Research Article

Dementia-related Bri2 BRICHOS is a versatile molecular chaperone that efficiently inhibits Aβ42 toxicity in Drosophila

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Formation of fibrils of the amyloid-β peptide (Aβ) is suggested to play a central role in neurodegeneration in Alzheimer’s disease (AD), for which no effective treatment exists. The BRICHOS domain is a part of several disease-related proproteins, the most studied ones being Bri2 associated with familial dementia and prosurfactant protein C (proSP-C) associated with lung amyloid. BRICHOS from proSP-C has been found to be an efficient inhibitor of Aβ aggregation and toxicity, but its lung-specific expression makes it unsuited to target in AD. Bri2 is expressed in the brain, affects processing of Aβ precursor protein, and increased levels of Bri2 are found in AD brain, but the specific role of its BRICHOS domain has not been studied in vivo. Here, we find that transgenic expression of the Bri2 BRICHOS domain in the Drosophila central nervous system (CNS) or eyes efficiently inhibits Aβ42 toxicity. In the presence of Bri2 BRICHOS, Aβ42 is diffusely distributed throughout the mushroom bodies, a brain region involved in learning and memory, whereas Aβ42 expressed alone or together with proSP-C BRICHOS forms punctuate deposits outside the mushroom bodies. Recombinant Bri2 BRICHOS domain efficiently prevents Aβ42-induced reduction in γ-oscillations in hippocampal slices. Finally, Bri2 BRICHOS inhibits several steps in the Aβ42 fibrillation pathway and prevents aggregation of heat-denatured proteins, indicating that it is a more versatile chaperone than proSP-C BRICHOS. These findings suggest that Bri2 BRICHOS can be a physiologically relevant chaperone for Aβ in the CNS and needs to be further investigated for its potential in AD treatment.

Introduction

Amyloid fibrils — polymers of polypeptide chains in cross-β-strand conformation — define one group of misfolding diseases, in which the pathology is caused by gain of toxic effects rather than by loss of protein function [1]. The amyloid proteins form insoluble extracellular deposits in tissue and/or soluble aggregates, which are toxic to cells [2]. Amyloid formation is coupled with 30 known diseases, of which pulmonary fibrosis linked to deposition of amyloid composed of lung surfactant protein C (SP-C) is the latest one described [3,4]. The most common form of dementia, Alzheimer’s disease (AD), is characterized by senile plaques containing amyloid fibrils of amyloid-β peptide (Aβ) and neurofibrillary tangles containing the tau protein [5]. It is suggested that the main cause of
neuronal dysfunction in AD is that pre-fibrillar intermediates present during the aggregation of Aβ cause toxicity to neurons, but the underlying mechanisms are poorly understood [2,6]. In spite of tremendous efforts, there is still no cure available for AD and the symptomatic therapies used have limited effects [7]. To date, therapeutic strategies focus on inhibition of Aβ generation and misfolding, and the elimination of toxic forms by immunotherapies. However, the results of clinical trials have so far been disappointing [8]. The BRICHOS domain of proSP-C is the first described example of a chaperone that specifically blocks the secondary nucleation event during Aβ42 fibril formation, and thereby markedly reduces the formation of toxic oligomers [9], and when transgenically expressed, it reduces the toxic effects of Aβ42 in the central nervous system (CNS) of Drosophila melanogaster, measured as improved longevity and locomotor activity [10]. This makes BRICHOS a novel candidate in the search for AD treatments [11,12]; however, proSP-C BRICHOS expression is restricted to alveolar type II cells [13], making the endogenous protein unable to interfere with Aβ deposition in the CNS. The BRICHOS domain is found in over 1000 proteins, including species variants, and 10 families of BRICHOS-containing proproteins have been defined [14]. In the CNS, two BRICHOS-containing proteins are expressed, Bri2 and Bri3, also referred to as integral transmembrane protein 2B (ITM2B) and ITM2C, respectively. Mutations in the Bri2 protein underlie familial British and Danish dementias (FBD and FDD, respectively) and result in peptides released from the C-terminal part of Bri2 that form amyloid [15,16]. Bri2 and Bri3 have previously been shown to interact with Aβ precursor protein (AβPP) and to play a role in its processing, affecting the levels of produced Aβ peptides [17,18], but whether the BRICHOS domain specifically has a physiological role in Aβ production, aggregation and/or toxicity is not known.

All BRICHOS-containing proproteins have a region of high β-sheet propensity located outside of the BRICHOS domain, either at the C-terminal end or in the transmembrane (TM) part in the case of proSP-C [19]. BRICHOS has broad substrate specificity, for example proSP-C BRICHOS interacts with SP-C, but also with Aβ [20], medin, associated with amyloid in the aortic wall [21], and with non-natural, designed amyloidogenic proteins [22]. Bri2, like other BRICHOS-containing proproteins, is a type II TM protein [23]. It is ubiquitously expressed with a significant expression in the neurons of the hippocampus and cerebellum in humans [15,24]. Normally, the C-terminal part of Bri2 is processed by proteolysis, which results in release of a 23-residue peptide, Bri23 [25]. Mutations in the Bri2 gene lead to production and release of extended 34-residue C-terminal peptides ABri and ADan, which form amyloid deposits linked to FBD and FDD, diseases that share pathological and clinical characteristics with AD [15,16]. Bri2 suppresses Aβ deposition and is also suggested to be a physiological inhibitor of AβPP processing, probably by masking the secretase cleavage sites. It has been proposed that the loss of proper Bri2 function, as in FBD and FDD, affects the levels of AβPP metabolites, resulting in similar pathobiology in FBD/FDD and AD [17,25–27].

Transgenic expression of a fusion protein composed of Aβ42 linked to Bri2 in mice results in delayed amyloid plaque formation, compared with mice transgenic for AβPP, and in the lack of detectable memory deficits [28]. This suggests that the presence of the BRICHOS domain can delay Aβ42 fibril formation and prevent the cognitive decline associated with it [10]. Both recombinant human proSP-C and Bri2 BRICHOS domains delay fibril formation of Aβ40 and Aβ42 in vitro, but Bri2 is more effective on a molar basis [29]. These observations, together with the fact that Bri2 and AβPP are expressed in neurons, and that the BRICHOS domain is proteolytically released from mature human Bri2 [30], suggest that Aβ could be a physiological target for Bri2 BRICHOS. For these reasons, Bri2 is an interesting novel potential target or effector in the search for treatments of AD. However, Bri2 is a multidomain proprotein and it has not been settled what region or regions exactly are involved in the interactions with Aβ and/or AβPP. Bri2 residues 46–106, a region encompassing the TM part but not the BRICHOS domain, are sufficient for interfering with AβPP processing [31]. It has been shown that the Bri23 peptide inhibits Aβ aggregation [32], but also a Bri2 construct lacking the Bri23 region can inhibit Aβ aggregation and toxicity [28]. It is therefore important to study the effects of Bri2 BRICHOS alone, that is in the absence of remaining parts of the Bri2 protein, on Aβ42 aggregation and toxicity. In the present study, we find that the Bri2 BRICHOS domain (corresponding to residues ∼130–230 of Bri2 [23]) is a more potent inhibitor of Aβ42 toxicity in vivo and has a broader mechanism of action than proSP-C BRICHOS.

**Experimental procedures**

**Expression and purification of recombinant proteins**

Met-Aβ1–42 or Met-Aβ1–40 (hereafter referred to as Aβ42 and Aβ40, respectively) was expressed in *Escherichia coli* BL21 from synthetic genes and purified in batch format using ion exchange and size exclusion steps as
described before, which results in highly pure monomeric peptides [9]. Purified Aβ42 was aliquoted in low-bind Eppendorf tubes (Axygene) and stored at −20°C. The Aβ42 and Aβ40 concentrations were determined by measuring the absorbance at 280 and 300 nm (A280 and A300) and were calculated by using an extinction coefficient of 1424 M⁻¹ cm⁻¹ for (A280 − A300).

