Protofibrillar and fibrillar amyloid β-binding proteins in cerebrospinal fluid

M. Mahafuzur Rahman[a], Gunilla T. Westermark[b], Henrik Zetterberg[c,d,e,f], Torleif Härd[a] and Mats Sandgren[a*]

[a]Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala BioCenter, Box 7015, SE-750 07 Uppsala, Sweden

[b]Department of Medical Cell Biology, Uppsala University, Box 571, SE-751 23 Uppsala Sweden

[c]Department of Psychiatry and Neurochemistry, the Sahlgrenska Academy at University of Gothenburg, SE-431 80 Mölndal, Sweden

[d]Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, SE-431 80 Mölndal, Sweden

[e]Department of Neurodegenerative Disease, UCL Institute of Neurology, Queen Square, London WC1N 3BG, United Kingdom

[f]UK Dementia Research Institute at UCL, London WC1N 3BG, United Kingdom

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[*]Corresponding author: Mats Sandgren

Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU),

Uppsala BioCenter, Box 7015, SE-750 07 Uppsala, Sweden

Phone: +46 (0)18 4714592

ABSTRACT

Aggregation and deposition of misfolded amyloid β (Aβ) peptide in the brain is central to Alzheimer's disease (AD). Oligomeric, protofibrillar and fibrillar forms of Aβ are believed to be neurotoxic and cause neurodegeneration in AD, but the toxicity mechanisms are not well understood and may involve Aβ-interacting molecular partners. In a previous study, we identified potential $Aβ_{42}$ protofibrillar-binding proteins in serum and cerebrospinal fluid (CSF) using an engineered version of $Aβ_{42}$ ($Aβ_{42}$CC) that forms protofibrils, but not fibrils. Here we studied binding of proteins to $Aβ_{42}$ fibrils in AD and non-AD CSF and compared these with protofibrillar $Aβ_{42}$CC-binding partners. $Aβ_{42}$ fibrils sequestered 2.4-fold more proteins than $Aβ_{42}$CC protofibrils. Proteins with selective binding to fibrillar aggregates with low nanomolar affinity were identified. We also found that protofibrillar and fibrillar Aβ-binding proteins represent distinct functional categories. $Aβ_{42}$CC protofibrils triggered interactions with proteins involved in catalytic activities, like transferases and oxidoreductases, whilst $Aβ_{42}$ fibrils were more likely involved in binding to proteoglycans, growth factors and neuron-associated proteins, *e.g.*, neurexin-1, -2 and -3. Interestingly, 10 brain-enriched proteins were identified among the fibril-binding proteins, whilst protofibril-extracted proteins had more general expression patterns. Both types of Aβ aggregates bound several extracellular proteins. Additionally, we list a set of CSF proteins that might have potential to discriminate between AD and non-AD CSF samples. The results may be of relevance both for biomarker studies and for studies of Aβ-related toxicity mechanisms.

Keywords: Amyloid β, protofibrils, fibrils, Alzheimer's disease, biomolecular interaction and cerebrospinal fluid

INTRODUCTION

Protein misfolding is associated with a broad range of human diseases [1]. Alzheimer's disease (AD), the most common cause of dementia affecting more than 40 million individuals worldwide, is the most well-known protein misfolding disease [2]. Misfolded tau and amyloid β (Aβ) peptide accumulation within and around the nerve cells are the major pathological hallmarks of AD. Aβ peptides are proteolytic cleavage products of the amyloid precursor protein (AβPP). An imbalance between production and clearance of Aβ results in misfolding and the subsequent formation of morphological and conformational distinct species ranging from Aβ dimers to insoluble fibrils [3, 4]. Although much attention has been given to Aβ in the field of AD research, the exact roles of various structural assemblies of Aβ in AD pathogenesis remain to be elucidated. Aβ peptides may be present in both non-AD and AD brains, indicating that Aβ alone might not be sufficient to cause AD [5]. Today, an important hypothesis is that interaction of Aβ with certain molecular partners may contribute to the development of AD [5-7].

