High molecular mass assemblies of amyloid-β oligomers bind prion protein in patients with Alzheimer’s disease

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Alzheimer’s disease is the most common form of dementia and the generation of oligomeric species of amyloid-β is causal to the initiation and progression of it. Amyloid-β oligomers bind to the N-terminus of plasma membrane-bound cellular prion protein (PrP\(^C\)) initiating a series of events leading to synaptic degeneration. Composition of bound amyloid-β oligomers, binding regions within PrP\(^C\), binding affinities and modifiers of this interaction have been almost exclusively studied in cell culture or murine models of Alzheimer’s disease and our knowledge on PrP\(^C\)-amyloid-β interaction in patients with Alzheimer’s disease is limited regarding occurrence, binding regions in PrP\(^C\), and size of bound amyloid-β oligomers. Here we employed a PrP\(^C\)-amyloid-β binding assay and size exclusion chromatography on neuropathologically characterized Alzheimer’s disease and non-demented control brains (n = 15, seven female, eight male, average age: 79.2 years for Alzheimer’s disease and n = 10, three female, seven male, average age: 66.4 years for controls) to investigate amyloid-β-PrP\(^C\) interaction. PrP\(^C\)-amyloid-β binding always occurred in Alzheimer’s disease brains and was never detected in non-demented controls. Neither expression level of PrP\(^C\) nor known genetic modifiers of Alzheimer’s disease, such as the PrP\(^C\) codon 129 polymorphism, influenced this interaction. In Alzheimer’s disease brains, binding of amyloid-β to PrP\(^C\) occurred via the PrP\(^C\) N-terminus. For synthetic amyloid-β\(_{42}\), small oligomeric species showed prominent binding to PrP\(^C\), whereas in Alzheimer’s disease brains larger protein assemblies containing amyloid-β\(_{42}\) bound efficiently to PrP\(^C\). These data confirm Alzheimer’s disease specificity of binding of amyloid-β to PrP\(^C\) via its N-terminus in a large cohort of Alzheimer’s disease/control brains. Differences in sizes of separated protein fractions between synthetic and brain-derived amyloid-β binding to PrP\(^C\) suggest that larger assemblies of amyloid-β or additional non-amyloid-β components may play a role in binding of amyloid-β\(_{42}\) to PrP\(^C\) in Alzheimer’s disease.

Keywords: prion protein; PrP\(^C\); Alzheimer’s disease; amyloid-β oligomers; amyloid-β neurodegeneration

Abbreviations: APIA = amyloid-β PrP interaction assay; PrP = prion protein

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Introduction

Alzheimer’s disease is the most common neurodegenerative disorder mainly affecting the elderly. Clinically it is characterized by progressive memory loss and cognitive impairment. In Alzheimer’s disease, generation and deposition of aggregated species of amyloid-β are linked to initiation and progression of dementia (Querfurth and LaFerla, 2010). The main alloforms of amyloid-β, proteolytically processed peptides of the larger amyloid precursor protein, range from 37 to 49 amino acids with amyloid-β42 showing the highest propensity to aggregate into oligomers and fibrils (Bergmans and De Strooper, 2010). Dimeric, trimeric, and other oligomeric assemblies of soluble amyloid-β42 aggregates correlate with brain dysfunction in Alzheimer’s disease (Walsh et al., 2002; Lesne et al., 2013).

The cellular prion protein (PrP), a glycosylphosphatidylinositol-anchored membrane protein, plays a key role in prion diseases such as Creutzfeldt-Jakob disease and in other neurodegenerative conditions such as Alzheimer’s disease. In prion disease, it is the substrate for the generation of its pathological isoform (PrPSc) which represents the principal component of prion infectivity and is causally involved in pathophysiology (Geissen et al., 2007; Colby and Prusiner, 2011). In cultured cells and murine Alzheimer’s disease models, PrPC binds oligomeric but not monomeric or fibrillar forms of amyloid-β42 leading to synaptotoxic effects (Lauren et al., 2009; Gimbel et al., 2010; Larson et al., 2012; Um et al., 2012). Although the principal finding that oligomeric forms of amyloid-β42 bind to PrPC is undisputed, there is an ongoing controversy on its pathophysiological relevance (reviewed in Benilova and De Strooper, 2010). Nevertheless, most recent studies reveal that the interaction of certain amyloid-β species with PrPC, most likely complexed with other proteins, leads to neuronal degeneration involving activation of the Src kinase Fyn (Larson et al., 2012; Um et al., 2012). Thus the amyloid-β42+PrPC interaction provides important mechanistic insights into the pathophysiology of Alzheimer’s disease-related neurodegeneration (Westaway and Jhamandas, 2012; Um and Strittmatter, 2013). It is possible that binding of amyloid-β42 to PrPC is not exclusive and other sterically similar assemblies of β-sheet rich oligomers may bind to and signal through PrPC (Resenberger et al., 2011).

Binding of synthetic amyloid-β42 occurs via the PrPC N-terminus with defined binding sites in PrPC residues 23–27 and 95–110 (Chen et al., 2010; Zou et al., 2011; Fluharty et al., 2013; Younan et al., 2013). The majority of studies investigating amyloid-β42+PrPC interaction employ cell-free systems, cultured cells or genetically modified mice (Lauren et al., 2009; Gimbel et al., 2010; Larson et al., 2012; Westaway and Jhamandas, 2012; Um and Strittmatter, 2013). Surprisingly little information is available on the binding of amyloid-β42 to PrPC in patient material. One study using Alzheimer’s disease brains and controls showed preferential binding of amyloid-β42 to PrPC in Alzheimer’s disease, which occurred mainly in the insoluble fraction of amyloid-β (Zou et al., 2011).