A fragment corresponding to positions 113–231 of human Bri2 (NP_068839) was expressed together with His6 and thioredoxin in E. coli strain Origami B (DE3) pLysS (Novagen, Madison, WI). Bacteria were cultured in LB medium with 70 μg/ml kanamycin at 30°C for 16 h. Protein expression was induced by adding 0.5 mM isopropylthiogalactoside. After another 4 h at 25°C, cells were harvested and resuspended in 20 mM sodium phosphate buffer, pH 7.5 and stored at −20°C. The cells were lysed by lysozyme (1 mg/ml) for 30 min and incubated with Dnase and 2 mM MgCl₂ for 30 min on ice, the lysate was centrifuged at 6000 × g for 20 min and the pellet was suspended in 2 M urea in 20 mM sodium phosphate buffer, pH 7.5, and sonicated for 5 min. After centrifugation at 24 000 × g for 30 min at 4°C, the supernatant was filtered through a 5 μm filter and loaded on a nickel-agarose column (Qiagen, Ltd, West Sussex, UK). The column was washed with 2 M urea in 20 mM phosphate buffer, pH 7.5, then with 1 M urea in the same buffer and finally with 50 μl of 20 mM sodium phosphate buffer and 20 mM imidazole, pH 7.5. The protein was then eluted with 200 mM imidazole in 20 mM sodium phosphate buffer, pH 7.5, dialyzed against 20 mM sodium phosphate buffer, pH 7.5, cleaved by thrombin for 16 h at 4°C (enzyme/substrate weight ratio of 0.002) to remove the thioredoxin and His₆ tag and then reapplied to a Ni²⁺ column to remove the released tag. Concentration was determined from A280 using a molar extinction coefficient of 9065 M⁻¹ cm⁻¹.

ProSP-C BRICHOS protein, corresponding to residues 59–197 in proSP-C, was expressed and purified as described previously [29]. Briefly, the construct was expressed in E. coli strain Origami B (DE3) pLysS (Novagen, Madison, WI) as a fusion protein with the thioredoxin–His₆ and S-tag and was purified using nickel-agarose column chromatography. After thrombin cleavage, an additional nickel-agarose purification step was used to remove the thioredoxin and His₆ tag. The protein was then further purified using ion exchange chromatography.

**Analysis of Aβ42 and Aβ40 fibril formation**

Fibril formation kinetics was studied by recording the thioflavin T (ThT) fluorescence intensity as a function of time in a plate reader (FLUOStar Galaxy, BMG Labtech, Offenbg, Germany). The fluorescence was recorded using bottom optics in half-area 96-well polystyrene plates with clear bottom (Corning Glass, 3881) using a 440 nm excitation filter and a 490 nm emission filter. Aβ42 and Aβ40 monomers were isolated by size exclusion chromatography (SEC) over a Superdex 75 column (GE Healthcare) in 20 mM sodium phosphate, 200 μM EDTA, 0.02% NaN₃ at pH 8 for Aβ42 and pH 7.2 for Aβ40, and the peptides were kept on ice. Every sample was supplemented with 10 μM ThT from a 1 mM stock solution. Ice-cold Aβ42 solution (80 μl) was added to each well, and the plate was immediately placed in the fluorescence reader at 37°C, and incubated under quiescent conditions with readings made every 4 min.

**Transmission electron microscopy**

Recombinant Aβ42 (5 μM), or Aβ40 (10 μM) with or without 2.5 or 0.15 μM of Bri2 BRICHOS, was incubated overnight (or for 60 h with Aβ40) at 37°C in a volume of 100 μl in the presence of 0.5 μM ThT for monitoring the fibril formation. The samples were thereafter centrifuged at 22 000 × g for 1 h, the supernatant was discarded and fibrils in the pellet were diluted in 20 μM Tris-buffered saline (TBS). Aliquots of 2 μl were loaded on nickel-coated grids, and excess of sample was removed. The grids were placed on a drop of 1% BSA in TBS, incubated for 30 min at room temperature and then washed three times for 10 min on drops of TBS. The grids were then placed on drops of goat anti-Bri2 BRICHOS antiserum diluted 1:500 in TBS and incubated overnight at +4°C. After washing three times for 10 min in TBS, the grids were placed on a drop of rabbit anti-goat IgG coupled with 10-nm gold particles diluted 1:40 in TBS and were incubated for 2 h at room temperature. The grids were then washed five times as before, followed by negatively staining with 2% uranyl acetate in 50% ethanol. The immunolabelled fibrils were examined using a Hitachi H7100 transmission electron microscopy (TEM) operated at 75 kV.

**Analysis of chaperone activity**

Pig heart mitochondrial citrate synthase (CS) was purified as described previously [33], firefly (Photinus pyralis) luciferase was purchased from Promega (Madison, USA) and bovine mitochondrial rhodanese was...
Electrophysiological studies in mouse hippocampal slice preparations

Experiments were carried out in accordance with the ethical approval granted by Norra Stockholm’s Djurförsöksetiska Nämnd to André Fisahn (N45/13). C57BL/6 mice of either sex (postnatal days 14–23, supplied from Charles River, Germany) were used in all experiments. The animals were deeply anaesthetized using isofluorane before being killed by decapitation.

The brain was dissected out and placed in ice-cold artificial cerebrospinal fluid (ACSF) modified for dissection. This solution contained (in mM): 80 NaCl, 24 NaHCO_3, 25 glucose, 1.25 NaH_2PO_4, 1 ascorbic acid, 3 NaPyruvate, 2.5 KCl, 4 MgCl_2, 0.5 CaCl_2 and 75 sucrose. Horizontal sections (350 μm thick) of the ventral hippocampi of both hemispheres were prepared with a Leica VT1200S vibratome (Microsystems, Stockholm, Sweden). Immediately after slicing, sections were transferred to a submerged incubation chamber containing standard ACSF (in mM): 124 NaCl, 30 NaHCO_3, 10 glucose, 1.25 NaH_2PO_4, 3.5 KCl, 1.5 MgCl_2 and 1.5 CaCl_2. The chamber was held at 34°C for at least 20 min after dissection. It was subsequently allowed to cool to ambient room temperature (∼22°C) for a minimum of 40 min. Proteins (Aβ42, Bri2 BRICHOS, lysozyme and combinations thereof) were added to the incubation solution for 15 or 180 min before transferring slices to the interface-style recording chamber for extracellular recordings. While incubating, slices were continuously supplied with carbogen gas (5% CO_2 and 95% O_2) bubbled into the ACSF.

Recordings were carried out in hippocampal area CA3 with borosilicate glass microelectrodes and pulled to a resistance of 3–5 MΩ. Local field potentials (LFPs) were recorded in an interface-type chamber (perfusion rate 4.5 ml/min) at 34°C using microelectrodes filled with ACSF placed in stratum pyramidale. LFP γ-oscillations were elicited by applying kainic acid (100 nM, Tocris) to the extracellular bath. The oscillations were allowed to stabilize for 20 min before any recordings were carried out. The interface chamber recording solution contained (in mM): 124 NaCl, 30 NaHCO_3, 10 glucose, 1.25 NaH_2PO_4, 3.5 KCl, 1.5 MgCl_2 and 1.5 CaCl_2.

Interface chamber LFP recordings were performed with a four-channel amplifier/signal conditioner M102 amplifier (Electronics Laboratory, Faculty of Mathematics and Natural Sciences, University of Cologne, purchased from Sigma (St. Louis, USA). All protein concentrations refer to the monomers. Thermal aggregation of model substrates was monitored by following the light scattering of the samples at 360 nm. Model substrates were diluted to an end concentration of 600 nM in 40 mM HEPES/KOH, pH 7.5, and were equilibrated at 43°C in the absence and also in the presence of different concentrations of BRICHOS proteins. Aggregation kinetics were measured in triplicates using an Ultrospec 3100 pro spectrophotometer (GE Healthcare, Uppsala, Sweden) with thermostated quartz cells.

Inactivation and refolding of CS was performed as previously described [34,35]. In short, thermal inactivation of 600 nM CS was assayed in the absence or presence of BRICHOS proteins in 40 mM HEPES/KOH, pH 7.5, at 43°C. To determine CS activity, aliquots were taken at time points indicated. The activity of CS, that is to catalyze condensation of oxaloacetate and acetyl coenzyme A, was measured in 50 mM Tris–HCl and 2 mM EDTA, pH 8.0, at 25°C. For refolding experiments, CS was unfolded in 40 mM HEPES/KOH (pH 7.5) with 6 M GdmCl. To initiate reactivation, CS was diluted 50-fold to an end concentration of 300 nM into 50 mM Tris–HCl and 2 mM EDTA, pH 8.0; samples were shifted to 25°C and the stabilizing ligand oxaloacetic acid was added to a final concentration of 1 mM. BRICHOS proteins (concentrations as indicated) were added subsequently.

