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Research article

Extracts from two ubiquitous Mediterranean plants ameliorate cellular and animal models of neurodegenerative proteinopathies

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HIGHLIGHTS

- Extract of Padina pavonica (EPP) and Opuntia ficus-indica (EOFI) investigated.
- EOFI or EPP improved viability of a yeast model of Alzheimer's disease (AD).
- EOFI or EPP treatment ameliorated lifespan and mobility defects in AD flies.
- Survival of Parkinson's disease but not wild-type flies is enhanced by EOFI or EPP.
- Either extract mitigates toxicity of Amyloid-β and α-synuclein aggregates.

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ABSTRACT

A signature feature of age-related neurodegenerative proteinopathies is the misfolding and aggregation of proteins, typically amyloid-β (Aβ) in Alzheimer's disease (AD) and α-synuclein (α-syn) in Parkinson's disease (PD), into soluble oligomeric structures that are highly neurotoxic. Cellular and animal models that faithfully replicate the hallmark features of these disorders are being increasing exploited to identify disease-modifying compounds. Natural compounds have been identified as a useful source of bioactive molecules with promising neuroprotective capabilities. In the present report, we investigated whether extracts derived from two ubiquitous Mediterranean plants namely, the prickly pear *Opuntia ficus-indica* (EOFI) and the brown alga *Padina pavonica* (EPP) alleviate neurodegenerative phenotypes in yeast (*Saccharomyces cerevisiae*) and fly (*Drosophila melanogaster*) models of AD and PD. Pre-treatment with EPP or EOFI in the culture medium significantly improved the viability of yeast expressing the Arctic Aβ42 (E22G) mutant. Supplementing food with EOFI or EPP dramatically ameliorated lifespan and behavioural signs of flies with brain-specific expression of wild-type Aβ42 (model of late-onset AD) or the Arctic Aβ42 variant (model of early-onset AD). Additionally, we show that either extract prolonged the survival of a PD fly model based on transgenic expression of the human α-syn A53T mutant. Taken together, our findings suggest that the plant-derived extracts interfere with shared mechanisms of neurodegeneration in AD and PD. This notion is strengthened by evidence demonstrating that EOFI and to a greater extent EPP, while strongly inhibiting the fibrillogenesis of both Aβ42 and α-syn, accumulate remodelled oligomeric aggregates that are less effective at disrupting lipid membrane integrity. Our work therefore opens new avenues for developing therapeutic applications of these natural plant extracts in the treatment of amyloidogenic neurodegenerative disorders.

1. Introduction

Neurodegenerative disorders are a major societal challenge since they cause a wide range of debilitating symptoms that inexorably worsen with age and are associated with a significant drop in life expectancy. Most of these maladies are proteinopathies

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since they share a signature biochemical event which involves the pathological misfolding and aggregation of proteins or peptides of different amino acid compositions into a neurotoxic species, eventually forming insoluble amyloid fibrils that deposit within the nervous system [5,17,53]. Two highly prevalent neurodegenerative proteinopathies are Alzheimer's disease (AD), by far the most common cause of dementia, and Parkinson's disease (PD), which represents the most common age-related movement disorder. Both are predominantly sporadic, with familial cases accounting for a small percentage of early-onset disease [6]. Key events in the pathology of AD and PD involve the aggregation of Amyloid-β (Aβ) peptides and the α-synuclein (α-syn) protein, respectively, in disease-specific brain regions. Aβ peptides, which are generated by differential proteolytic cleavage of the transmembrane Amyloid Precursor Protein (APP), are the main components of the extracellular amyloid plaques that characterise AD brains. Cleavage products include Aβ40 and Aβ42, and familial mutations in the APP and presenilin genes are known to increase the levels of the latter, indicating that Aβ42 is the most toxic form [50]. α-syn is the principal constituent of intracytoplasmic inclusions known as Lewy bodies. The latter are identified in neurons and glia in the brains of both sporadic and inherited forms of PD [43].

The development of cellular and animal models that faithfully replicate the hallmarks of AD and PD has been central to further our understanding of the mechanisms leading to neurodegeneration [16,29,55]. In the simple and well-characterised eukaryote *Saccharomyces cerevisiae*, also known as budding yeast, Aβ toxicity was modelled by directing the Aβ peptide to the secretory pathway [58]. Phenotypic severity depends on the allele expressed, hence, the familial Arctic mutant (E22G), which accelerates the rate of aggregation, is more harmful than wild-type Aβ and disturbance of cell traffic routes has mitigating consequences [20]. Turning to *Drosophila melanogaster* as a complex behaving animal with a sophisticated, centralised nervous system, AD and PD models include those based on the transgenic expression of the human Aβ42 peptide [19] and α-syn protein [19,23], respectively. Reminiscent of the situation in patients, flies develop aggregates and show a robust decline of neurons both of which are enhanced on expression of mutant familial variants. The neuronal decline correlates with reduced lifespan and locomotor dysfunction [33]. Through genetic screens that identify enhancers and suppressors of neurodegenerative phenotypes in model organisms, inroads have been made in uncovering pathogenic pathways [47,58,60]. Importantly, established models of neurodegeneration are being increasingly exploited to facilitate drug discovery by identifying compounds that have the potential to prevent protein aggregation or reduce oligomer neurotoxicity [10,38,39].