In our study we investigated the binding of amyloid-β42 to PrPC using brains of patients with Alzheimer’s disease and non-demented subjects. We developed a PrPC-binding assay using site-directed immobilization of recombinant full-length and N-terminally truncated PrPC to study binding of amyloid-β to PrPC in our patient cohorts. We show that amyloid-β binding to PrPC occurs only in Alzheimer’s disease. Moreover, this interaction is neither influenced by PrPC expression levels nor by PRNP codon 129 genotype, although this polymorphism is a known risk factor for Alzheimer’s disease. Using size exclusion chromatography, we determined sizes of amyloid-β aggregates with preferred binding to PrPC. For Alzheimer’s disease brains, prominent binding is uniformly restricted to larger protein assemblies whereas for synthetic amyloid-β42, prominent binding is uniformly restricted to smaller species of amyloid-β42 oligomers.

Materials and methods

Patients and control subjects

Fifteen patients with Alzheimer’s disease and 10 non-demented subjects from Northern Germany were chosen for this study. Alzheimer’s disease was clinically confirmed by applying clinical signs indicative for a dementia. This occurred in all patients with Alzheimer’s disease and in none of the healthy control subjects (Table 1). The use of specimens and basic clinical information were in agreement with the regulations and ethical standards at the contributing hospitals and written consent by patients or relatives was obtained where necessary.

Neuropathological investigations and immunohistochemistry

Brains were fixed in 4% formalin and paraffin-embedded tissue samples (frontal cortex) were cut into 3-μm thick serial sections, mounted on glass slides and processed according to published protocols (Glatzel et al., 2003). Immunohistochemical stainings with the following primary antibodies were performed: amyloid-β (1:100; 6E10, DBS Emergo) (Weidemann et al., 1989), Tau (1:1500; Thermo). Primary antibodies were visualized using a standard diaminobenzidine streptavidin-biotin horseradish peroxidase method (Ventana/Roche). Quantification of immunosignals was performed according to published methods (Sepulveda-Falla et al., 2011) by experienced morphologists (J.M., M.G., F.D.) blinded with respect to experimental groups. Briefly, for quantification of diffuse and cored amyloid-β plaques and neurofibrillary tangles, we counted the presence of positive events (plaques, tangles) in a representative area of the entire sample (at least 1 mm²) using a Zeiss DMD 108 large image area microscope. For quantification of immunopositive areas (amyloid-β, tau) 15 randomly chosen 0.2 mm² regions of each sample were assessed using Zeiss Axiovision quantification software on images taken with a Zeiss Axioscience S1000 microscope.

Polymorphisms

Genomic DNA was extracted from frozen brain tissue (frontal cortex) of patients with Alzheimer’s disease and control subjects using the DNeasy® Blood and Tissue Kit (Qiagen), according to the manufacturer’s recommendations. Codon 129 status was determined by restriction fragment length polymorphism analysis and ApoE status was determined as published (Debatin et al., 2008).
Table 1 Demographic and clinical data

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<th>Alzheimer’s</th>
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CERAD = Consortium to Establish a Registry for Alzheimer’s Disease; F = female; M = male.

Preparation of brain homogenates
Grey and white matter of frontal cortices was dissected. One millilitre of a 25% (w/v) brain homogenate (grey matter) was prepared in lysis buffer (10 mM Tris, 150 mM NaCl, 0.5% deoxycholate, 0.5% NP40, 5 mM EDTA, pH 7.4), containing a Protease Inhibitor Cocktail Mix (Roche). Brain tissue was homogenized with 25 strokes in a glass Dounce Homogenizer. Homogenates were incubated for 30 min on ice and centrifuged at 7500 g at 4°C for 10 min to remove cellular debris. The resulting supernatant (S1; total homogenate) was carefully transferred into a clean 1.5 ml test tube. S1 fractions (75 µl) were collected separately for subsequent experiments. After ultra-centrifugation of S1 (100 000g; 4°C; 1 h), the resulting supernatant (S2; detergent soluble fraction) was transferred into a new 1.5 ml test tube. The pellet (P2; detergent insoluble fraction) was gently washed twice with 500 µl lysis buffer and resuspended in 1 ml lysis buffer containing protease inhibitor. S1, S2 and P2 fractions were stored at −80°C until use.

Co-immunoprecipitation with 3F4 antibody
To demonstrate the interaction between PrP C and amyloid-β in vivo, co-immunoprecipitation experiments with the PrP C-specific 3F4 antibody (Covance) were performed on P2 fractions of Alzheimer’s disease and control samples. To avoid unspecific binding of proteins to Protein G Sepharose (GE Healthcare), a suitable amount of beads were centrifuged at 1000 g for 1 min. The supernatant was discarded and beads mixed in a 1:1 ratio with lysis buffer. This washing step was repeated three times. Five hundred microlitres of brain homogenate was incubated with 50 µl of a 1:1 mix (beads:lysis buffer) at 4°C for 1 h on a rotator. After incubation, beads were centrifuged as described above. The supernatant was used for co-immunoprecipitation experiments.

Immunoprecipitation and detection of amyloid-β
A 1:250 dilution of the 3F4 capture antibody was added to the P2 fraction of the brain homogenate (see above) and incubated at 4°C on a rotator overnight. The following day, a suitable amount of beads was prepared as described above and a volume of 50 µl was added to the homogenate and incubated for 4 h on a rotator at room temperature. After incubation, the beads were centrifuged at 1000g for 1 min and the resulting supernatant was discarded. Beads were washed three times with lysis buffer and resuspended in 25 µl of a SDS sample buffer containing β-mercaptoethanol. Samples were boiled at 95°C for 5 min
Dried peptide films were stored at \(-20\) °C for 30 s break in between sonication steps) to destroy pre-built peptide aggregates (amyloid-\(\beta\)) with 2% Sarkosyl. Samples were stored at \(-80\) °C until use. For western blot analysis and amyloid-\(\beta\) PrP interaction assay (APIA) experiments, samples were pooled, lyophilized and resuspended in 0.1 ml PBS. Twenty microlitres of eluted P2 fractions were separated on 4–12% Bis-Tris gradient gels (Novex) for western blot analysis with 6E10. The remaining 80 μl were tested with APIA.