For analytical SEC experiments, CS (end concentration of 300 nM) refolding samples at 5 min after initiation of refolding in the presence of 4.8 μM BRICHOS proteins as well as 4.8 μM BRICHOS proteins without the addition of CS were separated on a Diol 300 column (YMC, Dinslaken, Germany), pre-equilibrated in 50 mM sodium phosphate and 150 mM NaCl, pH 7.0, using a flow rate of 0.5 ml/min. Proteins were detected by fluorescence at an excitation wavelength of 280 nm and an emission wavelength of 330 nm using a FP 920 fluorescence detector (Jasco, Großumstadt, Germany). Standard proteins from the molecular-weight marker kit (carbonic anhydrase, albumin, alcohol dehydrogenase and thyroglobulin; Sigma, St. Louis, USA) were used for calibration. The recorded spectra were normalized setting the highest peak of the spectrum to 1.

Inactivation and reactivation of luciferase was assayed as described previously [36]. Luciferase was unfolded in 6 M GdmCl and refolding was initiated by dilution to a final concentration of 600 nM in assay buffer in the presence and absence of 2.4 μM BRICHOS proteins. The kinetics of refolding was monitored for 60 min at 25°C. The activity of luciferase was compared with the activity of untreated, native luciferase, which was set to 100%. Luciferase activity was measured using a Tecan GENios Microplate Reader (Tecan, Männedorf, Switzerland).
Cologne, Germany). Signals were sampled at 10 kHz, conditioned using a Hum Bug 50 Hz noise eliminator (LFP signals only; Quest Scientific, North Vancouver, BC, Canada), software low-pass filtered at 1 kHz, digitized and stored using a Digidata 1322A and Clampex 9.6 software (Molecular Devices, CA, USA).

Power spectral density plots (from 60 s long LFP recordings) were calculated in averaged Fourier-segments of 8192 points using Axograph X (Kagi, Berkeley, CA, USA). Oscillation power was calculated by integrating the power spectral density between 20 and 80 Hz. Data are reported as means ± standard errors of the means. For statistical analysis, the Student’s t-test (unpaired) was used. Significance levels are *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. All experiments were performed with parallel controls from the same animal/preparation.

**Transgenic D. melanogaster strains**

To obtain transgenic *Drosophila* lines, transcription factor GAL4 responsive pUAST vectors were used. The cDNA sequence of the Bri2 BRICHOS domain (Bri290–236, NP_068839), including an upstream sequence for the human surfactant protein B (SPB1-23, NP_000533) signal peptide (for expression through the secretory pathway), was inserted into the pUASTattB vector. Chromosome 3 86 Fb locus site-specific transgenesis was achieved using the φC31 system (BestGene, Inc., USA). The generation of proSP-C BRICHOS (proSPC59–197, NP_001165881) transgenic flies has been described recently [10].

Human Aβ42 including a signal peptide from the *Drosophila* necrotic gene (NP_524851) transgenic flies with transgene inserted into the second chromosome was used to achieve expression of Aβ42 [37]. These flies were crossed with BRICHOS-expressing flies to generate Aβ42 + BRICHOS flies. Expression of Aβ42 and BRICHOS proteins were obtained by crossing transgenic flies with the specific GAL4-driver strains — pan-neuronal elav$c^{155}$-GAL4 and eye-specific GMR-GAL4 driver [38].

For experiments, female flies expressing BRICHOS, Aβ42, Aβ42 + BRICHOS and control flies [yellow white mutant strain (y.w.), expressing GAL4 only] were collected after eclosion. Adult female flies with different genotypes were aged and used for experiments. Different *Drosophila* transgenic strains were reared and analyzed in parallel, thereby excluding confounding factors caused by small differences in environmental conditions. The flies were kept on standard cornmeal food, containing corn flour, potato flour, brewer’s yeast, agar and propionic acid. Flies for crosses and experiments were kept at 26°C and relative humidity of 55–60%.

**RNA extraction and quantitative real-time PCR**

At least 10 flies of each genotype at the age of 5 days were collected. Heads of the flies were isolated and mRNA was extracted using the Oligotex Direct mRNA Mini Kit (Qiagen) according to the manufacturer’s protocol except for using 100 μl of buffer OL1 (with β-mercaptoethanol) instead of 600 μl in first addition. Equal amounts (9–11 ng) of mRNA for each sample were reverse-transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). The resulting cDNA was used as a PCR template on a 7500 Fast Real-Time PCR System (Applied Biosystems) to determine gene-specific transcription levels. Reactions were carried out in a volume of 20 μl using the Power SYBR Green PCR Master Mix (Applied Biosystems) together with 500 nM forward and reverse primers, specific for the template. Each sample was run in triplicates. Expression of mRNAs was calculated using relative quantitation against an endogenous control with the $2^{-\Delta\Delta C_t}$ method [39].

αTub84B was selected as an internal reference for real-time quantitative PCR (RT-qPCR) data normalization. All primers were obtained from DNA Technology, Denmark.

Following primers were used:

αTub84B for: 5′-TGGGCCCCTCCTGGACACAA

αTub84B rev: 5′-TCGCCGTCACCCGGAGTCCAT

Aβ42 for: 5′-CAGCGGCTACGAAGTGCATC

Aβ42 rev: 5′-CGCCCACCATCAAGCCAATA

Bri2 for: 5′-GTGTGCTGTCCAGAGTGT

Bri2 rev: 5′-CCAGTAGGTTTCTGGGTGGC

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Longevity assay
Newly hatched offspring flies of each genotype under the influence of the pan-neuronal-driver elav<sup>155</sup>-GAL4 were collected and placed in at least 10 tubes with 10 flies in each and kept at 26°C. Flies were transferred to new tubes and the number of dead flies was counted every 2–3 days. The percentage of living flies was plotted against the age of the flies. Survival plots were calculated using the Kaplan–Meier method, and statistical analyses were done using the log-rank test (GraphPad Prism).

Locomotor activity
Offspring flies under the influence of the pan-neuronal-driver elav<sup>155</sup>-GAL4 were collected and five flies of each genotype were placed in separate tubes with food replaced every 2–3 days. For climbing assay, 25-day-old flies were placed in an empty tube with a marked line at 8 cm from the bottom. The tube was dropped from 11 cm height onto a hard surface, causing all flies to fall to the bottom, and the number of flies that passed the 8 cm line in 20 s was determined. Ten tubes containing five flies were analyzed for each genetic group. Each tube was analyzed five times and mean values obtained were used for calculation of locomotor activity. The Mann–Whitney test (GraphPad Prism) was used for statistical analyses.

Immunohistochemistry
Brains from 25-day-old flies were dissected out in cold phosphate-buffered saline (PBS) and fixated for 20 min with 4% paraformaldehyde (Affymetrix, USA) on ice. Samples were subsequently washed in PBS-Tri (0.2% Triton X-100 in PBS) for 3 × 5 min and blocked in 5% BSA in PBS-Tri for 1 h at room temperature. Subsequently, the brains were incubated overnight at 4°C with the primary antibody in blocking solution, followed by washing with PBS-Tri 3 × 30 min and subsequent 4°C overnight incubation with the appropriate fluorescent secondary antibody. Next day, samples were washed with PBS-Tri for 3 × 30 min and incubated with DAPI for 10 min (Sigma, 1:1000) at room temperature, rinsed with PBS and finally mounted in 1:1 PBS and 85% glycerol. At least three brains per genotype were examined and representative data are shown.

The following primary antibodies and dilutions were used: monoclonal mouse 6E10 (Nordic BioSite, Sweden; 1:2000), rabbit anti-Bri2 (ITM2B; Atlas Antibodies, Sweden; 1:500) and rabbit anti-surfactant protein C (SFTPC; Atlas Antibodies, Sweden; 1:500). Secondary antibodies, Alexa Fluor goat anti-mouse F633 (Invitrogen) and Alexa Fluor goat anti-rabbit F488 (Invitrogen), were used both diluted 1:1000.

Images were collected with a confocal microscope (Zeiss, LSM 520, Germany) with ×10 and ×40 magnification objective using Z-stack settings. Images were visualized, processed and 3D-reconstructed using ImageJ software (National Institutes of Health, MD).