Natural products are chemical compounds produced naturally by living organisms. They represent a vast resource of structurally diverse chemical entities that often feature biologically pre-validated molecular scaffolds. Natural product chemical space is therefore a 'privileged' starting point for inspired medicinal chemistry [49]. Extracts and compounds derived from plants have long been considered as an important source of drug discovery in the treatment of various conditions and they are increasingly being mined for natural product-inspired drug design [18]. Notably, in view that effective disease-modifying treatments for AD and PD are largely lacking, recent years have seen a surge in studies that identify plant-derived small molecules with promising neuroprotective activities (reviewed in [14,31,32,48,56,61]). The prickly pear *Opuntia ficus-indica* and the brown alga *Padina pavonica* are ubiquitous terrestrial and marine Mediterranean plants, respectively. Though long known for its antioxidant properties (reviewed in [42]), an extract from the skin of the *Opuntia ficus-indica* fruit (EOFI) has been shown to promote the synthesis of heat shock proteins in various organisms [8,45,54]. In humans, the consumption of EOFI before alcohol consumption was reported to reduce the hangover symptoms by dampening the inflammatory response [62]. Along similar lines, an extract of *Padina pavonica* (EPP) was found to be protective against both colon and liver injury in mice through anti-oxidant and anti-inflammatory activities [1,37].

In this study, we examine the *in vivo* neuroprotective properties of both EOFI and EPP. Our approach involved the sequential use of two different model systems, starting with the simpler *S. cerevisiae* cellular model and followed by validation in *Drosophila*, a more complex animal model. We show that both extracts had an alleviating effect on the viability of yeast cells expressing the Arctic Aβ42 peptide. In flies, we assess the impact of both extracts in models with varying severity of AD depending on the brain-specific expression of either wild-type Aβ42 (late-onset) or the Aβ42 Arctic variant (early-onset). EPP and EOFI improve both survival and mobility of the late- and early-onset AD model, respectively. Interestingly, we show that both extracts do not extend the lifespan of wild-type flies though they have a positive pharmacological effect on survival in a *Drosophila* model of PD based on the expression of α-syn A53T familial variant. Furthermore, EOFI and to a larger extent EPP interfere with the structural assembly of Aβ42 peptide and α-syn protein, and prevent disruption of neuronal-like membranes by oligomeric aggregates. Hence, we gain a mechanistic insight into the effect of either extract on neurodegenerative phenotypes in fly models.

2. Materials & methods

2.1. Extracts

EPP and EOFI were derived from *Padina pavonica* (whole plant) and *Opuntia-ficus indica* (fruit skin), respectively. In brief, following drying and milling of either whole or part of the plant, solid-liquid extraction using acetone as the solvent was carried out. The extracted product was filtered and then fed into a rotary evaporator where it was dried under vacuum at 55°C for several hours. Extracts were kindly provided by the Institute of Cellular Pharmacology (ICP)-Texinfine Laboratories (France/Malta) as crude extracts of the active fraction. Concentrations were selected after performing preliminary dose-finding studies to identify an effective but non-toxic concentration. Black tea extract (BTE, >80% theaflavins), was purchased from Sigma-Aldrich (Munich, Germany).

2.2. Yeast strains and culture

The *S. cerevisiae* BY4742 (*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0)* yeast strain was transformed with a plasmid carrying the Arctic Aβ42 construct under the GAL10 promoter, as described previously [20]. Transformants were grown overnight on dextrose (SD) medium (0.67% yeast nitrogen base, 2% dextrose) supplemented with 0.67% casamino acids. Upon reaching the exponential phase (OD_{600} = 1), the cells were placed in galactose (SG) medium supplemented with 0.67% casamino acids to induce transgene expression. Either SG or SD medium contained EPP or EOFI diluted in DMSO. Aerobic growth of yeast cells in liquid culture was maintained at 30°C with constant shaking at 300 rpm.

2.3. Assessment of yeast viability

Aliquots of yeast cells were collected at successive time points following inoculation in SG medium supplemented with either EPP or EOFI. Following dilution, they were plated on yeast extract peptone dextrose medium (YPD) and allowed to grow for 2 days at 30°C prior to counting the number of colonies or colony forming units (CFUs). Percentage viability was calculated by dividing the number

of CFUs observed on the respective time point by the those observed on day 0 and multiplying by 100.

2.4. Fly stocks and culture

Flies were allowed to develop on standard molasses/maizemeal and agar medium in 50 ml plastic vials. Following eclosion, female flies were allowed to mate for 48 h. They were then transferred to 50 ml plastic vials containing a sugar agar base and yeast paste (dry baker's yeast dissolved in milliQ water) and cultured under 12:12 h light-dark cycles, in a setup of 10–15 flies per vial. The vials were changed every second or third day. EPP or EOFI was dissolved in 91.2% ethanol and then diluted into yeast paste to result in a final concentration of 0.06% extract added per 1 g yeast. Where indicated, the extract was also supplemented during the larval stages, where flies were allowed to develop on a sugar agar base and yeast paste containing either extract in 50 ml plastic vials. The *UAS*/GAL4 system was utilised for transgene expression. The *elav*-GAL4 driver was used to express transgenes in the brain starting early during development. The *UAS* transgenes included human Aβ42 [19], $A\beta42^{E22G}$ [19], and $\alpha\text{-syn}^{A53T}$ [33] (generous gifts from Dr. Damian Crowther, University of Cambridge, UK and Dr. Aaron Voigt, RWTH Aachen University, Germany). The wild-type strain was w^{1118}. Incubation temperature was 25°C. Where indicated, temperature was raised to 29°C during the adult stage to enhance expression levels of transgenes.

2.5. Assessment of fly lifespan

Assessment of fly lifespan allows monitoring of the effect of genotype or environment on *Drosophila* survival throughout its lifespan. In brief, more than 100 mated female flies that were either wild-type or expressing transgenes were collected in groups of 10 in vials containing sugar agar base and yeast paste with either extract dissolved in ethanol or ethanol alone (control). Flies were exposed to the extract or ethanol throughout life, and the number of surviving flies was counted every second or third day when food was changed. Percentage survival at each time point was calculated to plot a Kaplan-Meier survival graph.