### Handling and preparation of synthetic amyloid-\(\beta\) species

Lyophilized synthetic amyloid-\(\beta\) peptides (amyloid-\(\beta\)-38, amyloid-\(\beta\)-40, amyloid-\(\beta\)-42, and scrambled amyloid-\(\beta\)-42) were purchased from BACHEM or GenicBio, dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Fluka) to obtain a final concentration of 1 mM. After incubation on a shaker under heavy agitation for 1.5 h at 4 °C the peptide solution was sonicated on ice (amplitude 50%; 3 \times 10 s of sonication; with 30 s break in between sonication steps) to destroy pre-built peptide aggregates. After sonication, HFIP was completely removed by Speed Vac. Dried peptide films were stored at \(-20\) °C. If mainly monomeric amyloid-\(\beta\)-42 species were needed, the peptide film was resuspended in distilled H\(_2\)O + 0.1% NH\(_4\) (ammonium) to obtain a final concentration of 100 mM. Remaining synthetic amyloid-\(\beta\)-42 aggregates were spun down (16000g, 15 min at 4 °C). After centrifugation, the supernatant of the peptide solution was used for experiments in which mainly monomeric forms of amyloid-\(\beta\) were needed. For ageing/oligomerization of synthetic amyloid-\(\beta\)-42 after the HFIP-treatment, dried peptide films were resuspended in PBS + 0.1% NH\(_4\) to obtain a final concentration of 1 mg/ml. For the ageing/oligomerization process of synthetic amyloid-\(\beta\) the peptide solution was incubated for at least 12 h at room temperature. Successful preparations of amyloid-\(\beta\)-42 monomers and oligomers were tested on western blots or dot blots, using a 1:2000 dilution of the amyloid-\(\beta\) specific 6E10 antibody (Covance) or the oligomer-specific A11 antibody (Invitrogen).

### Sample preparation of human brain tissue for Sarkosyl size exclusion chromatography

Brain homogenates were prepared as described above with some slight modifications in the protocol. Briefly, grey and white matter of brain tissue (frontal cortex) was dissected. Two hundred and fifty milligrams of grey matter were homogenized in a volume of 1 ml Lysis Buffer (10 mM Tris, 150 mM NaCl, 0.5% NP40, 0.5% deoxycholate, 5 mM EDTA, Complete Protease Inhibitor Cocktail mix, pH 7.4) by 25 strokes using a glass Dounce homogenizer. After centrifugation of the homogenate at 7500 g for 10 min, the supernatant (S1) was transferred into a fresh Eppendorf tube and an equal amount of 2% N-lauroylsarcosine sodium salt (Sarkosyl; Fluka) in H\(_2\)O was added. After incubation for 30 min on ice, the sample was centrifuged at 10000g for 1 h at 4 °C. The resulting supernatant (S2; detergent soluble fraction) was transferred into a fresh Eppendorf tube. The pellet (P2; detergent insoluble fraction) was gently washed twice in 0.5 ml lysis buffer to remove residual proteins and resuspended in 1 ml of a 1:1 solution of lysis buffer with 2% Sarkosyl. Samples were stored at \(-80\) °C until use.

### Size exclusion chromatography of human brain tissue

P2 fractions were centrifuged at 10000g at 4 °C for 10 min before injection of 1 ml of the sample to the Superose TM12 10/300 GL column on the Äkta Explorer System (GE Healthcare). Proteins were separated at a flow rate of 0.25 ml/min and detected at a wavelength of 280 nm. PBS (0.05 M phosphate, 0.15 M NaCl) + 1% Sarkosyl was used as elution buffer. Eluted protein fractions were collected in a volume of 0.5 ml and stored at \(-80\) °C until use. For western blot analysis and amyloid-\(\beta\) PrP interaction assay (APIA) experiments, samples were pooled, lyophilized and resuspended in 0.1 ml PBS. Twenty microlitres of eluted P2 fractions were separated on 4–12% Bis-Tris gradient gels (Novex) for western blot analysis with 6E10. The remaining 80 μl were tested with APIA.

### Size exclusion chromatography of synthetic amyloid-\(\beta\)-1-42

Synthetic amyloid-\(\beta\)-1-42 oligomers (GenicBio) were prepared as described above. Five hundred microlitres of a 1 mg/ml synthetic amyloid-\(\beta\)-1-42 preparation was injected in the Superose TM12 10/300 GL column, connected to a Äkta Explorer System (GE Healthcare). Synthetic amyloid-\(\beta\)-peptides were separated at a flow rate of 0.25 ml/min. PBS was used as elution buffer. Synthetic amyloid-\(\beta\)-1-42 peptides were observed at a wavelength of 216 nm. Eluted fractions were collected in a volume of 500 μl and stored at \(-80\) °C until use. For western blot analysis and APIA, three adjacent fractions were pooled and lyophilized in a Speed-Vac. Sample pools were resuspended in 0.1 ml PBS. Ten microlitres of the amyloid-\(\beta\) pools were separated on 4–12% Bis-Tris gradient gels (Novex) and tested by western blot analysis using the amyloid-\(\beta\) specific antibody 6E10. Fifty microlitres of peptide pools were used in APIA experiments.

### Amyloid-\(\beta\)-PrP\(C\) interaction assay

Based on the Flexi Vector System (Promega), human PrP\(C\) as full-length (rPrP\(C\)) and the human N-terminal truncated form (rPrP\(C\)-NT) was amplified and provided with necessary restriction sites via PCR, according to the manufacturer’s recommendations. In the case of full length rPrP\(C\), the following primers were used:

**Upstream:** 5′-AGGAGCGATCGCCATGAAGAAGCGCCCGAAGCCTGGAGGGAGAGATCCAGG3′

**Downstream:** 5′-AACCTGTGTTAAACTCCCACTATCGAGAGAAGGAGGAAAGAGATCAGG3′

In the truncated construct rPrP\(C\)-NT, the following primers were used:

**Upstream:** 5′-GGCGTGCCATCGCCACATGGGCTGTGCAGGACGAGCTGTTCTG-3′

**Downstream:** 5′-AACCTGTGTTAAACTCCCACTATCGAGAGAAGGAGGAAAGAGATCAGG-3′

PCR was performed under standard conditions.

