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 (PrPSc) initiating a series of events leading to synaptic degeneration. Composition of bound amyloid-β oligomers, binding regions within PrPSc, 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 PrPSc-amyloid-β interaction in patients with Alzheimer’s disease is limited regarding occurrence, binding regions in PrPSc, and size of bound amyloid-β oligomers. Here we employed a PrPSc-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-PrPSc interaction. PrPSc-amyloid-β binding always occurred in Alzheimer’s disease brains and was never detected in non-demented controls. Neither expression level of PrPSc nor known genetic modifiers of Alzheimer’s disease, such as the PrPSc codon 129 polymorphism, influenced this interaction. In Alzheimer’s disease brains, binding of amyloid-β to PrPSc occurred via the PrPSc N-terminus. For synthetic amyloid-β42, small oligomeric species showed prominent binding to PrPSc, whereas in Alzheimer’s disease brains larger protein assemblies containing amyloid-β42 bound efficiently to PrPSc. These data confirm Alzheimer’s disease specificity of binding of amyloid-β to PrPSc 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 PrPSc suggest that larger assemblies of amyloid-β or additional non-amyloid-β components may play a role in binding of amyloid-β42 to PrPSc in Alzheimer’s disease.

Keywords: prion protein; PrPSc; Alzheimer’s disease; amyloid-β oligomers; amyloid-β neurodegeneration

Abbreviations: APIA = amyloid-β PrP interaction assay; PrP = prion protein
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 allomorphs 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 (PrP⁰), 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, PrP⁰ 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 PrP⁰ 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 PrP⁰, 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-PrP⁰ 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 PrP⁰ is not exclusive and other sterically similar assemblies of β-sheet rich oligomers may bind to and signal through PrP⁰ (Resenberger et al., 2011).

Binding of synthetic amyloid-β₄₂ occurs via the PrP⁰ N-terminus with defined binding sites in PrP⁰ residues 23–27 and 95–110 (Chen et al., 2010; Zou et al., 2011; Fliharty et al., 2013; Younan et al., 2013). The majority of studies investigating amyloid-β₄₂-PrP⁰ 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 PrP⁰ in patient material. One study using Alzheimer’s disease brains and controls showed preferential binding of amyloid-β₄₂ to PrP⁰ 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 PrP⁰ using brains of patients with Alzheimer’s disease and non-demented subjects. We developed a PrP⁰-binding assay using site-directed immobilization of recombinant full-length and N-terminally truncated PrP⁰ to study binding of amyloid-β to PrP⁰ in our patient cohorts. We show that amyloid-β binding to PrP⁰ occurs only in Alzheimer’s disease. Moreover, this interaction is neither influenced by PrP⁰ 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 PrP⁰. 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 (Glazzer 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 Axiosver S100 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).
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 Î¼l) were collected separately for subsequent experiments. After ultra-centrifugation of S1 (100 000 g; 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 Î¼l 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 Î¼l 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 Î¼l 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 Î¼l of a SDS sample buffer containing β-mercaptoethanol. Samples were boiled at 95°C for 5 min

Table 1  Demographic and clinical data

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CERAD = Consortium to Establish a Registry for Alzheimer’s Disease; F = female; M = male.
before SDS-PAGE. Proteins were separated on Novex 10–20% Tris-tricine gradient gels (Invitrogen) and blotted for 70 min at 250 mA on an activated PVDF membrane. After blotting, the membrane was boiled for 3 min in TBS. Detection of immunoprecipitated amyloid-β was performed using a 1:2000 dilution of the amyloid-β specific 6E10 antibody (Covance).

**Handling and preparation of synthetic amyloid-β species**

Lyophilized synthetic amyloid-β peptides (amyloid-β1-38, amyloid-β1-40, amyloid-β1-42, and scrambled amyloid-β1-42) 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 × 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.

The amyloid-β peptide solution was used for experiments in which mainly monomeric amyloid-β1-42 was needed. For ageing/oligomerization of synthetic amyloid-β1-42 after the HFIP-treatment, dried peptide films were resuspended in distilled H2O + 0.1% NH4 (ammonium) to obtain a final concentration of 100 mM. Remaining synthetic amyloid-β1-42 aggregates were spun down (16000 g, 15 min at 4°C). After centrifugation, the supernatant of the peptide solution was used for experiments in which mainly monomeric forms of amyloid-β were needed. For ageing/oligomerization of synthetic amyloid-β1-42 the peptide solution was incubated for at least 12 h at room temperature.

