzuki, S. Taniguchi, T. Oikawa, T. Nonaka, T. Iwatsubo, S. Hisanaga, M. Goedert, M. Hasegawa, Small molecule inhibitors of alpha-synuclein filament assembly, Biochemistry 45 (2006) 6085–6094.

[38] X. Meng, L.A. Munishkina, A.L. Fink, V.N. Uversky, Effects of various flavonoids on the alpha-synuclein fibrillation process, Parkinsons Dis. 2010 (2010) 650794.

[39] G. Di Giovanni, A diet for dopaminergic neurons? J. Neural Transm. Suppl. (2009) 317–331.

[40] M. Caruana, T. Hogen, J. Levin, A. Hillmer, A. Giese, N. Vassallo, Inhibition and dis-aggregation of alpha-synuclein oligomers by natural polyphenolic compounds, FEBS Lett. 585 (2011) 1113–1120.

[41] M. Zhu, S. Rajamani, J. Kaylor, S. Han, F. Zhou, A.L. Fink, The flavonoid baicalein inhibits fibrillation of alpha-synuclein and disaggregates existing fibrils, J. Biol. Chem. 279 (2004) 26846–26857.

[42] D.E. Ehrnhoefer, J. Bieschke, A. Boeddrich, M. Herbst, L. Masino, R. Lurz, S. Engemann, A. Pastore, E.E. Wanker, EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers, Nat. Struct. Mol. Biol. 15 (2008) 558–566.

[43] J. Bieschke, J. Russ, R.P. Friedrich, D.E. Ehrnhoefer, H. Wobst, K. Neugebauer, E.E. Wanker, EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 7710–7715.

[44] J.N. Rao, V. Dua, T.S. Ulmer, Characterization of alpha-synuclein interactions with selected aggregation-inhibiting small molecules, Biochemistry 47 (2008) 4651–4656.

[45] P.I. Oteiza, A.G. Erlejman, S.V. Verstraeten, C.L. Keen, C.G. Fraga, Flavonoid–membrane interactions: a protective role of flavonoids at the membrane surface? Clin. Dev. Immunol. 12 (2005) 19–25.

[46] F. Ollila, K. Halling, P. Vuorela, H. Vuorela, J.P. Slotte, Characterization of flavonoid–biomembrane interactions, Arch. Biochem. Biophys. 399 (2002) 103–108.

[47] A. Arora, T.M. Byrem, M.G. Nair, G.M. Strasburg, Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids, Arch. Biochem. Biophys. 373 (2000) 102–109.

[48] S. Hüls, T. Hogen, N. Vassallo, K.M. Danzer, B. Hengerer, A. Giese, J. Herms, AMPA-receptor-mediated excitatory synaptic transmission is enhanced by iron-induced alpha-synuclein oligomers, J. Neurochem. 117 (2011) 868–878.

[49] O.M. El-Agnaf, K.E. Paleologou, B. Greer, A.M. Abogrein, J.E. King, S.A. Salem, N.J. Fullwood, F.E. Benson, R. Hewitt, K.J. Ford, F.L. Martin, P. Harriott, M.R. Cookson, D. Allsop, A strategy for designing inhibitors of alpha-synuclein aggregation and toxicity as a novel treatment for Parkinson's disease and related disorders, FASEB J. 18 (2004) 1315–1317.

[50] S. Takamori, M. Holt, K. Stenius, E.A. Lemke, M. Gronborg, D. Riedel, H. Urlaub, S. Schenck, B. Brugger, P. Ringler, S.A. Muller, B. Rammner, F. Grater, J.S. Hub, B.L. De Groot, G. Mieskes, Y. Moriyama, J. Klingauf, H. Grubmuller, J. Heuser, F. Wieland, R. Jahn, Molecular anatomy of a trafficking organelle, Cell 127 (2006) 831–846.

[51] A.J. Gauci, M. Caruana, A. Giese, C. Scerri, N. Vassallo, Identification of polyphenolic compounds and black tea extract as potent inhibitors of lipid membrane destabilization by Abeta42 aggregates, J. Alzheimers Dis. 27 (2011) 767–779.

[52] A.S. Hillmer, P. Putcha, J. Levin, T. Hogen, B.T. Hyman, H. Kretzschmar, P.J. McLean, A. Giese, Converse modulation of toxic alpha-synuclein oligomers in living cells by N'-benzylidene-benzohydrazide derivates and ferric iron, Biochem. Biophys. Res. Commun. 391 (2010) 461–466.