### Cloning

The amplified PrP\(C\) DNA sequences were first cloned into the N-terminal pFN18A Flexi Vector (Promega). To avoid steric hindrance of the N-terminal fused HaloTag\(TM\) with the proposed N-terminal PrP\(C\) binding sites for amyloid-\(\beta\) oligomers, the sequences were also cloned into the C-terminal pFC20K Flexi Vector (Promega) according to the manufacturer’s recommendations and tested by restriction analysis and sequencing.

### Expression

Both PrP\(C\)-HaloTag\(TM\) fusion proteins were expressed in bacterial *E.coli* Tuner\(TM\) (Invitrogen) cells by the addition of 1 mM IPTG and incubation on a shaker at 37 °C for 5 h. After expression, the
bacterial cultures were centrifuged at 15,000 g at 4°C for 15 min. Pellets were stored at −20°C until use.

Directed immobilization of PrPC fusion proteins
The covalent and directed immobilization of PrPC fusion proteins on the HaloLink™ Resin (Promega) was performed according to manufacturer’s recommendations with some modifications of the protocol. Pellets of 50 mL bacterial culture were resuspended in 2.5 mL PBS with Complete Protease Inhibitor Mix (Roche). Cell disruption and harvesting of the fusion protein was achieved through sonication of the bacterial culture with three pulses of 15 s at an amplitude of 60% on ice. The resin was prepared according to the manufacturer’s recommendations. Seventy-five microlitres of the fusion protein was incubated with 100 μL resin in binding buffer (100 mM Tris, 150 mM NaCl, 0.05% NP40) for 30 min on a rotator at room temperature. After centrifugation for 2 min at 800 g the supernatant was discarded and the resin washed three times with 1 mL of binding buffer. The procedure was repeated three times to saturate the resin with PrPC, because of poor expression of the C-terminal PrPC-HaloTag™ fusion protein. The last three washing steps were performed with 1 mL of washing buffer (100 mM Tris, 150 mM NaCl, 0.05% NP40, 1 mg/mL bovine serum albumin). After a final centrifugation step of 800 g, the supernatant was discarded. Subsequent interaction assays were performed in 100 μL of binding buffer.

Binding of synthetic amyloid-β oligomers to immobilized PrPC
One hundred microlitres of binding buffer was added to the resin. Aged synthetic amyloid-β oligomers (100 nM) were added and incubated for 1 hour on a rotator at room temperature. After incubation, the subsequent steps were performed according to the manufacturer’s recommendations. Peptides were separated on NuPAGE® Bis-Tris Mini Gels (Novex™). In case of binding studies of synthetic amyloid-β oligomers separated by size-exclusion chromatography, three consecutive fractions were pooled to one single fraction, lyophilized in a Speed-Vac to a volume of 100 μL and applied to APIA as described above.

Binding of human amyloid-β homogenates to immobilized PrPC
Different fractions (S1, S2, P2) of total homogenate from patients with Alzheimer’s disease or control subjects and the resin were prepared as described above. The lyophilized fraction (75 μL) was added to the resin with 100 μL of binding buffer. The following steps are described above. In case of interaction studies with fractions of Alzheimer’s disease and control samples separated by size exclusion chromatography, two consecutive fractions were pooled to one single fraction. Elution buffer of the pooled fraction was completely lyophilized by Speed-Vac. Lyophilized proteins were resuspended in 100 μL PBS and applied to APIA as described. To proof the specificity of the amyloid-β-PrPC binding, the interaction was blocked using the oligomer specific A11 antibody (Invitrogen). For this, synthetic amyloid-β preparations or human P2 fractions were incubated with a 1:10,000 dilution of this antibody for 1 hour on a rotator at room temperature before testing with APIA.

Coupling of Alexa488 reporter to PrPC
To detect the interaction with immobilized synthetic amyloid-β oligomers on a nitrocellulose membrane in dot blot experiments, the coupling of the PrPC-HaloTag™ fusion protein with a fluorescent reporter molecule was required. Briefly, expression of E. coli Tuner™ bacterial cultures (transformed with PrPC or PrPCΔNT) were induced at OD600 = 0.5 through addition of 1 mM IPTG into the LB-media. The bacterial cultures were incubated for 5 hours on a shaker at 37°C. After sonication of the bacteria and harvesting of the protein, the PrPC fusion protein was incubated with the fluorescent AlexaFluor® 488 Ligand (Promega) at 4°C overnight according to the manufacturer’s recommendations.

Dot blot analysis
Synthetic amyloid-β oligomers were prepared as described above and spotted on nitrocellulose membranes (Bio-Rad) using a vacuum-supported Dot Blot machine (TE70 ECL, Amersham Biosciences). After blocking of the membrane with 5% non-fat dry milk powder in Tris-buffered saline and Tween-20 (TBS-T, blocking solution) for 1 hour, 100 μL of a 1:50 dilution of the PrPC reporter molecules (in 5% blocking solution) were incubated for 40 min under agitation inside the wells of the dot blot apparatus. After three washing steps with TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 15 min, the membrane was washed for 3 × 5 min with TBS to remove residual Tween-20 from the membrane. For the detection of a fluorescent signal of the PrPC-AlexaFluor® 488 reporter molecule (excitation maximum 499 nm; emission maximum 518 nm) as a proof for interaction between PrPC and amyloid-β oligomers a Typhoon 9410 Scanner (GE Healthcare) with appropriate settings to the used fluorophor was used.