2.6. Assessment of fly climbing activity

A stringent assay was used to measure climbing ability every week throughout adulthood. In brief, 10 adult flies were transferred to a clean vertical column (25 cm long, 1.5 cm diameter) with a conical bottom end. After tapping, flies reaching the top and remaining at the bottom of the column after a 45 s period were counted separately. Vertical climbing entails a strenuous activity because flies have to work against gravity to climb to the top of the column. Four trials were performed at 1-min interval for each experiment. Only the last 3 trials were taken into consideration. Scores recorded were the mean number of flies at the top (n_{top}), the mean number of flies at the bottom (n_{bottom}) and the total number of flies assessed (n_{total}). The performance index (PI) defined as $1/2(n_{total} + n_{top} - n_{bottom}/n_{total})$ was calculated and utilised to assess climbing performance.

2.7. Preparation of Aβ42 and α-syn aggregates

Wild-type α-syn recombinant protein (kindly provided by Prof. Armin Giese, Ludwig-Maximilians-University of Munich, Germany) was aggregated as described previously [11]. Briefly, α-syn oligomers were prepared by incubating 7 μM monomeric α-syn with 1% (*v*/*v*) DMSO and 10 μM $FeCl_3$ in sterile phosphate-buffered saline (PBS) buffer (pH 7.4) for 4 h at 37°C, under constant agitation at 500 rpm. Stock solutions (200 μM) of human Aβ42 (rPeptide, UK) were obtained by dissolving the hexafluoroisopropanol (HFIP)-lyophilized peptide in 10 mM NaOH, followed by gentle vortexing for 30–60 s. Aliquots were stored at −80 °C and used only once. The oligomerisation reaction of Aβ42 monomer was initiated by incubating 45 μM Aβ42 in sterile PBS (pH 7.4) for 2 h at 37°C [26,40].

2.8. Preparation of small unilamellar vesicles (SUVs)

SUVs had a net negative charge and lipid moieties native to the neuronal environment [34] and were composed 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. Preparation was carried out as previously described [26]. In brief, chloroform was evaporated by nitrogen gas and the resulting dried lipid films hydrated with buffer (100 mM KCl, 10 mM MOPS/Tris, 1 mM EDTA, pH 7.0) containing Oregon Green® 488 BAPTA-1 (OGB-1; Invitrogen, Life Technologies, UK). The resulting milky lipid suspensions were used to make unilamellar lipid vesicles encapsulating OGB-1 by the detergent dialysis method. The size distribution of the vesicles was analysed by dynamic light scattering (DLS) with Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Vesicles had a homogenous size distribution with an average diameter of 81 ± 19 nm, and were hence classified as small unilamellar vesicles (SUVs).

2.9. Liposome permeabilisation assay

Liposome permeabilisation assays were carried out as described previously [26] using OGB-1 as the encapsulated fluorophore. Briefly, disruption of the vesicle membrane was indicated by an increase in OGB-1 fluorescence intensity (excitation, 485 nm; emission, 528 nm) as a function of time. Aggregates (1 μM Aβ42, 0.5 μM α-syn) 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) whilst fluorescence intensity data was acquired using KC Junior™ software (BioTek, Germany). Disruption of lipid vesicles by aggregates in the presence of compound extract was calculated as a percentage of permeabilisation caused by aggregates alone. At the concentrations tested, none of the extracts had any significant destabilising effect on the liposomal membranes (data not shown). Autofluorescence (where necessary) was measured for each compound and was subtracted from the sample well values.

2.10. Western blotting

Recombinant Aβ42 was dissolved in sterile PBS (pH 7.4) at 45 μM and aggregated by incubating, either alone or with compound extracts, at 37°C for 24 h. Recombinant α-syn was aggregated, either alone or with extracts, in the presence of 1% DMSO and 20 μM Fe^{3+} at room temperature for 7 days in parafilm-sealed Eppendorf tubes. Samples were prepared for electrophoresis by mixing with NuPAGE 4x LDS buffer and heating at 70°C for 10 min. Samples were loaded (Aβ42, 2 μg; α-syn, 1.5 μg) and separated on NuPAGE 4–12% Bis-Tris minigels (Invitrogen, Life Technologies, UK), then transferred onto Hybond nitrocellulose membrane (0.45 μm; GE Healthcare Life Sciences, Germany) in an XCell II™ semi-wet blotting chamber. After blocking overnight at 4°C with 5% Marvel dried skimmed milk in PBS containing 0.05% Tween-20 (TBS-T), membranes were probed for 1 h at room temperature with the following primary antibodies: anti-human amyloid-β 6E10 (1:4000; Covance, Cambridge, UK) and anti-human α-synuclein 15G7 (1:20,000; Abcam, Cambridge, UK). After several washings

with TBS-T and incubation with horseradish peroxidase-linked secondary antibodies (1:50,000 in TBS-T), the immunoreactive bands were visualized using the chemiluminescence-based Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Germany) according to manufacturer's instructions.

2.11. Statistical analysis

Statistical analyses were performed using GraphPad™ Prism 7 software (GraphPad Software, Inc.). Unless expressly indicated in the figure legend, results were expressed as the means and the standard error of the mean (S.E.M.) values, with n as the number of experiments. Differences between means were determined by the unpaired *t*-test whereas differences in survival between two groups was determined by the log-rank test. In all analyses, the null hypothesis was rejected at the 0.05 level.