Successful preparations of amyloid-β1-42 monomers and oligomers were tested on western blots or dot blots, using a 1:2000 dilution of the amyloid-β 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 at 4°C for 10 min, the supernatant (S1) was transferred into a fresh Eppendorf tube and an equal amount of 2% N-lauroyl sarcosine sodium salt (Sarkosyl; Fluka) in H2O was added. After incubation for 30 min on ice, the sample was centrifuged at 10000 g 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 10000 g 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-β PPI 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.
Amyloid-β and prion protein in Alzheimer’s disease

Directed immobilization of PrP<sup>C</sup> fusion proteins

The covalent and directed immobilization of PrP<sup>C</sup> fusion proteins on the HaloLink<sup>TM</sup> 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 1 × 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 PrP<sup>C</sup>, because of poor expression of the C-terminal PrP<sup>C</sup>-HaloTag<sup>TM</sup> 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 800g, the supernatant was discarded. Subsequent interaction assays were performed in 100 μl of binding buffer.

Binding of synthetic amyloid-β oligomers to immobilized PrP<sup>C</sup>

One hundred microlitres of binding buffer was added to the resin. Aged synthetic amyloid-β oligomers (100 nM) were added and incubated for 1 h 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<sup>TM</sup>). 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 PrP<sup>C</sup>

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, lyophilized in a Speed-Vac to a volume of 100 μl and applied to APIA as described above. To prove the specificity of the amyloid-β-<i>iPrP<sup>C</sup></i> binding, the interaction was blocked with 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 h on a rotator at room temperature before testing with APIA.

Coupling of Alexa488 reporter to PrP<sup>C</sup>

To detect the interaction with immobilized synthetic amyloid-β oligomers on a nitrocellulose membrane in dot blot experiments, the coupling of the PrP<sup>C</sup>-HaloTag<sup>®</sup> fusion protein with a fluorescent reporter molecule was required. Briefly, expression of <i>E. coli</i> Tuner<sup>TM</sup> bacterial cultures (transformed with PrP<sup>C</sup> or PrP<sup>C-ΔNT</sup>) were induced at OD<sub>595</sub> = 0.5 through addition of 1 mM IPTG into the LB-media. The bacterial cultures were incubated for 5 h on a shaker at 37°C. After sonication of the bacteria and harvesting of the protein, the PrP<sup>C</sup> fusion protein was incubated with the fluorescent AlexaFluor<sup>®</sup>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 h, 100 μl of a 1:50 dilution of the PrP<sup>C</sup> 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 PrP<sup>C</sup>-AlexaFluor<sup>®</sup>488 reporter molecule (excitation maximum 499 nm; emission maximum 518 nm) as a proof for interaction between PrP<sup>C</sup> 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 PrP<sup>C</sup>-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 in 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), LR1 with 8G1 (1:10,000; Abcam), Fyn1 with Fyn (1:1000; Cell Signalling), and the appropriate secondary antibody (1:2500; 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-β<sub>1-40</sub> and amyloid-β<sub>1-42</sub> ELISA

The amyloid-β<sub>1-40</sub> and amyloid-β<sub>1-42</sub> 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.

PrP<sup>C</sup> ELISA

To compare the PrP<sup>C</sup> levels of S2 and P2 in human brain tissue of Alzheimer’s disease and control samples, the PrP<sup>C</sup> 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 (***)

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<sup>C</sup> 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 amounts of deposited amyloid-β and plaque type morphometrically.

As expected patients with Alzheimer’s disease had significantly higher plaque loads than non-demented controls in the frontal cortex (Fig. 1A and B). We then grouped patients with Alzheimer’s disease into cohorts with predominantly neuritic, predominantly diffuse plaques or patients where both plaque types were equally frequent. This analysis showed equal distribution between the three cohorts (Fig. 1A and C).