Investigation of mushroom body lobe structure using CD8-GFP fluorescence
Elav<sup>155</sup>-GAL4 flies of Aβ2 and control flies expressing the membrane-associated form of GFP (CD8-GFP; a fusion protein between the TM mouse lymphocyte marker CD8 and the green fluorescence protein) under UAS control were reared. Brains from 26-, 31- and 42-day-old flies were dissected out in cold PBS and fixated for 15 min on ice with 4% paraformaldehyde (Affymetrix, USA). Samples were subsequently washed in PBS-Tri (0.2% Triton X-100 in PBS) for 2 × 5 min, rinsed with PBS and then mounted in 1:1 PBS and 85% glycerol.

Images were collected within 2 h after mounting with an LSM 520 confocal microscope (Zeiss, Germany) with ×40 magnification objective and applying Z-stack settings. Images were visualized, processed and 3D-reconstructed using ImageJ software (National Institutes of Health, MD).

TEM analyses of fly eyes
Fly heads of 30-day-old flies of each genotype under the influence of the GMR-GAL4 driver were fixated in 2.5% glutaraldehyde at 4°C overnight. Fly heads were washed in PBS for 3 × 5 min and incubated in 1% osmium for 3 h at room temperature. Subsequently, the samples were washed in PBS for 3 × 5 min. Then, samples were dehydrated using a stepwise gradient of ethanol at 70, 95 and 100% for 3 × 5 min followed by incubation in propylene oxide for 10 min at room temperature. The samples were then incubated for 1 h in 50% propylene oxide mixed with 50% agar-based plastic, followed by incubation in 100% plastic overnight. The following day, the samples were mounted in plastic, cut into sections and put on copper grids. The sections were contrasted with 5% uranyl acetate for 10 min, rinsed in H2O, further contrasted for 3 min with lead citrate and rinsed again in H2O. The grids were then dried and analyzed by a Hitachi H7100 TEM (Hitachi,
Analyses of fibrillation kinetics

Aggregation traces of 2 μM Aβ42 at different Bri2 BRICHOS concentration were primarily fitted to an empirical sigmoidal equation \( F = F_0 + A/(1 + \exp(r_{max}(r_{1/2} - t))) \), where \( r_{1/2} \) is the aggregation half-time and \( r_{max} \) the maximal growth rate, \( A \) the amplitude and \( F_0 \) the base value of the curve. Then, the aggregation kinetics was globally fitted to a kinetic model including primary \( (k_\infty) \) and secondary nucleation \( (k_\beta) \) as well as fibril-end elongation \( (k_e) \) [40,41]. In this model, the time dependence of the fibril mass \( M(t) \) is described by:

\[
\frac{M(t)}{M(\infty)} = 1 - \left( \frac{B_+ + C_+ \cdot \exp(\kappa t)}{B_+ + C_+ \cdot \exp(\kappa t)} - \frac{B_- + C_- \cdot \exp(\kappa t)}{B_- + C_-} \right)^{\frac{1}{\lambda}} \cdot \exp(-k_\infty t)
\]

with the global fit \( \lambda \) parameters for primary

\[
\lambda = \sqrt{2 \cdot k_\infty k_\infty \cdot m(0)^n_c}
\]

and \( \kappa \) secondary nucleation, respectively.

\[
k_\infty = \sqrt{2 \kappa^2/(n_2(n_2 + 1)) + 2 \lambda^2/n_c}
\]

\[
\tilde{k}_\infty = \sqrt{k_\infty^2 - 4 C_\infty C_\infty \cdot \kappa^2}
\]

\[
B_\pm = (k_\infty \pm \tilde{k}_\infty)/2/\kappa
\]

The parameter \( k_\infty \) reflects the polymer number concentration, \( P(t) \), for \( t \to \infty \) and the elongation rate, \( k_e \), and is given by \( k_\infty = 2k_\infty_0 P(\infty) \) [42].

The reaction orders for primary and secondary nucleation were set to \( n_c = n_2 = 2 \), since these numbers revealed the best fits for Aβ42 aggregation as reported previously [40]. To investigate the effect on the individual nucleation rates, for \( k_\infty \) and \( k_\beta \), either \( \sqrt{k_\infty k_\beta} \) or \( \sqrt{k_\infty^2 - 4 C_\infty C_\infty \cdot \kappa^2} \) as a fitting parameter was constrained to the same value, whereas the other one was allowed to vary across different chaperone concentrations. For the elongation rate \( k_e \), the fitting parameter was redefined as \( \sqrt{k_\infty^2}/k_\beta \) and \( \sqrt{k_\infty^2 - 4 C_\infty C_\infty} \), where the first one was fixed to the same value, resulting in \( k_e \) as the sole effective fitting parameter [43,44].

Results

Bri2 BRICHOS efficiently prevents Aβ42-induced reduction in γ-oscillations in vitro

γ-Oscillations were induced in horizontal hippocampal slices from C57BL/6 mice by superfusing slices with 100 nM kainate (KA). LFP recordings in area CA3 revealed control γ-oscillations (no prior incubation with Aβ) of \( 6.81 \times 10^{-9} \pm 2.39 \times 10^{-9} \text{V}^2\text{cm}^{-2} \) power (\( n = 9 \); Figure 1A). Neither incubation with 100 nM nor with 1 μM Bri2 BRICHOS had a significant effect on KA-induced γ-oscillations in the absence of Aβ42 (100 nM Bri2: γ-oscillation power 91.9% of control, \( n = 8 \), \( P > 0.05 \); 1 μM Bri2: γ-oscillation power 95.2% of control, \( n = 8 \), \( P > 0.05 \)). Incubating hippocampal slices with 50 nM or 1 μM Aβ42 for 15 or 180 min prior to superfusion with 100 nM KA resulted in that induction of γ-oscillations was impaired in a concentration- and incubation time-dependent manner ([45]; Figure 1A–C). Incubation with 50 nM Aβ42 for 15 min resulted in significantly reduced γ-oscillation power to 54.2% of control (\( n = 8 \), \( P < 0.0001 \)) and even stronger reductions were recorded after incubation with 1 μM Aβ42 for 15 min (to 9.1% of control, \( n = 9 \), \( P < 0.0001 \)), 50 nM Aβ42 for 180 min...
To test the abilities of Bri2 and proSP-C BRICHOS domains to prevent the Aβ42-induced reduction in γ-oscillations, we co-incubated hippocampal slices with Aβ42 and the respective BRICHOS domain. The presence of 100 nM Bri2 BRICHOS prevented the Aβ42-induced reduction in γ-oscillations by 50 nM Aβ42 present for 15 min ($\gamma$-oscillation power 90% of control, $n = 8$, $P > 0.05$; Figure 1A,B). There was a tendency of Bri2 BRICHOS being more efficient than proSP-C BRICHOS at the same concentration ($\gamma$-oscillation power 85.4% of control, $n = 8$, $P > 0.05$; Figure 1B, $P = 0.083$ for comparison between Bri2 and proSP-C BRICHOS).

Likewise, the γ-oscillation reduction in 50 nM Aβ42 present for 180 min was reduced by 100 nM Bri2 ($\gamma$-oscillation power 33.4% of control, $n = 9$), with a trend to be more efficient than 100 nM proSP-C ($\gamma$-oscillation power 26.8% of control, $n = 8$, $P = 0.088$ for comparison between Bri2 and proSP-C BRICHOS). Either 100 nM Bri2 or proSP-C BRICHOS was unable to block the effect of 1 μM Aβ42 present for 15 min (100 nM Bri2: γ-oscillation power 16.7% of control, $n = 8$; 100 nM proSP-C: γ-oscillation 12.3% of control, $n = 8$, $P < 0.0001$ compared with controls) and of 1 μM Aβ42 present for 180 min (100 nM Bri2: γ-oscillation power 9.3% of control, $n = 8$; 100 nM proSP-C: γ-oscillation power 6.7% of control $P < 0.0001$; Figure 1C). As 100 nM Bri2 or proSP-C BRICHOS did not prevent the reduction in γ-oscillation power caused by 1 μM Aβ42 and/or longer incubation time (180 min), we tested the effect of 1 μM Bri2 BRICHOS under the same experimental conditions as previously reported for proSP-C BRICHOS [45]. The presence of 1 μM Bri2, or proSP-C, BRICHOS prevented the Aβ42-induced reduction in γ-oscillations by 50 nM Aβ42 present for 15 min (1 μM Bri2: γ-oscillation power 106.9% of control, $n = 12$; 1 μM proSP-C: γ-oscillation power 97% of control $P > 0.05$), by 1 μM Aβ42 present for 15 min (1 μM Bri2: γ-oscillation power 81.4% of control, $n = 13$; 1 μM proSP-C: γ-oscillation power 62% of control, $n = 12$, $P > 0.05$) and by 50 nM Aβ42 present for 180 min (1 μM

Figure 1. Bri2 BRICHOS prevents Aβ42-induced reduction in γ-oscillation power.