Western blot analysis
Brain homogenates and samples derived from PrPC-amyloid-β binding assays were prepared as described above. S1, S2 or P2 fractions, or washed resin of IP/APIA experiments, were mixed with a suitable amount of 10 × sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.008% bromphenol blue), boiled at 95°C for 5 min, and separated by size on 10–20% Tris-Tricine or 4–12% Bis-Tris gradient gels (Novex) according to the manufacturer’s recommendations. Proteins were blotted on nitrocellulose membranes (0.2 μm; Bio-Rad) using a constant amperage of 250 mA for 70 min. After blotting of proteins, membranes were boiled for 3 min in TBS to expose protein antibody epitopes on the membrane. Detection of proteins was performed with the following antibodies: amyloid-β with 6E10 (1:200; Covance), LR1P with BG1 (1:10,000; Abcam), Fyn1 with Fyn (1:1,000; Cell Signalling), and the appropriate secondary antibody (1:2,500; Promega). Blots were developed by using a mixture of SuperSignal West Pico/Femto (Pierce) in a 4:1 ratio. Detection and quantification of the signals was performed by the Universal Hood II and Quantity One 4.6.2 software (Biorad).

Amyloid-β1-40 and amyloid-β1-42 ELISA
The amyloid-β40 and amyloid-β42 sandwich ELISA kits were purchased from Invitrogen and applied to S2 and P2 fractions of Alzheimer’s disease and control samples according to the manufacturer’s recommendations.

PrPC ELISA
To compare the PrPC levels of S2 and P2 in human brain tissue of Alzheimer’s disease and control samples, the PrPC ELISA Kit from SPI-Bio was used according to the manufacturer’s recommendations.
Statistical analysis

Statistical comparison of western blot quantifications and ELISA results between patients with Alzheimer’s disease and non-demented control subjects was performed using Student’s t-test with consideration of statistical significance at P-values <0.05 (**); <0.01 (***), and <0.0001 (****). Logistic regression was calculated for immobilized synthetic amyloid-β42 oligomers probed with rPrP:C-AlexaFluor488 on a nitrocellulose membrane in dot blot assays.

Results

Description of patient cohorts

Details on the two patient groups are given in Table 1. All patients with Alzheimer’s disease (seven female, eight male, average age: 79.2 years, standard deviation (SD): 7.3 years) showed characteristic clinical signs and symptoms of Alzheimer’s disease including prominent memory deficits. For non-demented control subjects (three female, seven male, average age: 66.4 years, SD: 5.4 years), no obvious signs of dementia, including memory loss or loss of executive functions, were documented. Brains from subjects of both cohorts were assessed neuropathologically, and for patients with Alzheimer’s disease CERAD and Braak criteria were applied (Braak and Braak, 1991; Fillenbaum et al., 2008). Additionally, genetic modifiers for Alzheimer’s disease such as the ApoE4 genotype and the methionine/valine polymorphism at codon 129 of PRNP were analysed. The codon 129 polymorphism in PRNP is a susceptibility gene for Alzheimer’s disease but did not modulate binding efficiency between PrP C and amyloid-β in vitro (Bertram et al., 2007; Chen et al., 2010). For patients with Alzheimer’s disease, as expected, a high ratio of ApoE4 carriers could be documented (66.6%) whereas non-demented control subjects only showed 10% ApoE4 carriers (Querfurth and LaFerla, 2010). For PRNP codon 129 there were no significant differences between groups with 46.6% (Alzheimer’s disease) and 50% (controls) methionine homozygotes. We quantified these data give an explanation for prominent binding of amyloid-β to PrP C in the insoluble (P2) fraction where differences between Alzheimer’s disease brains and controls were highest for amyloid-β42. In Alzheimer’s disease brains, levels of SDS-stable amyloid-β dimers, trimers or low molecular and high molecular weight oligomers were much lower than those of amyloid-β monomers (Supplementary Fig. 4). Interestingly, we could identify weak binding of amyloid-β to PrP C in the supernatant (S1) fraction of Alzheimer’s disease brains, although this has never been observed in control brains (data not shown) even though amyloid-β40 and amyloid-β42 concentrations are comparable.

Amyloid-β binds to PrP C only in Alzheimer’s disease but not in control brains

Data from cell culture experiments and transgenic Alzheimer’s disease mouse models have shown that amyloid-β binds to PrP C (Um et al., 2012). Published data suggest that amyloid-β binding to PrP C also occurs in patients with Alzheimer’s disease (Zou et al., 2011; Um et al., 2012). We studied this binding in vivo by performing co-immunoprecipitation assays using brain homogenates from 15 patients with neuropathologically confirmed Alzheimer’s disease and 10 non-demented control subjects. For this purpose we used detergent insoluble (P2) fractions and bead-conjugated 3F4 anti-PrP monoclonal antibody and probed eluted proteins by western blotting using the anti-amyloid-β monoclonal antibody 6E10. We could only detect 6E10-positive signals in Alzheimer’s disease brains with no signal in controls (Fig. 2A and B, 15 positive in Alzheimer’s disease, zero positive in controls).

Amyloid-β binding to PrP C in Alzheimer’s disease is a function of amyloid-β concentration