Amyloid-β binds to PrP<sup>C</sup> 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<sup>C</sup> (Um et al., 2012). Published data suggest that amyloid-β binding to PrP<sup>C</sup> 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<sup>C</sup> in Alzheimer’s disease is a function of amyloid-β concentration
PrP<sup>C</sup> has been shown to preferentially bind to amyloid-β (Zou et al., 2011). We measured concentrations of amyloid-β and amyloid-β<sub>42</sub> in supernatants (S2) and pellet fractions (P2) of Alzheimer’s disease brains and controls using amyloid-β and amyloid-β<sub>42</sub> 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-β<sub>42</sub>, 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-β<sub>42</sub> (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<sup>C</sup>, 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). These data give an explanation for prominent binding of amyloid-β to PrP<sup>C</sup> in the insoluble (P2) fraction where differences between Alzheimer’s disease brains and controls were highest for amyloid-β<sub>42</sub>. In Alzheimer’s disease brains, levels of SDS-stable amyloid-β dimers, trimers or low molecular weight 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<sup>C</sup> 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-β<sub>40</sub> and amyloid-β<sub>42</sub> concentrations are comparable.

Determination of amyloid-β–PrP<sup>C</sup> binding efficiency using a novel prey-bait assay
Given that quantification of binding efficiency between amyloid-β and PrP<sup>C</sup> 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<sup>C</sup> binding. For this we coupled human recombinant full-length (rPrP<sup>C</sup>) or N-terminally truncated PrP<sup>C</sup> lacking amyloid-β binding domains (rPrP<sup>C</sup><sub>ΔN</sub>) to a resin (Fig. 4A and B) or to fluorescent reporters (Fig. 4D) using the Halotag<sup>TM</sup> technology.
C-terminal attachment of the HALOtag™ allowed for a directed immobilization of rPrP C and rPrPC/C1 without affecting amyloid-β-binding sites. The functionality of this new APIA was investigated using well defined preparations of synthetic oligomeric amyloid-β and human Alzheimer’s disease brain (Supplementary Fig. 2 and Fig. 4C). Binding of synthetic oligomeric amyloid-β to rPrP C but not to rPrPC/C1 confirmed N-terminal binding of oligomeric amyloid-β (Fig. 4C and E). In Alzheimer’s disease we could only observe binding of amyloid-β to rPrP C but not to rPrPC/C1 and thus demonstrate for the first time the necessity of the PrP N-terminus for binding of amyloid-β in diseased brains (Fig. 4C). Using serially diluted synthetic oligomeric amyloid-β immobilized on a membrane by dot blot and probed with fluorescently tagged rPrP C allowed for quantitative assessment of the interaction between PrP C and amyloid-β showing a linear correlation between PrP C-binding and concentrations of synthetic oligomeric amyloid-β_{42} (r² = 0.9385; Fig. 4D). Binding of synthetic oligomeric amyloid-β_{42} and amyloid-β oligomers found in patients with Alzheimer’s disease to rPrP C could be blocked by the oligomer-specific antibody A11, and neither amyloid-β_{38} nor amyloid-β_{40} or amyloid-β_{43} and scrambled amyloid-β_{42} were able to interact with our immobilized rPrP 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 rPrP C and amyloid-β oligomers significantly (Supplementary Fig. 2E; *P < 0.05, with relative densitometric intensity of 235700 ± 51950 for P2-rPrP C and 83520 ± 23200 for P2-rPrP C + A11).

**Figure 1** Histology, plaque load and classification of patient cohorts. Frontal cortices of Alzheimer’s disease (AD) and control brains were stained with haematoxylin and eosin (HE) and the amyloid-β specific 6E10 antibody. (A) Cortical plaque distribution is shown at ×5 and ×10 magnification with detailed plaque morphology shown at ×20 magnification. 6E10 staining shows examples of neuritic and diffuse amyloid-β plaques (scale bar = 50 mM). (B) Quantification of the plaque load of Alzheimer’s disease compared to control subjects as assessed by 6E10 immunostaining (**P < 0.001). (C) Distribution of patients with Alzheimer’s disease with predominately neuritic plaque pathology (n = 6), predominately diffuse plaque pathology (n = 5) or patients with mixed plaque pathology (n = 4).