(A) Example traces and example power spectra of γ-oscillations under control conditions (black), in the presence of 50 nM Aβ42 (red) and in the presence of 50 nM Aβ42 plus 100 nM Bri2 BRICHOS (green). (B and C) Summary plots of γ-oscillation power in the absence (controls) or presence of 50 nM (B) or 1 μM (C) Aβ42 applied for 15 min or 180 min (red columns), and corresponding effects on γ-oscillations when Bri2 BRICHOS (light and dark green columns) and proSP-C BRICHOS (light and dark blue columns) were present at 100 nM or 1 μM. (D) Summary plot of γ-oscillation power in the absence (control) or presence of 50 nM Aβ42 applied for 15 min alone, or together with 1 μM lysozyme.

(to 15.8% of control, $n = 8$, $P < 0.0001$) or 1 μM Aβ42 for 180 min (to 2.6% of control, $n = 7$, $P < 0.0001$; Figure 1B,C).
Bri2: γ-oscillation power 110.1% of control, n = 11; 1 μM proSP-C: γ-oscillation power 93% of control, n = 12, P > 0.05; Figure 1B,C). However, both 1 μM Bri2 and proSP-C BRICHOS were unable to block the effect of 1 μM Aβ42 present for 180 min (1 μM Bri2: γ-oscillation power 6.6% of control, n = 8; 1 μM proSP-C: γ-oscillation power 4.1% of control, n = 9, P < 0.0001; Figure 1C).

To test whether the prevention of Aβ42-induced reduction in γ-oscillations by BRICHOS could be an unspecific protein effect, we conducted a control experiment in which BRICHOS was replaced by a structurally unrelated protein, lysozyme. In the experiment, 50 nM Aβ42 was co-incubated for 15 min with 1 μM lysozyme before γ-oscillations were induced by 100 nM KA. The power of the resulting γ-oscillations was significantly reduced compared with KA-only control oscillations and not significantly different from γ-oscillations obtained after incubation with Aβ42 alone (102.7%, n = 8, P > 0.05; Figure 1D), indicating that lysozyme is unable to prevent Aβ42-induced reduction in γ-oscillations. We conclude that the observed ability of Bri2 and proSP-C BRICHOS to prevent Aβ42-induced reduction in γ-oscillations is not caused by an unspecific protein effect on Aβ aggregation.

We also wished to test whether the ability of Bri2 BRICHOS to prevent the Aβ42-induced decrease in γ-oscillations was dependent on the conformational state of Aβ42 at the start of incubation. To do this, we first incubated hippocampal slices for 15 min in the presence of 50 nM of monomeric, mixed or fibrillar Aβ42 (all concentrations calculated for the Aβ monomer). This resulted in a decrease in γ-oscillations that increased in correlation with the fibrillar content (monomeric Aβ42: to 66.4% of control, n = 14; mixed Aβ42: to 35.1% of control, n = 12; fibrillar Aβ42: to 18.6% of control, n = 12; Supplementary Figure S1) [45]. When co-incubating monomeric, mixed and fibrillar Aβ with 1 μM Bri2 BRICHOS for 15 min, we found that whereas Bri2 was able to prevent reduction in γ-oscillations caused by monomeric Aβ42 (108.8% of control, n = 8, P > 0.05) and mixed Aβ42 (96.0% of control, n = 8, P > 0.05), it only partially prevented the reduction in γ-oscillations caused by fibrillar Aβ (52.3% of control, n = 8, P = 0.0003; Supplementary Figure S1). These results suggest that the amount of fibrils correlates directly with the severity of the Aβ42 effect on γ-oscillations, and inversely with the ability of Bri2 (Supplementary Figure S1) and proSP-C BRICHOS to prevent it.

**Transgenic D. melanogaster expressing Aβ42 and/or BRICHOS**

Flies containing a signal peptide-Aβ42 gene, a signal peptide-proSP-C BRICHOS gene [10,37] or a signal peptide-Bri2 BRICHOS gene were generated, followed by crossings of relevant flies to generate Aβ42 + Bri2 BRICHOS and Aβ42 + proSP-C BRICHOS flies (Supplementary Figure S2). The signal peptides are inserted to direct expression to the endoplasmic reticulum (ER), which is necessary since both the BRICHOS domain of Bri2 and the Aβ region of AβPP are found on the luminal side of the ER under physiological conditions. Expression of inserted genes was obtained by crossing transgenic flies with driver flies producing the GAL4 protein, either in all neurons (elav<sup>155</sup>-GAL4) or in the eyes (GMR-GAL4). Control flies were generated by crossing yellow white strain (y.w.) flies with the relevant GAL4-driver line.

To ensure that expression levels of signal peptide-Aβ42 are not affected by the co-expression of signal peptide-BRICHOS proteins, or the other way around, the different transgenic flies were analyzed by RT-qPCR. The results show that the levels of Aβ42 mRNA relative to the housekeeping gene, αTub84B, do not differ between flies expressing Aβ42 only and flies that co-express Aβ42 and the Bri2 BRICHOS domain (Supplementary Figure S3). The same results were obtained for the Bri2 BRICHOS domain, that is Bri2 BRICHOS expression is not affected by Aβ42 expression (Supplementary Figure S3). The levels of proSP-C BRICHOS mRNA are not affected by Aβ42 expression, nor does proSP-C BRICHOS expression affect the Aβ42 mRNA levels [10].

**Aβ42 effects on longevity, locomotor activity and eye phenotype with or without Bri2 or proSP-C BRICHOS**

To analyze effects on longevity and locomotor activity, expression of Aβ42 alone, or with co-expression of the Bri2 or proSP-C BRICHOS domains, under the pan-neuronal-driver elav<sup>155</sup>-GAL4 was used. The longevity of flies expressing Bri2 BRICHOS, Aβ42, Aβ42 + Bri2 BRICHOS and control flies was analyzed using 120–130 flies for each group. The median life span of the control flies was 80 days, for Bri2 BRICHOS-expressing flies 79 days, Aβ42 flies 50 days and Aβ42 + Bri2 BRICHOS 70 days (Figure 2A and Supplementary Table S1). This shows that Bri2 BRICHOS has a marked effect on reducing the toxic effects of Aβ42 on *Drosophila* life span, but has no effect on the life span on its own. To allow comparisons of the effects of Bri2 and proSP-C
BRICHOS on Aβ42-induced reduction in longevity, flies were analyzed in parallel. The results show that Bri2 BRICHOS is significantly more potent than proSP-C BRICHOS in reducing the Aβ42-induced reduction in fly longevity (Figure 2B and Supplementary Table S2).

The locomotor activities for all fly lines were determined at the age of 25 days (an age just prior to when the flies started to die, see Figure 2A,B) using a climbing assay (Figure 2C). The expression of Bri2 BRICHOS domain alone had no significant effect on the climbing ability, compared with control flies (Figure 2C and Supplementary Table S3). Expression of Aβ42, in contrast, reduced the locomotor performance dramatically.
Co-expression with Bri2 BRICHOS increased the climbing ability significantly compared with flies expressing Aβ42 alone (Figure 2C and Supplementary Table S3). When comparing the locomotor activity of flies expressing Aβ42 together with Bri2 or proSP-C BRICHOS, there was a significantly stronger improvement for Bri2 BRICHOS (Figure 2C).