3. Results

3.1. EPP and EOFI improve viability in a yeast AD model

We sought to first investigate the effect of EPP and EOFI on a yeast cellular model of AD. In this respect, we assessed whether the extracts can ameliorate the reduction in viability induced upon heterologous expression of the Arctic Aβ42 mutant fused to the mating factor α (MFα) prepro-leader sequence secretion signal. In this yeast model, the toxicity of the Arctic mutation is comparable to its wild-type Aβ counterpart [20]. Transformed cells were cultured in the appropriate medium supplemented with either extract dissolved in DMSO. Aliquots collected at various time points were then diluted prior to plating and the number of colonies were counted following a 2-day growth period. The percentage viability reflects the number of colonies counted on a particular time-point relative to the number on the first time-point assessed. Considering EOFI, although there was no effect on day 5, percentage viability of yeast expressing Arctic Aβ42 was significantly improved compared to the vehicle control on the subsequent time points assessed (Fig. 1). In case of EPP, the viability of the yeast AD model was improved on all days assessed with the most significant amelioration occurring on day 5 (Fig. 1). Overall, these findings indicate that, in spite of their different dynamics, both extracts have the potential to inhibit Aβ42 induced cytotoxicity *in vivo*.

3.2. EPP and EOFI improve survival and mobility in Drosophila models of AD

We next assessed whether either extract is capable of alleviating AD-like symptoms in transgenic *Drosophila* models of the disease. To this end, we made use of lines with brain-specific expression of the human wild-type and Arctic mutant Aβ42 transgene which represent a late-onset and early-onset AD model, respectively. Compared to wild-type, these flies show reduced longevity and exhibit a gradual decline in locomotive ability, two robust phenotypes that are typically used to screen for neuroprotective compounds or extracts [10,25,39]. Upon eclosion, adult flies expressing a double copy of the Aβ42 transgene were transferred to vials containing fly food supplemented with either the extract or the carrier as a control and their survival was continuously monitored. No effect was observed for EOFI (Fig. 2A). In contrast, flies fed EPP throughout adulthood showed a significant increase in lifespan compared to flies in the control group ($p = 0.0033$) (Fig. 2A). In this respect, EPP exposed flies had a median lifespan of 46 days compared to 44 days in control flies. Addressing the potency of the extracts on Arctic Aβ42-expressing flies, we observed that although EPP increased median lifespan from 29 to 31 days, overall change in lifespan was not statistically significant (Fig. 2B). Strikingly, we show that EOFI supplementation had a dramatic impact on the lifespan of flies expressing Arctic Aβ42 ($p = 0.0005$) (Fig. 2B). Consequently, the median lifespan is shifted from 29 days to 33 days upon treatment with EOFI. We subsequently investigated whether the observed improvement in lifespan correlated with a suppression of the mobility deficits inherent to AD fly models. In this respect, we note that on EOFI treatment, the early-onset AD fly model showed a marked improvement in climbing ability during the first week of adulthood. In this regard, EOFI restored the climbing ability of AD flies from 48% to 67% (Fig. 2C). EPP also had a significant outcome on mobility as demonstrated by an elevated performance in the late-onset AD model during the later stages of adult life. The strongest effect was detected on week 4 where EPP increased the climbing performance from 29% to 46% in AD flies (Fig. 2C). In summary, we expose a neurodegenerative suppressor ability by either EPP or EOFI in transgenic *Drosophila* models of AD.

3.3. EPP treatment improves survival of a Drosophila model of PD

We next queried whether the amelioration in neurodegenerative phenotypes observed in AD fly models is specific to an effect on aggregate neurotoxicity. To this end, we first cultured adult wild-type flies on an extract-supplemented diet to observe if either their survival is improved throughout their lifespan. We found that this was not the case, hence, wild-type flies treated with either EOFI or EPP did not show increased longevity throughout their adult life (Fig. 3). Furthermore, we hypothesised that an aggregate-specific effect would imply that the treatment might also work on other models of neurodegenerative proteinopathies. In this regard, we tested the extracts on an established fly model of PD based on the expression of the human α-syn carrying the A53T missense mutation that is associated with an early-onset familial form of PD and known to accelerate α-syn aggregate formation [43]. Interestingly, the survival of adult PD flies cultured on food supplemented with EOFI exhibited an enhanced lifespan compared to their control counterparts ($p = 0.0086$; Fig. 3B). We get the same effect when we swap EOFI with EPP. Thus, flies cultured on EPP supplemented diet starting early during development also show significantly improved survival compared to the control group ($p = 0.003$; Fig. 3B). In summary, both extracts are capable of shifting the median lifespan of PD flies by a day.

3.4. EPP or EOFI attenuate toxicity of Aβ42 and α-syn oligomers

We wished to correlate an ameliorative effect of either extract with a decrease in the toxicity of aggregates. We have recently demonstrated that Aβ42 and α-syn oligomers robustly compromised membrane integrity of SUVs having a net negative charge and lipid moieties native to the neuronal environment. Importantly, polyphenolic compounds including black tea extract (BTE) reversed this outcome [13,26]. We made use of this established assay to test the inhibitory effects of EPP and EOFI using BTE as a positive control. In case of Aβ42, either extract was allowed to interact with 1 μM pre-aggregated Aβ42 before adding to the liposomes, an approach that favours destabilisation of the toxic oligomers. We find that EPP or EOFI supplementation protected against lipid vesicle disruption by the membrane-active oligomeric species. Compared to BTE, which is a strong inhibitor of membrane permeabilisation, EOFI and EPP had a mild-to-moderate effect (Fig. 3C). In case of α-syn, either extract was allowed to interact with 0.5 μM pre-aggregated α-syn species prior to incubation with the liposomes and monitoring permeabilisation. Both EOFI and to a larger extent EPP interfered with α-syn-induced membrane perturbation to levels comparable to those of BTE (Fig. 3C). These findings corroborate the hypothesis of an ameliorative effect