**Amyloid-β binding to PrP C in Alzheimer’s disease occurs mainly in the detergent insoluble fraction**

Binding of amyloid-β to 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% ± 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% ± 3.31% of total bound amyloid-β for S1; 5.79% ± 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-β_{42} have shown that not all synthetic oligomeric amyloid-β_{42} species bind to PrPC, although the exact size of optimally binding synthetic oligomeric amyloid-β_{42} was not established (Lauren et al., 2009). Using size exclusion chromatography with well-defined synthetic oligomeric amyloid-β_{42} adjusted to equal protein (amyloid-β_{42} amounts, Fig. 6 and Supplementary Fig. 2C) and APIA on pooled size exclusion chromatography fractions, we specified sizes of optimally binding synthetic oligomeric amyloid-β_{42} to fractions ranging between 21 and 8 kDa, corresponding to pentamers to dimers of synthetic oligomeric amyloid-β_{42} (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–PrP<sup>C</sup> interaction is a function of amyloid-β load in the brain and not dependent on PrP<sup>C</sup> levels. ELISAs for amyloid-β<sub>40</sub> (Aβ40) and amyloid-β<sub>42</sub> (Aβ42) as well as for PrP<sup>C</sup> loads in the brain were applied to supernatant (S2) and pellet (P2) homogenate fractions of Alzheimer’s disease (AD) and control patients. (A) Amyloid-β<sub>40</sub> concentrations in S2 fractions showed no significant difference in Alzheimer’s disease compared to the control group, whereas in P2 fractions the amyloid-β<sub>40</sub> level was significantly elevated. (B) Amyloid-β<sub>42</sub> concentrations in S2 fractions showed no significant difference in Alzheimer’s disease compared to the control group, whereas in P2 fractions the amyloid-β<sub>42</sub> level was significantly elevated. (C) No difference in the PrP<sup>C</sup> loads could be observed between patients with Alzheimer’s disease and control subjects in S2 and P2 homogenate fractions (*<i>P < 0.01</i>, **<i>*P < 0.001</i>). (D) Scatter plots of amyloid-β<sub>42</sub> concentrations against PrP<sup>C</sup> loads of S2 and P2 fractions. There is no 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<sup>C</sup> 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<sup>C</sup> 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<sup>C</sup> (amino acid 23–27 and amino acid 95–110; bars and black arrows). rPrP<sup>C</sup> consists of full length PrP<sup>C</sup> 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<sup>C</sup>ΔN (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<sup>C</sup> 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<sub>42</sub> (sAβ<sub>42</sub>) were applied to APIA as described in (B). As negative control, rPrP<sup>C</sup>ΔN and beads without immobilized rPrP<sup>C</sup> were used. An interaction could only be detected by using rPrP<sup>C</sup> with Alzheimer’s disease homogenate and synthetic amyloid-β<sub>42</sub> peptides. The truncated form rPrP<sup>C</sup>ΔN 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<sup>C</sup> 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<sup>C</sup> 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-β<sub>42</sub> and implies that in Alzheimer’s disease, amyloid-β oligomers with the highest binding propensity to PrP<sup>C</sup> are not low but rather high molecular weight assemblies. It is possible that PrP<sup>C</sup>-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 1 (LRP1) or the Fyn kinase (Lauren et al., 2011; Fluharty et al., 2013). Thus we assessed if either LRP1 or Fyn kinase are present in size exclusion chromatography fractions with highest amyloid-β-PrP<sup>C</sup> 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<sup>C</sup> 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<sup>C</sup> 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<sup>C</sup> (Chen et al., 2010; Zou et al., 2011; Fluharty et al., 2013; Younan et al., 2013).

Binding of amyloid-β<sub>42</sub> to PrP<sup>C</sup> 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