Using TEM, the eyes of 30-day-old flies expressing Aβ42, Bri2 BRICHOS, Aβ42 + Bri2 BRICHOS, proSP-C BRICHOS or Aβ42 + proSP-C BRICHOS under the GMR-GAL4 driver, as well as control flies expressing GMR-GAL4 only, were analyzed. The eyes were examined for overall damage and the diameter of photoreceptors was measured (Figures 3 and 4 and Supplementary Figure S4). Expression of either Bri2 (Supplementary Figure S4A1–A2) or proSP-C BRICHOS alone (Supplementary Figure S4B1–B2) did not cause any visible malformation or reduced size of the rhabdomeres (photoreceptors) when compared with the control flies (Figure 4 and Supplementary Table S4). Expression of Aβ42, in contrast, resulted in malformation of the rhabdomeres when compared with the control flies (Figure 3B1–B3,F1–F3 compared with A1–A3,E1–E3). Aβ42 expression also results in reduced diameters of rhabdomeres (Figure 4 and Supplementary Table S4). Expressing Bri2 BRICHOS together with Aβ42 (Figure 3C1–C3,G1–G3) inhibited these effects and gave an eye phenotype closely resembling the control flies. Expression of proSP-C BRICHOS together with Aβ42 (Figure 3D1–D3,H1–H3) resulted in significantly larger rhabdomere diameters than in Aβ42-only expressing flies, but rhabdomeres are not fully restored to control levels as in Aβ42 + Bri2 BRICHOS flies. The effects obtained by Bri2 and proSP-C BRICHOS, respectively, are statistically different (Figure 4 and Supplementary Table S4).

### Localization of Aβ42 with and without Bri2 or proSP-C BRICHOS co-expression

To examine the effect of BRICHOS on the Aβ42 deposition in fly brains, immunofluorescence analyses were performed. For this purpose, whole brains of 25-day-old flies with pan-neuronal expression were stained with the monoclonal anti-Aβ antibody 6E10. This showed immunoreactive deposits around the area of antennal lobes in the Drosophila brain (Figures 5A,J and 6A), which were not seen in flies without Aβ42 expression, where only some diffuse tissue staining can be observed (Figures 5D and 6D). Co-expression with Bri2 BRICHOS led to strong and diffuse Aβ42 staining that co-localizes with Bri2 BRICHOS in mushroom bodies of the brain (highlighted with a white line in the figures), as detected with double immunofluorescence staining (Figure 5G–LM–O).

In proSP-C BRICHOS + Aβ42 transgenic flies, Aβ42 was not detected in the mushroom bodies when co-expressed with proSP-C BRICHOS, when analyzed by confocal microscopy [10]. These analyses were, however, performed using an Aβ42 antibody that is no longer available and in order to allow comparisons of the respective effects on Aβ42 localization by the two BRICHOS domains, we stained brains from flies expressing proSP-C BRICHOS and Aβ42 with the same antibody as now used for Bri2 BRICHOS + Aβ42 transgenic flies. The result still showed that Aβ42 was not observed in the mushroom bodies in Aβ42 + proSP-C BRICHOS flies, instead the staining pattern resembled the one seen in Aβ42-only expressing flies (Figure 6A–C,G–I). The mushroom body structure was, however, seen when proSP-C BRICHOS was immunostained, in particular using ×10 magnification (Figure 6E,K). The Aβ42 and proSP-C BRICHOS co-expressing flies had deposits of Aβ42 in the midbrain area (Figure 6G), which were found to co-localize with proSP-C BRICHOS (Figure 6I), as observed previously [10].

To investigate the possibility that Aβ42 expression affects the structure of the mushroom body lobes, transgenic Aβ42, and yellow white strain (y.w.) flies as controls, were crossed with elav4255-GAL4 flies bearing an UAS-CD8-GFP transgene. The CD8-GFP transgene allows facile detection of normal tissue structure [46]. The patterns of GFP distribution showed strong GFP signal in the mushroom bodies and no visible atrophy or degradation caused by Aβ42 expression in flies aged 26–42 days could be observed (Supplementary Figure S5), confirming that Aβ42 expression does not destroy the mushroom body structure.

### Bri2 and proSP-C BRICHOS have different molecular mechanisms

The observed different effects of Bri2 and proSP-C BRICHOS on Aβ42 toxicity and distribution prompted us to compare their molecular mechanisms. Bri2 BRICHOS delays Aβ42 fibril formation in a concentration-dependent manner (Figure 7A and Supplementary Figure S6A) and the Aβ42 fibril formation kinetics in the presence of recombinant Bri2 BRICHOS differs from those recorded under identical conditions in the presence of the proSP-C BRICHOS domain [9]. The appearances of the kinetic traces reflect the mechanisms that
Figure 3. BRICHOS improves the eye phenotype of Aβ42-expressing flies.

Expressing Aβ42 in the Drosophila eyes (B1–B3, F1–F3) led to malformation and reduced diameter of the rhabdomeres (R, indicated with arrow in A1 and E1) compared with control flies (A1–A3, E1–E3). Co-expression of Bri2 BRICHOS with Aβ42 (C1–C3, G1–G3) inhibits these effects and gives an eye phenotype close to that of the control flies. Expressing proSP-C BRICHOS together with Aβ42 (D1–D3, H1–H3) results in reduced diameters of the rhabdomeres compared with controls, although to a lesser extent than when Aβ42 is expressed alone. Sections of Drosophila eyes were stained with osmium and analyzed by TEM. Scale bars represent 1 μm in ×15 000 magnification images and 500 nm in ×50 000 magnification images.
underlie the inhibition of Aβ42 fibril formation [40]. Specifically, Bri2 BRICHOS is more efficient in increasing the duration of the lag phase, but it is less efficient in reducing the maximum growth rate, \( r_{\text{max}} \), of the aggregation traces compared with proSP-C BRICHOS, indicating that the mechanisms whereby the two BRICHOS domains affect Aβ42 fibril formation differ. For Bri2 BRICHOS, the aggregation half-time \( \tau_{1/2} \), as obtained from fits to an empirical sigmoidal equation (Supplementary Figure S6A), exhibits a linear dependence on the chaperone concentration (Supplementary Figure S6C). Furthermore, the two fitting parameters, \( \tau_{1/2} \) and \( r_{\text{max}} \), feature an interdependence with a slope of \(-1.16 \pm 0.06\) in a double logarithmic plot (Supplementary Figure S6B), indicating the presence of secondary aggregation pathways. To obtain detailed information on which nucleation event(s) is/are primarily affected by the presence of Bri2 BRICHOS, the kinetic data were fitted to a model that includes the rate constants for primary, \( k_n \), and secondary nucleation, \( k_2 \), as well as fibril-end elongation, \( k_+ \) (Figure 7A) [40,41]. This analysis is performed using the combined rate constants \( \sqrt{k_n k_2} \) and \( \sqrt{k_n k_+} \) as free fitting parameters, and the fit describes well the observed aggregation traces (Figure 7A). To check whether a change in solely one nucleation rate can explain the aggregation behaviour, we restricted one fitting parameter to the same value, while letting the second one vary across all chaperone concentrations, resulting in only one rate constant as the sole effective fitting parameter. Following this approach, we found that a change in exclusively \( k_n \) is not able to capture the observed kinetics, whereas both \( k_n \) and \( k_2 \) as sole fitting parameters describe well the aggregation traces (Figure 7C), with a similar dependence on Bri2 BRICHOS concentration (Figure 7D). Moreover, as in the fit with two free fitting parameters (Figure 7A), both combined rate constants show a distinct dependence on the chaperone concentration (Figure 7B), these results indicate that presumably both \( k_n \) and \( k_2 \) are affected by Bri2 BRICHOS. This is in good agreement with a recent analysis performed on a similar Bri2 BRICHOS construct [47]. Thus, compared with the effect of proSP-C BRICHOS on the nucleation mechanism of Aβ42, which primarily affects exclusively the secondary nucleation [9], Bri2 BRICHOS seemingly features a more comprehensive inhibition effect, involving different nucleation events.

Analyzing effects of Bri2 and proSP-C BRICHOS on Aβ40 fibril formation under quiescent conditions shows highly efficient prolongation of the lag time and attenuation of the maximum growth rate, and that the Bri2 BRICHOS domain is more efficient compared with the proSP-C BRICHOS domain (Supplementary Figure S7). Similar results on Aβ40 fibril formation were obtained previously when the solutions were agitated during fibril formation, and a Bri2 construct containing a longer part of the linker region was included (residues 90–235 compared with residues 113–231 in the presently used construct) [29]. When sub-stoichiometric amounts of BRICHOS were present during fibril formation, abundant Bri2 BRICHOS protein bound to Aβ40
and Aβ42 fibrils could be visualized after completion of fibril formation, using immunogold labelling and TEM (Supplementary Figure S8).