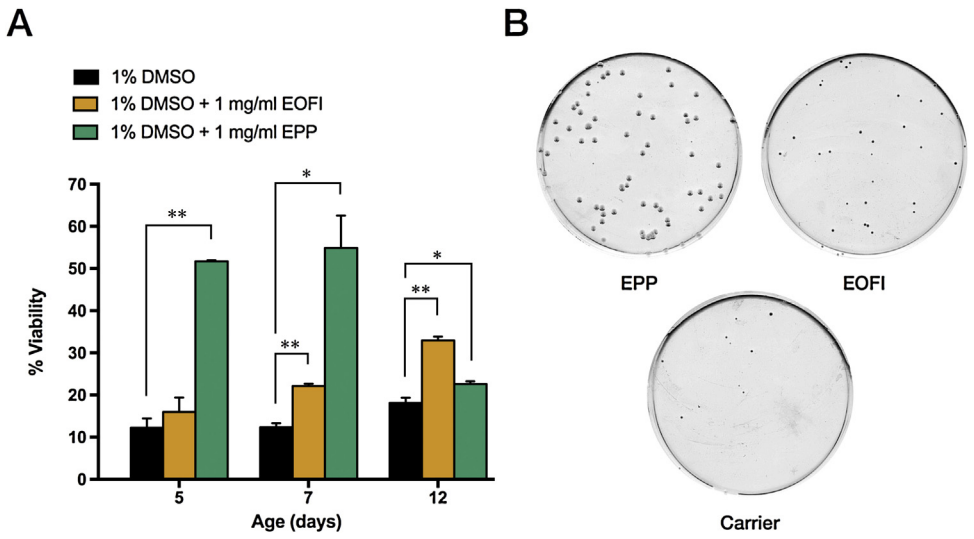

Fig. 1. Effect of EPP and EOFI on the viability of yeast expressing Arctic Aβ42. (A) EPP improved the viability of yeast cells at all time points measured whereas EOFI was observed to do so on day 7 and 12. Data presented are the mean percentage viability ± S.E.M. ($n = 2$ per condition). Significance was tested by the unpaired t-test, and for all data, $^{*}p < 0.05$, and $^{**}p < 0.01$. (B) Representative plates showing effect of both extracts on yeast colony growth assessed at day 7. EOFI and, to a greater degree EPP, increased number of viable yeast colonies.

by either EPP or EOFI that is dependent on antagonising prefibrillar aggregate toxicity.

3.5. EPP or EOFI demonstrate anti-amyloidogenic potential against Aβ42 and α-syn aggregate formation

In order to probe the direct anti-amyloidogenic potential of the two extracts, we evaluated their inhibitory activity on Aβ42 and α-syn aggregation *in vitro*. With this aim, aggregate assembly was monitored in the presence of EOFI and EPP using SDS-PAGE and immunoblotting. Regarding Aβ42 fibrillisation, both EOFI (400 μg/ml) and EPP (800 μg/ml) significantly slowed down Aβ42 aggregation over 24 h in a dose-dependent manner. This was evidenced by a marked increase in Aβ monomeric and low-order oligomeric band intensities upon extract incubation, compared to 24 h aggregation in absence of extracts (Fig. 4A). Moreover, we tested EOFI and EPP at a higher concentration (2000 μg/ml), and although the anti-aggregation activity of EOFI did not increase any further indicating a possible effect saturation, EPP at 2000 μg/ml resulted in a complete disappearance of the Aβ protofibril band (Fig. 4A). With regards to α-syn, EOFI and EPP, both at a concentration of 100 μg/ml, suppressed fibril aggregation over 7 days, as shown by an increase in α-syn monomer and low-molecular-weight oligomers (LMWO) concomitant with a marked reduction in high-molecular-weight oligomers (HMWO) (Fig. 4B). We additionally compared EOFI and EPP inhibition of α-syn fibrillisation with that exhibited by black tea extract (BTE), a well-characterised inhibitor of α-syn aggregation [12,28]. Indeed, at the same extract concentration of 100 μg/ml, BTE was less potent at interfering with the assembly of α-syn HMWO (Fig. 4B). Overall, these studies demonstrate that EOFI and EPP have a direct antagonising effect on the aggregation dynamics of both Aβ42 and α-syn.

4. Discussion

In the present work we report that, extracts derived from ubiquitous Mediterranean plants *Opuntia ficus-indica* and *Padina pavonica*, alleviate neurodegenerative phenotypes in cellular and animal models of AD and PD. Such an outcome can be partly explained by the anti-amyloidogenic potential of either extract which mitigates the disruption of lipid membranes by Aβ42 or α-syn aggregates. Considerable evidence postulates a critical role of toxic prefibrillar oligomers as the main pathological agents in neurodegenerative proteinopathies [24]. Hence, preventing oligomer toxicity may represent a key strategy for therapeutic intervention in these devastating disorders. Our study joins several others in indicating that natural products are an invaluable source of bioactive compounds which robustly interfere with aggregate toxicity [10,13,25,26,51]. The main active molecules in both EPP and EOFI have already been isolated. The activity of EPP results from a $C_{28}H_{44}O_2$ molecule whose structure bears resemblance to 17-β oestradiol despite having a non-steroidal scaffold [15]. In case of EOFI, a $C_{29}H_{49}O_2$ molecule has been identified by mass spectrometry though resolution of its structure requires further work [4]. Nonetheless, we do not exclude the possibility that minor components, either alone or in synergy, may be responsible for either extract's neuroprotective activities. Crucially, in view of their ameliorative effect on both AD and PD fly models, and their positive impact on either Aβ42 or α-syn aggregates, EPP or EOFI have a 'cross-species' effect, a characteristic that enhances their potential as drug candidates targeting a wider range of neurodegenerative disorders of the amyloid type.