**Figure 5** Amyloid-β-PrP<sup>C</sup> interaction is mainly found in P2 fractions of patients with Alzheimer’s disease. (A) Total homogenate (S1) and its resulting supernatant (S2) and pellet (P2), after ultracentrifugation at 100,000g, of four Alzheimer’s disease (AD) patients and two control cases (Ctrl) were tested using APIA. Samples were separated on 4–12% Bis-Tris gradient gels. After blotting, nitrocellulose membranes were tested with the rPrPC:AlexaFlour<sup>488</sup> reporter molecule (green circle). In this assay, amyloid-β oligomers were immobilized on a nitrocellulose membrane by using dot blot. The rPrP<sup>C</sup>−AlexaFlour<sup>488</sup> fusion protein was used as a fluorescent probe to detect amyloid-β oligomers on the membrane. (E) Proof of principle with the PrP<sup>C</sup>−AlexaFlour<sup>488</sup> reporter molecule. Synthetic, aged amyloid-β<sub>42</sub> oligomers were spotted onto a nitrocellulose membrane and incubated with the fluorescent rPrP<sup>C</sup>−AlexaFlour<sup>488</sup> or rPrP<sup>C</sup>−ΔN−AlexaFlour<sup>488</sup>. Significantly higher binding could be observed for rPrP<sup>C</sup>−AlexaFlour<sup>488</sup> when compared with rPrP<sup>C</sup>−ΔN−AlexaFlour<sup>488</sup> (**P < 0.001). (F) Concentration-dependent assay using PrP<sup>C</sup>−AlexaFlour<sup>488</sup> reporter molecule. Synthetic, aged amyloid-β<sub>42</sub> oligomers were spotted onto a nitrocellulose membrane in a concentration-dependent fashion, AlexaFlour<sup>488</sup> fluorescent signal decreased accordingly following a linear regression pattern (r<sup>2</sup> = 0.9385).
(i) significant binding of amyloid-β to PrP^C only occurs in Alzheimer’s disease; (ii) binding of amyloid-β aggregates to PrP^C is restricted to the N-terminus of PrP^C; (iii) optimal binding occurs in the insoluble fraction of amyloid-β; and (iv) neither expression levels of PrP^C nor the codon 129 polymorphism of PRNP influence this binding. Thus, our results are in agreement with studies using recombinant PrP^C and synthetic oligomeric amyloid-β_{42} 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_{42}-PrP^C binding was described. We also found optimal binding of amyloid-β_{42} to PrP^C in insoluble fractions (Chen et al., 2010). This contradicts...
studies where optimal binding was found in the soluble fractions of amyloid-β_{42} 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 PrP^C consistently occurs in Alzheimer’s disease brains via the PrP^C N-terminus and cannot be observed in non-demented controls.

Given that only amyloid-β_{12} levels are significantly different between Alzheimer’s disease cases and controls, binding may be a direct function of the amount of amyloid-β_{12} present in Alzheimer’s disease brains. Neither PrP^C expression nor known genetic modifiers such as the PrP^C codon 129 polymorphism influenced this binding.

To study amyloid-β–PrP^C binding, we established a prey-bait based assay (APIA), using human recombinant full-length or N-terminally truncated PrP^C 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 quantitatively in an in situ format.

Using APIA and synthetic oligomeric amyloid-β_{32}, we determined sizes of synthetic oligomeric amyloid-β_{32} with optimal binding to PrP^C 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-β_{32} and these species were shown to impair synaptic plasticity in a PrP^C-dependent fashion (Lauren et al., 2009; Larson et al., 2012; Um et al., 2012). In contrast to data with synthetic oligomeric amyloid-β_{32}, in patients with Alzheimer’s disease we found optimal binding to PrP^C in protein fractions ranging from 150 and 300 kDa in size with no detectable PrP^C-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-β_{32} bind to PrP^C. Our data would suggest 35–70mers, which is in line with recent studies showing binding of 20–100mers of amyloid-β to PrP^C (Freir et al., 2011; Younan et al., 2013). Interestingly, the study by Younan et al. (2013) shows that binding of amyloid-β_{32} to PrP^C 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 (Esparza 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-β_{32}–PrP^C complex (Noguchi et al., 2009). In support of this possibility, we found presence of PrP^C in higher molecular weight fractions eluting earlier than the predicted volume for monomeric PrP^C, indicating the possibility of presence of PrP^C-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 PrP^C-interacting proteins such as the Fyn-kinase to be present in size exclusion chromatography fractions with highest amyloid-β–PrP^C 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 PrP^C 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 PrP^C (Fig. 4A) but likely also prevents access of amyloid-β oligomers to PrP^C and subsequent neurotoxic signalling. Moreover, it generates a soluble neuroprotective N-terminal PrP^C 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 PrP^CΔN 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 PrP^C 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 PrP^C only occurs in Alzheimer’s disease brains and is neither influenced by expression levels of PrP^C nor by the codon 129 polymorphism of PRNP. Interestingly, in contrast to previous in vitro studies where small oligomeric species showed prominent binding to PrP^C, we found that in Alzheimer’s disease brains larger protein assemblies containing amyloid-β_{32} efficiently bound to PrP^C. Our study clearly emphasizes the relevance of amyloid-β–PrP^C binding in Alzheimer’s disease.

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Supplementary material

Supplementary material is available at Brain online.

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