[53] S.J. Wood, J. Wypych, S. Steavenson, J.C. Louis, M. Citron, A.L. Biere, Alpha-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease, J. Biol. Chem. 274 (1999) 19509–19512.

[54] M. Stockl, M.M. Claessens, V. Subramaniam, Kinetic measurements give new insights into lipid membrane permeabilization by alpha-synuclein oligomers, Mol. Biosyst. 8 (2011) 338–345.

[55] I.F. Tsigelny, P. Bar-On, Y. Sharikov, L. Crews, M. Hashimoto, M.A. Miller, S.H. Keller, O. Platoshyn, J.X. Yuan, E. Masliah, Dynamics of alpha-synuclein aggregation and inhibition of pore-like oligomer development by beta-synuclein, FEBS J. 274 (2007) 1862–1877.

[56] K.M. Danzer, D. Haasen, A.R. Karow, S. Moussaud, M. Habeck, A. Giese, H. Kretzschmar, B. Hengerer, M. Kostka, Different species of alpha-synuclein oligomers induce calcium influx and seeding, J. Neurosci. 27 (2007) 9220–9232.

[57] T. Högen, J. Levin, F. Schmidt, M. Caruana, N. Vassallo, H. Kretzschmar, K. Botzel, F. Kamp, A. Giese, Two different binding modes of alpha-synuclein to lipid vesicles depending on its aggregation state, Biophys. J. 102 (2012) 1646–1655.

[58] R. Bussell Jr., D. Eliezer, Effects of Parkinson's disease-linked mutations on the structure of lipid-associated alpha-synuclein, Biochemistry 43 (2004) 4810–4818.

[59] L.C. Tan, W.P. Koh, J.M. Yuan, R. Wang, W.L. Au, J.H. Tan, E.K. Tan, M.C. Yu, Differential effects of black versus green tea on risk of Parkinson's disease in the Singapore Chinese Health Study, Am. J. Epidemiol. 167 (2008) 553–560.

[60] Y. Levites, M.B. Youdim, G. Maor, S. Mandel, Attenuation of 6-hydroxydopamine (6-OHDA)-induced nuclear factor-kappaB (NF-kappaB) activation and cell death by tea extracts in neuronal cultures, Biochem. Pharmacol. 63 (2002) 21–29.

[61] L.R. Feng, H.J. Federoff, S. Vicini, K.A. Maguire-Zeiss, Alpha-synuclein mediates alterations in membrane conductance: a potential role for alpha-synuclein oligomers in cell vulnerability, Eur. J. Neurosci. 32 (2010) 10–17.

[62] B.D. van Rooijen, M.M. Claessens, V. Subramaniam, Membrane interactions of oligomeric alpha-synuclein: potential role in Parkinson's disease, Curr. Protein Pept. Sci. 11 (2010) 334–342.

[63] J. Hert, J.J. Irwin, C. Laggner, M.J. Keiser, B.K. Shoichet, Quantifying biogenic bias in screening libraries, Nat. Chem. Biol. 5 (2009) 479–483.

[64] C. Manach, A. Scalbert, C. Morand, C. Remesy, L. Jimenez, Polyphenols: food sources and bioavailability, Am. J. Clin. Nutr. 79 (2004) 727–747.

[65] T. Hamaguchi, K. Ono, A. Murase, M. Yamada, Phenolic compounds prevent Alzheimer's pathology through different effects on the amyloid-beta aggregation pathway, Am. J. Pathol. 175 (2009) 2557–2565.

[66] M. Bagheri, M.T. Joghataei, S. Mohseni, M. Roghani, Genistein ameliorates learning and memory deficits in amyloid beta(1–40) rat model of Alzheimer's disease, Neurobiol. Learn. Mem. 95 (2011) 270–276.

[67] R.K. Chaturvedi, S. Shukla, K. Seth, S. Chauhan, C. Sinha, Y. Shukla, A.K. Agrawal, Neuroprotective and neurorescue effect of black tea extract in 6-hydroxydopamine-lesioned rat model of Parkinson's disease, Neurobiol. Dis. 22 (2006) 421–434.

[68] K. Rezai-Zadeh, G.W. Arendash, H. Hou, F. Fernandez, M. Jensen, M. Runfeldt, R.D. Shytle, J. Tan, Green tea epigallocatechin-3-gallate (EGCG) reduces beta-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice, Brain Res. 1214 (2008) 177–187.

[69] X. Mu, G.R. He, X. Yuan, X.X. Li, G.H. Du, Baicalein protects the brain against neuron impairments induced by MPTP in C57BL/6 mice, Pharmacol. Biochem. Behav. 98 (2011) 286–291.