PrP C has been shown to preferentially bind to amyloid-β (Zou et al., 2011). We measured concentrations of amyloid-β and amyloid-β42 in supernatants (S2) and pellet fractions (P2) of Alzheimer’s disease brains and controls using amyloid-β and amyloid-β42 specific ELISA. We found no significant differences in supernatants (S2) between Alzheimer’s disease brains and controls for amyloid-β (1.92 ± 0.03 pM for Alzheimer’s disease and 1.86 ± 0.05 pM for controls, Fig. 3A) and amyloid-β42, respectively (2.01 ± 0.09 pM for Alzheimer’s disease and 1.90 ± 0.10 pM for controls, Fig. 3B), whereas the concentrations for amyloid-β (2.72 ± 0.07 pM for Alzheimer’s disease and 2.18 ± 0.14 pM for controls, Fig. 3A) and amyloid-β42 (28.61 ± 6.29 pM for Alzheimer’s disease and 0.77 ± 0.14 pM for controls, Fig. 3B) were significantly higher in pellet fractions (P2) of Alzheimer’s disease brains when compared with control subjects. For PrP C, comparable expression levels for supernatants (S2) and pellet fractions (P2) were found in both cohorts (S2: 2.908 relative absorption ± 0.05520 for Alzheimer’s disease and 2.932 ± 0.05392 for controls, P2: 2.658 ± 0.1887 for Alzheimer’s disease and 2.676 ± 0.1571 for controls, Fig. 3C). Given that quantification of binding efficiency between amyloid-β and PrP C can only be poorly assessed by co-immunoprecipitation assays using bead-conjugated antibodies because of variations in antibody-coupling efficiencies and unspecific background signals (Lal et al., 2005), we developed a suitable assay to investigate amyloid-β–PrP C binding. For this we coupled human recombinant full-length (rPrP C) or N-terminally truncated PrP C lacking amyloid-β binding domains (rPrPCΔN42) to a resin (Fig. 4A and B) or to fluorescent reporters (Fig. 4D) using the Halotag® technology.
C-terminal attachment of the HALOtag™ allowed for a directed immobilization of r\(\text{PrP}^C\) and r\(\text{PrP}^{C\text{ANT}}\) without affecting amyloid-\(\beta\) binding sites. The functionality of this new APIA was investigated using well defined preparations of synthetic oligomeric amyloid-\(\beta\) and human Alzheimer’s disease brain (Supplementary Fig. 2 and Fig. 4C). Binding of synthetic oligomeric amyloid-\(\beta\) to r\(\text{PrP}^C\) but not to r\(\text{PrP}^{C\text{ANT}}\) confirmed N-terminal binding of oligomeric amyloid-\(\beta\) (Fig. 4C and E). In Alzheimer’s disease we could only observe binding of amyloid-\(\beta\) to r\(\text{PrP}^C\) but not to r\(\text{PrP}^{C\text{ANT}}\) and thus demonstrate for the first time the necessity of the \(\text{PrP}^C\) N-terminus for binding of amyloid-\(\beta\) in diseased brains (Fig. 4C). Using serially diluted synthetic oligomeric amyloid-\(\beta\) immobilized on a membrane by dot blot and probed with fluorescently tagged r\(\text{PrP}^C\) allowed for quantitative assessment of the interaction between \(\text{PrP}^C\) and amyloid-\(\beta\) showing a linear correlation between \(\text{PrP}^C\)-binding and concentrations of synthetic oligomeric amyloid-\(\beta_{42}\) (\(r^2 = 0.9385\); Fig. 4D). Binding of synthetic oligomeric amyloid-\(\beta_{42}\) and amyloid-\(\beta\) oligomers found in patients with Alzheimer’s disease to r\(\text{PrP}^C\) could be blocked by the oligomer-specific antibody A11, and neither amyloid-\(\beta_{38}\) nor amyloid-\(\beta_{40}\) or amyloid-\(\beta_{43}\) and scrambled amyloid-\(\beta_{42}\) were able to interact with our immobilized r\(\text{PrP}^C\), thus confirming the specificity of the assay (Supplementary Fig. 2C and D). In patients with Alzheimer’s disease, we were able to block the interaction between r\(\text{PrP}^C\) and amyloid-\(\beta\) oligomers significantly (Supplementary Fig. 2E; *\(P < 0.05\), with relative densitometric intensity of 235 700 ± 51 950 for P2-r\(\text{PrP}^C\) and 83 520 ± 23 200 for P2-r\(\text{PrP}^C + A11\)).

Amyloid-\(\beta\) binding to \(\text{PrP}^C\) in Alzheimer’s disease occurs mainly in the detergent insoluble fraction

Binding of amyloid-\(\beta\) to \(\text{PrP}^C\) in transgenic mouse models of Alzheimer’s disease and in Alzheimer’s disease brains has been
described in soluble and insoluble fractions of brain homogenates (Zou et al., 2011; Larson et al., 2012). APIA allowed us to quantify binding of amyloid-β to PrPC in supernatants (S1), soluble (S2) and insoluble (P2) fractions of 15 Alzheimer’s disease brain homogenates. Highest binding efficiency was detected in insoluble P2 fractions (77.06% C6 3.74 of total bound amyloid-β, Fig. 5A and B). Since freeze thaw cycles of S1 fractions may influence binding efficiencies, further processing was directly carried out. However we tested this effect also and could not observe significant differences in binding properties (Supplementary Fig. 3A and B). Significantly, lower binding efficiencies could be observed for supernatants or soluble S1 and S2 fractions of Alzheimer’s disease brain homogenates (16.97% C6 3.31% of total bound amyloid-β for S1; 5.79% C6 1.20% of total bound amyloid-β for S2, Fig. 5A and B).

High molecular mass assemblies containing amyloid-β bind to PrPC in patients with Alzheimer’s disease

Studies using synthetic oligomeric amyloid-β42 have shown that not all synthetic oligomeric amyloid-β42 species bind to PrPC, although the exact size of optimally binding synthetic oligomeric amyloid-β42 was not established (Lauren et al., 2009). Using size exclusion chromatography with well-defined synthetic oligomeric amyloid-β42 adjusted to equal protein (amyloid-β42 amounts, Fig. 6 and Supplementary Fig. 2C) and APIA on pooled size exclusion chromatography fractions, we specified sizes of optimally binding synthetic oligomeric amyloid-β42 to fractions ranging between 21 and 8 kDa, corresponding to pentamers to dimers of synthetic oligomeric amyloid-β42 (Fig. 6A and B). Following a published protocol for brain tissue (Zou et al., 2011), we assessed distribution of amyloid-β and PrPC in eluted size exclusion chromatography fractions. This showed that amyloid-β is present as SDS-instable oligomers in all tested high molecular weight fractions whereas PrPC is mainly present in its monomeric form from 44 kDa onwards with smaller amounts of PrPC eluting at higher molecular weight fractions (Fig. 7A–C).