To assess the molecular chaperone activity of Bri2 BRICHOS and proSP-C BRICHOS in more detail, we also investigated the influence of the BRICHOS proteins on the amorphous aggregation of CS (48 kDa), firefly luciferase (61 kDa) and rhodanese (30 kDa) as model substrates [48–53]. First, we tested the ability of the BRICHOS proteins to suppress the aggregation of the different model proteins at elevated temperatures, which

Figure 5. Co-expression of Aβ42 and Bri2 BRICHOS leads to co-localization in mushroom bodies of the fly brain. Immunostaining of 25-day-old Drosophila brains with anti-Aβ antibody 6E10 (green), anti-Bri2 (ITM2B) antibody (red) and DAPI (blue) for staining of cell nuclei under ×10 (A–I) or ×40 (J–O) magnification objectives. Co-localization of Aβ42 and Bri2 BRICHOS appears as yellow colour in the merged pictures. Arrows indicate Aβ42 deposits around antennal lobes (AL). The α- and β-lobes of a mushroom body are indicated with a white line (I, O). Scale bars represent 50 μm.
Figure 6. Aβ42 co-localizes with proSP-C BRICHOS in the fly brain area around the antennal lobes.

Immunostaining of midbrain area of 25-day-old Drosophila flies with anti-Aβ antibody 6E10 (green), SFTPC antibody (red) and DAPI (blue) for staining of cell nuclei under ×10 (J–L) or ×40 (A–I) magnification objectives. Co-localization of Aβ42 and proSP-C BRICHOS around AL appears as yellow colour in merged pictures. Arrows indicate Aβ42 deposits around ALs. Scale bars represent 50 μm.
represents a key trait of molecular chaperones [51]. Interestingly, Bri2 BRICHOS was able to suppress the thermal aggregation of all substrates in a concentration-dependent manner (Figure 8A,B and Supplementary Figure S9). Aggregation suppression was highly effective already at sub-stoichiometric ratios (e.g. 1:8, Bri2 BRICHOS:luciferase) and remained so up to stoichiometric ratios of Bri2 BRICHOS (Figure 8A,B and Supplementary Figure S9). In contrast, proSP-C BRICHOS showed no such chaperone activity at sub-stoichiometric ratios and only at excess of proSP-C BRICHOS was the aggregation of the model substrates suppressed (Figure 8A,B and Supplementary Figure S9). Thus, Bri2 BRICHOS is an efficient molecular chaperone in terms of the suppression of thermally induced, amorphous aggregation of different model substrates. In comparison with proSP-C BRICHOS, Bri2 BRICHOS is much more potent in this respect, because much lower amounts were sufficient to fully suppress the aggregation of the substrate proteins.

To further characterize the chaperone function, we used CS inactivation and reactivation reactions [35]. Monitoring the kinetics of the thermal inactivation of CS allows to describe and characterize the interaction of a chaperone with unfolding intermediates of CS and the stability of the formed chaperone–substrate complex in more detail [34]. For Bri2 BRICHOS as well as for proSP-C BRICHOS, we observed no deceleration of CS inactivation (Figure 8C) even at high excess of the BRICHOS proteins, indicating that early unfolding intermediates of CS are not stabilized [54,55]. Accordingly, the activity of Bri2 BRICHOS (Figure 8A,B and Supplementary Figure S9) hints at a rather stable interaction between the BRICHOS proteins and nearly completely unfolded CS. To further characterize the interaction of the BRICHOS proteins with the model substrate, refolding of chemically unfolded CS was investigated. First, samples of early refolding reactions (5 min after the start of the refolding reaction) were applied to SEC. Analyzing Bri2 BRICHOS without CS, we observed three

![Figure 7. Bri2 BRICHOS affects secondary nucleation and/or fibril-end elongation as revealed by a global fit analysis.](image-url)

(A) Aggregation traces of 2 μM Aβ42 in the presence of 0 (black), 0.07 (red), 0.13 (green), 0.27 (blue) and 0.65 (pink) molar equivalents of Bri2 BRICHOS fitted globally to a model including primary, \( k_n \), and secondary nucleation, \( k_s \), as combined rate constants. (B) Dependence of the relative combined rate constants from the global fit in (A) on Bri2 BRICHOS concentration. (C) Dependence of relative rate constants, \( k_2 \) and \( k_+ \), on the chaperone concentration from fits shown in (D). (D) Global fit of aggregation traces where only \( k_n \), \( k_2 \) or \( k_+ \) is the sole effective fitting parameter. While \( k_n \) is not able to capture alone the observed aggregation behaviour (RSS 3.02), \( k_2 \) and even more \( k_+ \) as a free fitting parameter describe well the effect of Bri2 BRICHOS on the aggregation kinetics (RSS 1.05 and RSS 0.39, respectively).
Figure 8. Bri2 BRICHOS suppresses the aggregation of model substrates by the formation of stable complexes with its substrate.

Chaperone function was assessed by the ability to suppress the thermal aggregation of 600 nM luciferase (A) and CS (B) at 43°C. The kinetics of aggregation was determined by recording the light scattering of the samples at 360 nm. The influence of the addition of 600 nM Bri2 BRICHOS (▪; green); 600 nM proSP-C BRICHOS (▴, blue) or in the absence of BRICHOS (●, black) is illustrated. Mean values of three independent assays are depicted. (C) Influence of BRICHOS proteins on the thermal inactivation of CS. Aliquots from a thermostated solution (43°C) of 600 nM CS (●, black; straight line) alone or in the presence of 9.6 μM Bri2 BRICHOS (▪, green; short dashed line) or 9.6 μM proSP-C BRICHOS (▴, blue; dashed line) were extracted at the indicated time points and the enzymatic activity was determined. (D) SEC of Bri2 BRICHOS in presence (black) and absence (blue) of CS. The peaks representing the substrate complex as well as monomeric unfolded CS are indicated. (E) Refolding of 300 nM chemically denatured CS (●; black) in the presence of 4.8 μM Bri2 BRICHOS (▪; green) or 4.8 μM proSP-C BRICHOS (▴; blue). (F) Refolding of 600 nM chemically denatured luciferase (●; black) in the presence of 2.4 μM Bri2 BRICHOS (▪; green) or 2.4 μM proSP-C BRICHOS (▴; blue). Error bars indicate the standard deviation of three independent experiments.
prominent peaks with apparent molecular masses corresponding to a dimer, a tetramer and a high-molecular mass complex (~32 mer). Interestingly, in the presence of unfolded CS, a peak corresponding to the monomeric CS and an additional high-molecular-weight complex of CS and Bri2 BRICHOS with unknown stoichiometry were observed (Figure 8D). For proSP-C BRICHOS, SEC showed two peaks, and the majority of the protein elutes under the peak corresponding to a larger molecular mass. In the presence of unfolded CS, no complex formation between proSP-C BRICHOS and CS was detected (Supplementary Figure S1S0), indicating that this BRICHOS protein forms no stable complex with the unfolded CS. Second, refolding of CS and luciferase in the presence and absence of BRICHOS proteins was investigated kinetically to verify if the BRICHOS-bound substrate is in a refolding-competent state. Interestingly, only the presence of Bri2 BRICHOS resulted in a very low reactivation of the substrate proteins and no influence of proSP-C BRICHOS on refolding was observed (Figure 8E,F).

**Discussion**

Bri2 and Bri3 proteins are associated with AD, AβAPP processing and Aβ deposition [18,32]. Moreover, increased Bri2 levels and deposition in AD hippocampus have recently been found [56]. Bri2, like BRICHOS-containing proteins in general, is a multidomain protein that is composed of an N-terminal cytosolic part, a TM region, a linker region, a BRICHOS domain and a C-terminal region [14,23]. What region, or regions, of Bri2 mediate the aforementioned effects is not fully understood. To design potential Bri2-based interventions of AD-related phenomena, it is necessary to identify what functions individual parts have, and herein we analyze specifically the Bri2 BRICHOS domain.