Both protofibrillar and fibrillar form of Aβ are neurotoxic (reviewed in [1]) and the toxicity might be due to interaction of Aβ with other proteins, including membrane proteins and intracellular and extracellular components [8-10]. Several proteins, *e.g.*, α-1 antichymotrypsin, apolipoprotein E and J, complement components, collagen, heparin sulfate proteoglycan and serum amyloid P, have been reported to colocalize with Aβ [8, 11-14], and may contribute to Aβ-related toxicity due to loss of function of the interacting proteins [15] or gain of toxic function of Aβ [16]. Moreover, interaction of Aβ with other proteins may activate tissue reactions of relevance to neurodegeneration [9], *e.g.*, microglial and astrocytic activation in the plaque-affected brain tissue [17]. Aβ-interacting partners may target Aβ for internalization into the cell or sequestration in the extracellular matrix, instead of clearance of

Aβ into the blood. Several studies have been conducted to explore Aβ-binding partners [11, 18] and some Aβ-binding partners alongside with other aggregation inhibitor compounds are also tested for their ability to modulate Aβ aggregation (reviewed in [8, 19]). However, our knowledge about which proteins in body fluids, *e.g.*, cerebrospinal fluid (CSF, the biofluid that is most similar to the brain interstitial fluid where Aβ aggregates), associate with Aβ is limited. Such knowledge would provide new potential molecules that may be targeted to prevent amyloid formation and its associated toxicity.

We have recently investigated the binding of serum and CSF proteins to Aβ protofibrils formed by an Aβ variant called $Aβ_{42}CC$ [20]. Protofibrils formed by wild-type Aβ peptide are unstable and propagate rapidly into mature fibrils [21]. Thus, the wild-type protofibril is not optimal in studies of protofibrillar interaction with human fluid proteins. Protofibrils formed by $Aβ_{42}CC$ variant are stable and do not convert into mature fibrils [22], and the protofibrils are indistinguishable in structure and cell toxicity from the protofibrillar aggregates generated by wild-type $Aβ_{42}$ [23, 24]. We have identified approximately 100 proteins in serum and CSF that bind to $Aβ_{42}CC$ protofibrils, including known Aβ-binding amyloid proteins, proteins involved in complement system and hemostasis, as well as in lipid transport and metabolism. The aims of this study were: to investigate which proteins in CSF associate to mature $Aβ_{42}$ fibrils and to examine if such proteins are different from those found to associate with $Aβ_{42}CC$ protofibrils; to analyze the molecular function and cellular location of fibrillar Aβ-targeted proteins and to explore if protein-binding is changed upon Aβ aggregation from protofibrils to fibrils.

MATERIALS AND METHODS

Cerebrospinal fluid samples

Samples were from patients who sought medical advice because of cognitive impairment. Patients were designated as AD or non-AD according to CSF biomarker levels that are >90% sensitive and specific for AD, as previously described [25]. Demographics are summarized in Table 1. The ethics committee at the University of Gothenburg approved the study.

Peptide production and aggregate formation

$A\beta_{42}$CC and $A\beta_{42}$ peptides were produced by co-expression with an Affibody molecule, and the purification was performed as described previously [22, 23, 26]. The peptides were separated from the Affibody by denaturation in 7 M guanidine hydrochloride (GdnHCl) followed by an immobilized metal affinity chromatography (IMAC) purification under denaturing condition.

$A\beta_{42}$CC protofibrils were obtained by dialysis of the peptide solution against 20 mM Na-phosphate, pH 7.4, 50 mM NaCl with 1 mM EDTA overnight followed by a second dialysis for 7 h in the same buffer without EDTA. The sample was heated to 60 °C for 10 min [23].

Wild-type $A\beta_{42}$ peptide was loaded onto a Superdex 75 16/600 column (GE Healthcare) equilibrated with 20 mM Na-phosphate, pH 10.5, 150 mM NaCl to change pH (from 8 to 10.5) as well as to confirm monomeric species. To produce fibrils, monomeric $A\beta_{42}$ was spun down at 17,000 ×g using Heraeus Pico 17 centrifuge (Thermo Scientific) for 10 minutes to pellet any existing insoluble aggregates. The supernatant was transferred to a new tube. Fibrils formation was induced by adjusting the pH of the alkaline (pH ~10.5)

solution to pH 7.4 (with 1 M HCl) in 20 mM Na-phosphate, 50 mM NaCl [21]. Fibrils (25 µM assay concentration) were allowed to form at 37 °C for 96 h without agitation [27].

Microscopy analysis

Twenty µL $A\beta_{42}$CC protofibril or $A\beta_{42}$ fibril solutions were applied onto formvar-coated copper grids and negatively stained with 2% uranyl acetate in 50% ethanol for 1 min. Air-dried samples were analyzed at 75 kV in a Hitachi 7100 transmission electron microscopy (TEM) and images were obtained with a Gatan 832 Orius SC1000.