It is noteworthy that the alleviating effect of either extract is associated with the genotype which determines the severity of the disease model. In this respect, EPP has the strongest effect on flies expressing wild-type Aβ42, which represent a model of late-onset AD. Furthermore, the disease-modifying ability of this extract was pronounced in the later stages of adulthood. These results might indicate that EPP has poor bioavailability, hence, its therapeutically effective dose is reached only after several weeks of repeated oral administration precluding an effect on models with more severe phenotypes. EOFI gave opposite results, thereby having a marked impact only on an early-onset or aggressive AD fly model based on the expression of the Arctic Aβ42 variant. In this respect, EOFI is thought to have good bioavailability properties and possibly is more effective in situations with heighted levels of cellular stress [54,62]. In the yeast AD model, both EPP and EOFI were found to have an ameliorative effect on the viability of cells expressing Arctic Aβ42. Improved bioavailability in yeast could well explain the apparent discrepancy between fly and yeast with regards to the effect of EPP.

Interestingly, we find that although EPP or EOFI did not influence the survival of wild-type flies, either extract was capable of

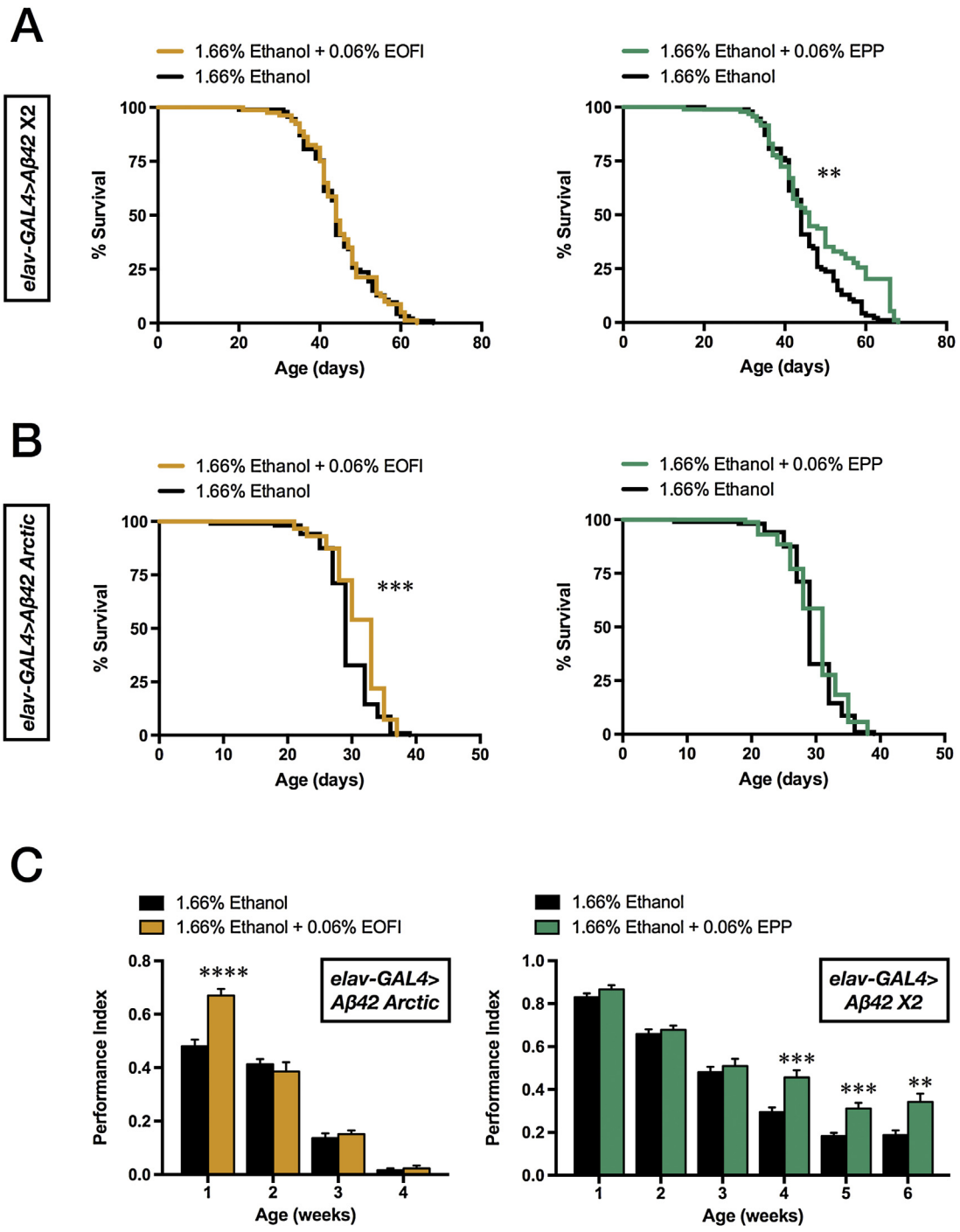

Fig. 2. EPP and EOFI improve lifespan and locomotor ability in fly models of AD. (A) EPP, but not EOFI, has a positive impact on the longevity of flies with pan-neuronal expression of a double dose of Aβ42 representing a model of late-onset AD. (B) As a model of early-onset AD, flies expressing the Arctic Aβ42 variant showed an increased overall survival on exposure to EOFI but not EPP. Significance was tested by the log-rank test, and for all data, $^{**}p < 0.01$, and $^{***}p < 0.001$ ($n \geq 100$ per condition). (C) EPP and EOFI suppressed locomotor dysfunction during different stages of adulthood in the late- and early-onset AD fly model, respectively. Data are presented as the mean percentage climbing performance of flies ± S.E.M. of 10 independent experiments, and $n \geq 100$ per condition. Significance was tested by the unpaired *t*-test, and for all data, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