BRICHOS domains from several different proproteins are potent inhibitors of Aβ fibril formation and toxicity *in vitro* [9,29,57]. Amyloid fibril formation consists of several different microscopic processes that affect differently the macroscopic aggregation profile. By analyzing Aβ42–BRICHOS fibril formation kinetic profiles, it was recently demonstrated that specific inhibition of the secondary nucleation process underlies the effect of proSP-C BRICHOS on Aβ42 fibril formation [9]. While proSP-C BRICHOS selectively inhibits one aggregation step, the Bri2 BRICHOS influences simultaneously several events in Aβ42 aggregation, both the elongation and the secondary nucleation processes (Figure 7) [47]. Bri2 BRICHOS effects on surface catalyzed secondary nucleation, are also supported by its ability to avidly bind to the surface of Aβ42 fibrils (Supplementary Figure S8). The molecular chaperone activity measurements show that Bri2 is the more efficient chaperone in terms of preventing heat-induced aggregation, and seems to work mechanistically differently compared with the proSP-C BRICHOS domain (Figure 8 and Supplementary Figure S9). It has been observed before that related proteins can show distinct but overlapping mechanisms against amyloid fibril forming proteins. For example, the yeast small heat shock proteins (sHsps) Hsp26 and Hsp42 inhibit fibril formation of Sup35 by different mechanisms [58].

γ-Oscillations were recorded after incubation with Aβ42 in the absence or in the presence of Bri2 BRICHOS. The results were compared with the effects of proSP-C BRICHOS under the same conditions. The strength of γ-oscillations is an important functional biomarker for brain disorders that involve cognitive decline, since this brain rhythm plays a central role in higher processes, such as learning, memory and cognition [59]. Clinical data show that the cognitive decline observed in AD patients goes hand-in-hand with a decrease in γ-oscillations [60]. It is assumed that the reduction in the γ-oscillation network rhythms in AD underlies the negative effects on learning, memory, perception and cognition typical for AD, and increased Aβ levels have been shown to disrupt the timing of evoked action potentials in a mouse model of AD [61,62]. The Bri2 and proSP-C BRICHOS domains are equally efficient in preventing Aβ42 from reducing γ-oscillations (Figure 1). We, however, find significant differences when analyzing the effects of Bri2 and proSP-C BRICHOS domains on aggregation and toxicity of Aβ42 in *Drosophila* brain and eyes. The Bri2 BRICHOS domain is more efficient in inhibiting Aβ42-induced reduction in longevity, locomotor activity and destruction of eye morphology (Figures 2–4). Previous studies have shown different degrees of Aβ42 toxicity in the *Drosophila* eye depending on Aβ dose and fly age [37,63]. Here, we find that Aβ42 expressed in the eyes of the flies, gives rise to malformation of the rhabdomeres, as analyzed by TEM, and that co-expression of Bri2 BRICHOS prevents this effect of Aβ42. ProSP-C BRICHOS also significantly represses the toxic effects of Aβ42 expression in the fly eye, but to a lesser extent compared with Bri2 BRICHOS. The two BRICHOS domains also have strikingly different effects on Aβ42 distribution in the fly CNS (Figures 5 and 6). The divergence of the two BRICHOS proteins in their mechanistic effects on Aβ42 aggregation and their different profiles as molecular chaperones may translate into the now observed stronger potency of Bri2 BRICHOS to suppress Aβ42 aggregation and toxicity *in vivo*.
The different effects of Bri2 and proSP-C BRICHOS domains on Aβ42 distribution in the fly brain (Figures 5 and 6) are another indication that they work differently in vivo. Using immunohistochemistry, we found that Aβ42 distributes uniformly in the mushroom bodies when co-expressed with Bri2 BRICHOS (Figure 5), which is not seen with proSP-C BRICHOS co-expression (Figure 6), although the two BRICHOS domains are detected in the mushroom bodies when expressed alone. To our knowledge, Aβ42 expressed by the elav<sup>155</sup> driver has previously not been detected in the mushroom bodies. This suggests that Bri2 BRICHOS has a profound effect on Aβ42 solubility and/or distribution in the CNS. ProSP-C BRICHOS might be less efficient than Bri2 BRICHOS in keeping Aβ42 soluble in the mushroom body, because it exclusively interferes with the secondary nucleation step. The mushroom body in the *Drosophila* brain plays an important role in olfactory learning and memory [64-66] and APPL, the *Drosophila* AβPP family member required for formation of long-term memory, is enriched in mushroom bodies [67]. Previously, it has been shown that expression of AβPP together with the β-secretase BACE (which in concert with endogenous γ-secretase generates Aβ) gives rise to degeneration of the mushroom bodies, which was proposed to be due to generation of Aβ from AβPP [68]. In the present study, using CD8-GFP as a marker, expression of human Aβ42 alone does not seem to affect the mushroom body structure (Supplementary Figure S5). In fact, the structure observed by GFP fluorescence is indistinguishable between controls and Aβ42-expressing flies, even when 42-day-old flies are examined (Supplementary Figure S5E,F). A similar result was obtained for expression of Aβ42 with the Arctic mutation, which likewise did not affect mushroom body appearance as observed by GFP fluorescence [69].

We also analyzed the molecular chaperone activities of the Bri2 and proSP-C BRICHOS domain, that is, whether they potently inhibit aggregation of unfolded proteins and/or are able to promote refolding of denatured proteins. A previous study [21] showed that proSP-C BRICHOS was unable to reduce aggregation of denatured alcohol dehydrogenase and insulin. We likewise found that proSP-C BRICHOS is not able to significantly affect aggregation or refolding of any of the three model substrates used in our study (Figure 8 and Supplementary Figure S9). The low proSP-C BRICHOS chaperone activity in these assays might be due to unspecific stabilizing effects on the model substrates at higher protein concentrations. Bri2 BRICHOS on the other hand is capable of efficiently suppressing the aggregation of different model substrates, to form rather stable complexes with its substrates, and shows only a slight spontaneous release of folding-competent substrate molecules. Overall, the observed activity of Bri2 BRICHOS is intriguingly similar to the chaperone function of many sHsps, for example Hsp26 or Hsp42, from baker's yeast [50,70]. sHsps are ATP-independent molecular chaperones, which bind non-selectively to proteins with non-native conformations, thereby preventing aggregation of the substrate. They are most often found to form large oligomers, from a dimeric building block, and the complexes are highly dynamic and exchange their subunits [70,71]. In this context, it seems possible that different quaternary structures of Bri2 and proSP-C BRICHOS are related to their different molecular chaperone activities. Recombinant proSP-C BRICHOS is predominantly a trimer [4,72]. In contrast, Bri2 BRICHOS forms mainly large complexes of at least 30-mers (Figure 8D). Bri2 BRICHOS might fulfil a similar role in the ER (where usually no sHsps are present) as members of the sHsp family in the cytosol, acting against AβPP and Aβ in the secretory pathway. Additionally, the BRICHOS domain of Bri2 can be released by regulated proteolysis [30], so it appears likely that the Bri2 BRICHOS domain is present in the extracellular space as well. It is conceivable that the BRICHOS domain of Bri2 is involved in regulation of AβPP processing intracellularly [73,74], as well as in preventing aggregation and toxicity of Aβ extracellularly. Finally, since several of the sHsps, such as αB-crystallin, Hsp20 and Hsp27, have been shown to bind to Aβ and affect its aggregation and toxicity [75-77], BRICHOS and sHsp chaperone machineries may together constitute a defence system that suppresses or regulates the fibril formation of amyloidogenic substrate proteins in different locations.

**Abbreviations**

ACSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; Aβ, amyloid-β peptide; AβPP, Aβ precursor protein; APPL, amyloid protein precursor-like; BACE, β-secretase; CNS, central nervous system; CS, citrate synthase; ER, endoplasmic reticulum; FBD, familial British dementia; FDD, familial Danish dementia; ITM2B, integral transmembrane protein 2B; KA, kainate; LFP, local field potential; PBS, phosphate-buffered saline; PBS-Tri, 0.2% Triton X-100 in PBS; proSP-C, prosurfactant protein C; SEC, size exclusion chromatography; SFTPC, surfactant protein C; sHsp, small heat shock protein; SP-C, surfactant protein C; TBS, Tris-buffered saline; TEM, transmission electron microscopy; ThT, thioflavin T; TM, transmembrane; UAS, upstream activation sequence.
Author Contribution

H.P., M.H., F.R.K., E.H., G.C., G.K., H.B., S.C., A.A. and J.P. performed experiments and analyzed data. M.H., A. F., J.P. and J.J. planned experiments and analyzed data. J.J. and J.P. conceived and designed the study. All authors took part in writing the manuscript.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

References


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