To determine appropriate sizes of protein aggregates with optimal binding to PrPC in P2 fractions, we performed size exclusion chromatography on four Alzheimer’s disease brains, pooled fractions eluted in size exclusion chromatography and assayed for their PrPC-binding efficiency using APIA (Fig. 7A, D and E). This showed that protein aggregates containing amyloid-β oligomers
Figure 3  Amyloid-β–PrPC interaction is a function of amyloid-β load in the brain and not dependent on PrPC levels. ELISAs for amyloid-β40 (Aβ40) and amyloid-β42 (Aβ42) as well as for PrPC loads in the brain were applied to supernatant (S2) and pellet (P2) homogenate fractions of Alzheimer’s disease (AD) and control patients. (A) Amyloid-β40 concentrations in S2 fractions showed no significant difference in Alzheimer’s disease compared to the control group, whereas in P2 fractions the amyloid-β40 level was significantly elevated. (B) Amyloid-β42 concentrations in S2 fractions showed no significant difference in Alzheimer’s disease compared to the control group, whereas in P2 fractions the amyloid-β42 level was significantly elevated. (C) No difference in the PrPC loads could be observed between patients with Alzheimer’s disease and control subjects in S2 and P2 homogenate fractions (*P < 0.01, **P < 0.001). (D) Scatter plots of amyloid-β42 concentrations against PrPC loads of S2 and P2 fractions. There is not significant correlation between these variables in Alzheimer’s disease or controls. Conc. = concentration.
Figure 4  APIA allows a qualitative and semi-quantitative proof of the amyloid-β-PrP<sub>C</sub> interaction. Schematic representation of relevant forms of the prion protein and proof of principle of APIA. (A) Linear representation of the primary sequence of human PrP<sub>C</sub> showing relevant protein domains. After removal of the N-terminal signal sequence (amino acid 1–22; red box) and the C-terminal signal sequence for the attachment of the GPI-anchor (amino acid 231–253; white box) by signal peptidases in the endoplasmic reticulum, the mature prion protein comprises an octameric repeat region (amino acid 51–91; black box), a neurotoxic domain (amino acid 106–126; yellow box), a disulphide bridge (between amino acid 179 and 214), and two variably occupied N-glycosylation sites (amino acid 181 and 197). The two proposed binding sites for amyloid-β-oligomers (AβO) are located at the N-terminal end of PrP<sub>C</sub> (amino acid 23–27 and amino acid 95–110; bars and black arrows). rPrP<sub>C</sub> consists of full length PrP<sub>C</sub> excluding the signal sequence (amino acid 1–22) and is fused on its C-terminal end with the HaloTag<sup>®</sup>/C213 (blue box) to avoid sterical hindrance with proposed N-terminal specific amyloid-β oligomer binding sites. As a negative control a N-terminally truncated form, named rPrP<sub>C</sub>/C<sub>1</sub>N (amino acid 111–230) also fused on its C-terminus with the HaloTag<sup>®</sup>, was generated, lacking the complete N-terminus with its proposed amyloid-β oligomer binding sites. (B) Schematic representation of the APIA principle. rPrP<sub>C</sub> can be immobilized directed over its C-terminally fused HaloTag<sup>®</sup>/HaloLink<sup>®</sup> Ligand on a resin and is able to bind amyloid-β oligomers (light blue circles) derived from different origins. (C) APIA proof of principle. Total homogenate of one patient with Alzheimer’s disease (AD), one control subject and a solution of aged synthetic amyloid-β-42 (sAβ<sub>42</sub>) were applied to APIA as described in (B). As negative control, rPrP<sub>C</sub>/C<sub>1</sub>N and beads without immobilized rPrP<sub>C</sub> were used. An interaction could only be detected by using rPrP<sub>C</sub> with Alzheimer’s disease homogenate and synthetic amyloid-β-42 peptides. The truncated form rPrP<sub>C</sub>/C<sub>1</sub>N did not interact with amyloid-β oligomers from brain tissue or synthetic peptides. Likewise, in the case of control subjects no interaction could be observed. (D) rPrP<sub>C</sub> can also be coupled over its fused HaloTag<sup>®</sup>/HaloLink<sup>®</sup> Ligand with a fluorescent AlexaFlour<sup>®</sup>488 (continued)
with optimal binding to PrP C are present in fractions between 300 and 158 kDa (Fig. 7D and E). Similar findings with considerably lower binding efficiencies could be found in a S2 fraction of an Alzheimer’s disease brain (Supplementary Fig. 3C and D). This is in contrast with data obtained with synthetic oligomeric amyloid-β42 and implies that in Alzheimer’s disease, amyloid-β oligomers with the highest binding propensity to PrP C are not low but rather high molecular weight assemblies. It is possible that PrP C-interacting proteins such as the low-density lipoprotein receptor-related protein 1 (LRP1) or the Fyn kinase may explain higher molecular mass proteins such as the low-density lipoprotein receptor-related protein (LRP1) or the Fyn kinase being present in size exclusion chromatography fractions with highest amyloid-β-PrP C binding properties, but this was not the case (Supplementary Fig. 6).

**Discussion**

The mechanisms of neurodegeneration in Alzheimer’s disease are not fully understood. Much attention was attracted by the recent finding that PrP C is a high affinity receptor for amyloid-β oligomers (Lauren et al., 2009). Although all relevant studies agree on this interaction and structural features of PrP C with its highly flexible N-terminus would clearly support this role (Beland and Roucou, 2012), its consequences are controversially discussed (Benilova and De Strooper, 2010). A number of papers using synthetic amyloid-β have shown that oligomeric amyloid-β binds to residues 23-27 and 95-110 of PrP C (Chen et al., 2010; Zou et al., 2011; Fluharty et al., 2013; Younan et al., 2013).