prolonging the lifespan of flies expressing α-syn^{A53T}, which constitute a genetic model of inherited PD. These findings suggest an intrinsic effect by the extracts on the pathology underlying neurodegenerative aggregopathies. In support of this notion, we show that on incubation with Aβ42 or α-syn aggregates, EOFI and, to a greater degree, EPP demonstrate direct anti-aggregation potential and lower the toxicity of either species as manifested by a reduction in their deleterious effect on neuronal-like membranes. The function of neurons is highly dependent on maintenance of membrane integrity and perturbation of membranes intra- and extra-cellularly is recognised as a key pathophysiological event in both AD and PD [9,24,27]. In this regard, we demonstrate that both extracts prevent aggregation into fibrillar assemblies and force Aβ42 and α-syn to accumulate in oligomeric and protofibrillar conformations. Given that extract-incubated aggregates are significantly less damaging to membranes, presumably EOFI and EPP are redirecting

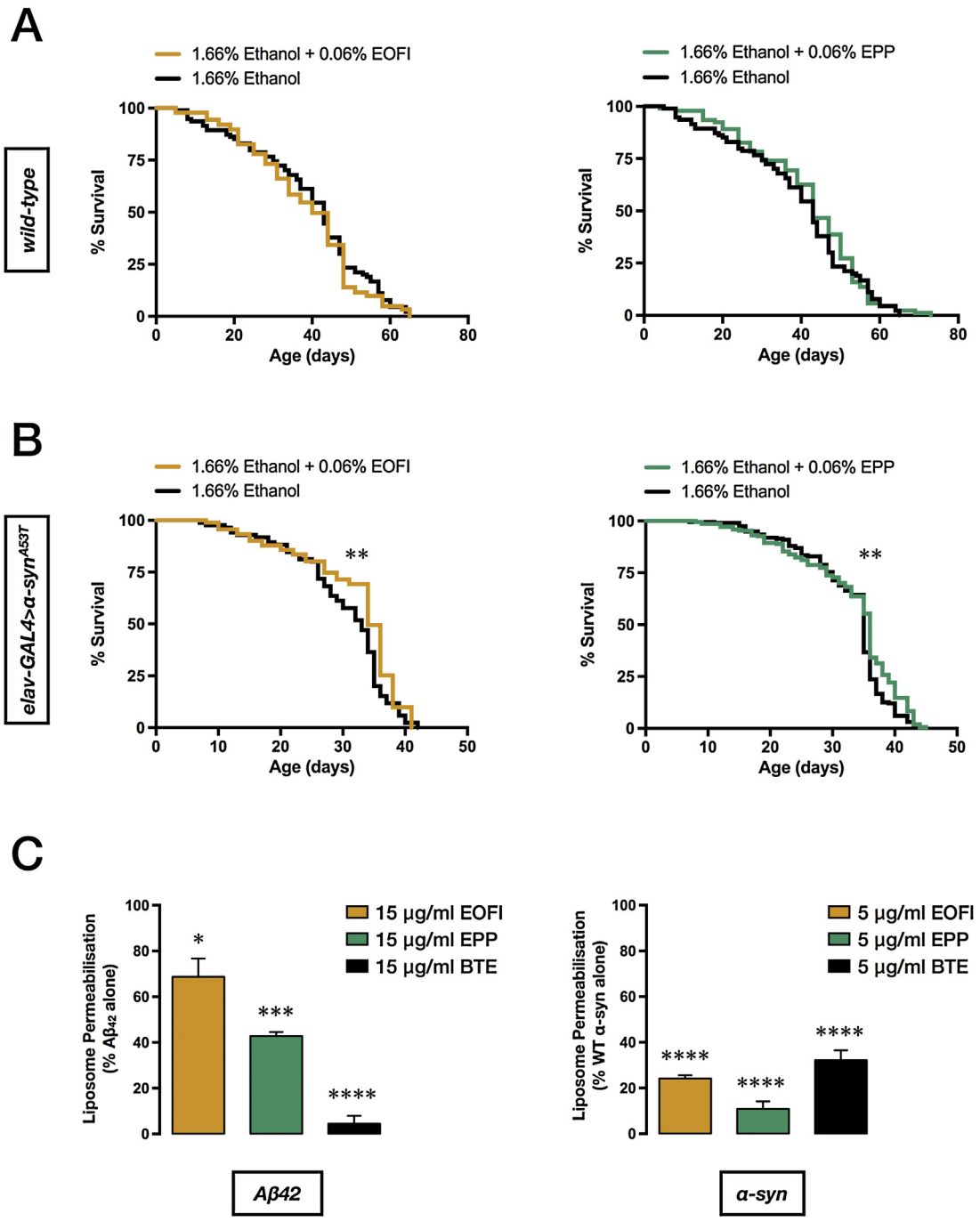

Fig. 3. EPP or EOFI impacts lifespan of PD flies and suppresses aggregate-induced membrane damage. (A) Wild-type flies reared on EPP or EOFI supplemented medium do not show an increase in longevity. (B) Flies with a pan-neuronal expression of α-syn^{A53T}, representing a model of PD, show an appreciable improvement in survival throughout adulthood upon treatment with either EOFI or EPP. Significance was tested by the log-rank test, and $^{**}p < 0.01$ ($n \geq 100$ per condition). PD flies were cultured at a temperature of 29°C and, for EPP, extract supplementation started from early development. (C) Liposome permeabilisation induced by prefibrillar aggregates of Aβ42 (left) or α-syn (right) was significantly reduced when each aggregate species was allowed to interact with either EPP or EOFI prior to liposome exposure. BTE, which is previously known to stimulate the assembly of α-syn into nontoxic aggregates. Data presented are the mean percentage of the permeabilisation effect of Aβ42 or α-syn aggregates alone in the absence of extracts ± S.E.M. ($n \geq 3$). Significance was tested by the unpaired *t*-test, and for all data, $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