Binding of amyloid-β42 to PrP C in Alzheimer’s disease has been studied before (Zou et al., 2011; Um et al., 2012). Our study uses a large patient cohort to further investigate this important question. This allows us to make statistical calculations and show that...
(i) significant binding of amyloid-β to PrP<sup>C</sup> only occurs in Alzheimer’s disease; (ii) binding of amyloid-β aggregates to PrP<sup>C</sup> is restricted to the N-terminus of PrP<sup>C</sup>; (iii) optimal binding occurs in the insoluble fraction of amyloid-β; and (iv) neither expression levels of PrP<sup>C</sup> nor the codon 129 polymorphism of PRNP influence this binding. Thus, our results are in agreement with studies using recombinant PrP<sup>C</sup> and synthetic oligomeric amyloid-β<sub>42</sub> where N-terminal binding of amyloid-β was shown (Chen et al., 2010; Fluharty et al., 2013; Younan et al., 2013) and where the negligible role of codon 129 polymorphism on amyloid-β<sub>42</sub>-PrP<sup>C</sup> binding was described. We also found optimal binding of amyloid-β<sub>42</sub> to PrP<sup>C</sup> in insoluble fractions (Chen et al., 2010). This contradicts...
studies where optimal binding was found in the soluble fractions of amyloid-β42 oligomers (Barry et al., 2011; Larson et al., 2012) and may be due to subtle differences in methodologies. We show that binding of amyloid-β to PrPC consistently occurs in Alzheimer’s disease brains via the PrPC N-terminus and cannot be observed in non-demented controls.

Given that only amyloid-β42 levels are significantly different between Alzheimer’s disease cases and controls, binding may be a direct function of the amount of amyloid-β42 present in Alzheimer’s disease brains. Neither PrPC expression nor known genetic modifiers such as the PrPC codon 129 polymorphism influenced this binding.

To study amyloid-β–PrPC binding, we established a prey-bait based assay (APIA), using human recombinant full-length or N-terminally truncated PrPC as bait. A C-terminally situated tag allows for directed immobilization, so that APIA can be used in ELISA format or as a matrix for binding experiments. Additionally, this tag was used to covalently bind fluorescent molecules enabling detection of bound amyloid-β oligomers quantitatively in an in situ format. Using APIA and synthetic oligomeric amyloid-β42, we determined sizes of synthetic oligomeric amyloid-β42 with optimal binding to PrPC in a range between 8 and 16 kDa (Barry et al., 2011; Larson et al., 2012). This correlates with dimers up to tetramers of amyloid-β42 and these species were shown to impair synaptic plasticity in a PrPC-dependent fashion (Lauren et al., 2009; Larson et al., 2012; Um et al., 2012). In contrast to data with synthetic oligomeric amyloid-β42, in patients with Alzheimer’s disease we found optimal binding to PrPC in protein fractions ranging from 150 and 300 kDa in size with no detectable PrPC-binding in protein fractions ranging from 8 to 21 kDa.

How could this be explained? One possibility is that in patients with Alzheimer’s disease larger oligomeric assemblies of amyloid-β42 bind to PrPC. Our data would suggest 35–70mers, which is in line with recent studies showing binding of 20–100mers of amyloid-β to PrPC (Freir et al., 2011; Younan et al., 2013). Interestingly, the study by Younan et al. (2013) shows that binding of amyloid-β42 to PrPC was linked to a disassembly of aggregates. These data fit to recent studies showing that amyloid-β oligomers found in patients with Alzheimer’s disease are primarily of high molecular weight (Espanza et al., 2013). Another possibility suggests that amyloid-β oligomers act as a scaffold bringing together various proteins binding to either amyloid-β oligomers alone or the amyloid-β42–PrPC complex (Noguchi et al., 2009). In support of this possibility, we found presence of PrPC in higher molecular weight fractions eluting earlier than the predicted volume for monomeric PrPC, indicating the possibility of presence of PrPC-containing complexes. In this scenario, candidates include amyloid-β binding proteins related to lipid metabolism (Ray et al., 1998) and chaperones (Fonte et al., 2002). However, we did not find membrane constituents such as the low-density lipoprotein receptor-related protein 1 or other PrPC-interacting proteins such as the Fyn-kinase to be present in size exclusion chromatography fractions with highest amyloid-β–PrPC binding properties arguing against the possibility that complexes with these proteins are responsible for the higher molecular mass of amyloid-β assemblies (De Felice et al., 2007; Rushworth et al., 2013).

Interestingly, under physiological conditions, a subset of PrPC is subject to constitutive proteolytic processing, termed α-cleavage, in the middle of its amino acid sequence (reviewed in Altmeppen et al., 2012). By releasing the N-terminal domain, α-cleavage not only destroys the neurotoxic domain of PrPC (Fig. 4A) but likely also prevents access of amyloid-β oligomers to PrPC and subsequent neurotoxic signalling. Moreover, it generates a soluble neuroprotective N-terminal PrPC fragment that has been shown to physically block amyloid-β oligomers (Guillot-Sestier et al., 2009, 2012; Altmeppen et al., 2013). We decided to generate the rPrPCΔN construct used in our interaction assay to mimic this physiological cleavage. Our findings obtained with Alzheimer’s disease brains confirm the selectivity of amyloid-β binding to the PrPC N-terminus thus supporting a protective role of α-cleavage and indicating a potential therapeutic option (reviewed in Biasini et al., 2012; Altmeppen et al., 2013).

In conclusion, we show here in a large patient cohort that binding of amyloid-β to the N-terminus of PrPC only occurs in Alzheimer’s disease brains and is neither influenced by expression levels of PrPC nor by the codon 129 polymorphism of PRNP. Interestingly, in contrast to previous in vitro studies where small oligomeric species showed prominent binding to PrPC, we found that in Alzheimer’s disease brains larger protein assemblies containing amyloid-β42 efficiently bound to PrPC. Our study clearly emphasizes the relevance of amyloid-β–PrPC binding in Alzheimer’s disease.

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Supplementary material

Supplementary material is available at Brain online.

References