amyloidogenic polypeptide self-assembly into non-toxic, potentially off-pathway, oligomeric structures. They can also prevent membrane leakage by binding to exposed toxic structural motifs on the oligomers. Indeed, the mechanism of remodelling amyloid fibril assembly into the generation of off-pathway oligomers that are non-toxic has been amply demonstrated for several natural aromatic molecules [2,41]. These include the main phenolic constituent of green tea, (−)-epigallocatechin gallate (EGCG) [7,21], and theaflavins, a group of catechin polymers enriched in BTE [28]. EGCG and theaflavins were shown to stimulate the assembly of Aβ42 and α-syn into spherical aggregates that were both non-toxic and incompetent at seeding amyloid formation. Interestingly, we show by immunoblotting that EOFI and EPP had very similar effects on α-syn aggregation as BTE. Similarly, other small-molecule polyphenols like myricetin, nordihydroguaiaretic acid (NDGA), gallic acid (3,4,5-trihydroxybenzoic acid), a fisetin analogue (3,3′,4′,5′-tetrahydroxyflavone), and biflavonoids, have all been shown to drive Aβ42 or α-syn aggregation into the production

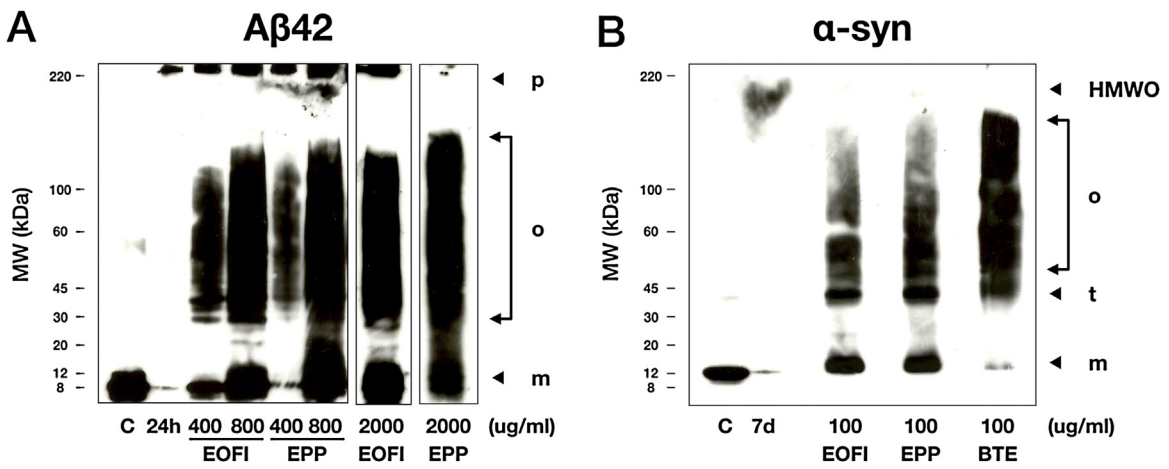

Fig. 4. EOFI and EPP attenuate aggregation of Aβ42 and α-syn *in vitro*. SDS-PAGE followed by Western blotting was used to determine the effects of EOFI or EPP on fibrillisation of Aβ42 peptide and α-syn protein. (A) 45 μM Aβ42 was incubated alone or in the presence of EOFI or EPP at concentration ratios of Aβ:extract 1:2 (0.4 mg/ml), 1:4 (0.8 mg/ml) and 1:10 (2 mg/ml) for 24 h at 37°C. Bands were visualized by immunoblot analysis probed with the anti-human amyloid-β 6E10 antibody. m, o, and p indicate monomer, oligomer and protofibril bands, respectively. (B) EOFI, EPP or BTE (0.1 mg/ml) were added to α-syn (7 μM) polymerisation reactions. Samples were incubated for 7 days at 25°C, and the bands visualized using the anti-human α-synuclein 15G7 antibody. m, t, o and HMWO indicate monomer, trimer, oligomer, and high-molecular-weight-oligomer bands, respectively. C (control) represents samples without incubation. Each gel is representative of results obtained in each of three independent experiments.

of atypical oligomeric aggregates that manifest reduced toxicity [3,36,57,59]. Finally, our findings on the anti-aggregation potential of EPP are congruent with those recently reported by Shanmuganathan and colleagues [52] who demonstrate that an extract derived from *Padina gymnospora*, a marine seaweed similar to *Padina pavonica*, had anti-aggregation and destabilising activities on Aβ peptides.

Our results are in agreement with other reports that highlight the neuroprotective effects of marine plants and terrestrial cactus species (reviewed in [22,44,46]). In particular, Kim et al. [34] report that the preventive administration of an extract derived from *Opuntia ficus-indica* fruits alleviated the excitotoxic neuronal damage induced by cerebral ischemic injury in gerbils. Similar findings were described in rats upon treatment with components derived from *Opuntia dillenii*, another species of prickly pear [30]. Furthermore, administration of a stem-derived extract from *Opuntia ficus-indica* enhanced long-term memory in mice, an effect partially dependent on enhanced survival of immature neurons [35].

5. Conclusion

The present study demonstrates that two extracts derived from ubiquitous plants in the Mediterranean basin alleviate signature phenotypes in models of AD and PD, an effect that is partly dependent on an attenuation of aggregate toxicity. It is noteworthy that in view of their safety profiles, both EPP and EOFI are already present on the market as nutraceuticals and cosmeceuticals targeting non-neuronal processes. Future work should therefore further explore the use of these extracts as promising drug candidates in the treatment of classical neurodegenerative proteinopathies.

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