Protein pull-down assay

The assay was performed as previously described [20]. Briefly, 100 µg ligands ($A\beta_{42}$CC protofibrils or $A\beta_{42}$ fibrils) were incubated with 5 mg Tosyl-activated Dynabeads M-280 beads (Invitrogen) in 0.1 M Na-phosphate pH 7.4 at 37 °C overnight for covalent binding. The beads were then incubated for 1 h in PBS buffer at pH 7.4 with 0.5% Tween-20 to block free binding sites. Beads incubated with 5 µg/mL glycine were used as control. $A\beta_{42}$CC protofibril and $A\beta_{42}$ fibril coupled beads (0.5 mg) and control beads (0.5 mg) were incubated with 200 µL CSF at 37 °C for 1 h. After incubation, beads were washed three times in PBS buffer at pH 7.4 with 0.1% Tween-20. Proteins bound to $A\beta_{42}$CC protofibrils, $A\beta_{42}$ fibrils or control beads were eluted in 62.5 mM Tris-HCl, 25% glycerol, 2% SDS by heating at 70 °C for 10 min.

Mass spectrometry analysis

The mass spectrometry analysis was carried out as described by Rahman *et al.* [20]. In brief, proteins were reduced in 45 mM dithiothreitol, alkylated in 100 mM iodoacetamide and in-solution (proteins bound to $A\beta_{42}$ fibrils) or in-gel (proteins bound to $A\beta_{42}$CC protofibrils) digested by 50 ng trypsin per µg of proteins. Thereafter, trypsinized peptides were desalted on a ZipTip C18 column, dried and resolved in 0.1% formic acid. The peptides

were separated in reversed-phase on a C18-column with a 60 minutes gradient and electro-sprayed on-line to a Q Exactive Plus mass spectrometer (Thermo Finnigan). Tandem mass spectrometry was performed applying higher energy collisional dissociation (HCD). Peptide database searches were performed using the Mascot algorithm towards human proteins in the SwissProt database (released Nov-2016).

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) analysis was performed on a Biacore X100 instrument (GE Healthcare). The $A\beta_{42}$CC protofibril and $A\beta_{42}$ fibril (30 µg/mL) were immobilized onto a CM5-sensor chip (GE Healthcare) as described previously [28]. A stable final immobilization level of ca. 3000 response unit (RU) was achieved.
Recombinant human proteins, agrin (cat. 6624-AG-050), dickkopf-related protein 3 (cat. 1118-DK-050), neurocan (cat. 6508-NC-050), osteopontin (cat. 1433-OP-CF) and SPARC-like protein (cat. 2728-SL-050), were purchased from R&D Systems, USA. Lyophilized proteins were dissolved in HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20, pH 7.4) buffer. The analytes were diluted to concentrations of 10 nM, 20 nM, 40 nM and 60 nM in HBS-EP and injected over the immobilized $A\beta_{42}$CC protofibrils and $A\beta_{42}$ fibrils surface for 180 s. The dissociation phase was monitored for 600 s in HBS-EP buffer. The analysis was implemented as a multiple cycle setup with a flow rate of 30 µL/min at 25 °C. The surface was regenerated after each injection of analyte with 15 mM NaOH which completely remove bound analyte without disturbing the surface [20, 28]. Collected SPR data was evaluated using the Biacore X100 Evaluation 2.0.1 software.

RESULTS

Characterization of Aβ aggregates

Fibrillar appearances of $A\beta_{42}$CC protofibrils and $A\beta_{42}$ fibrils were confirmed by TEM after negative staining of samples with 2% uranyl acetate. In accordance with typical protofibrils morphology, the $A\beta_{42}$CC protofibrils appeared as spherical shapes with an average diameter of 5 nm [22] (Fig. 1a). Also, the preparation contained some smooth curvature. The $A\beta_{42}$ fibrils appeared with an average diameter of 7-9 nm (Fig. 1b) as expected for amyloid fibrils [1, 29].

Binding of CSF proteins to $A\beta_{42}$ fibrils

Fibrils of $A\beta_{42}$ were ligated on Tosyl-activated Dynabeads M-280 and incubated with CSF samples. The complex was then washed several times to remove unbound proteins. Proteins bound to $A\beta_{42}$ fibrils were eluted and analyzed by LC-MS to identify which proteins that had been captured by $A\beta_{42}$ fibrils. Through the LC-MS analysis, we identified a total of 202 proteins that bound to $A\beta_{42}$ fibrils from the 11 CSF samples analyzed (Supplementary Table S1). The number of identified proteins in individual CSF samples ranged from 53 to 152. The number of identified proteins did not correlate with total proteins content of the individual samples or sex. However, a positive correlation between age and identified protein number was detected in samples from patients diagnosed with AD (Supplementary Table S2).

As controls, beads coated with glycine and incubated with CSF were used. We have previously tested a set of different controls, including tryptophan and non-disease related Sup35 nanofibers [20]. In the present investigation, we only used glycine as control. One non-AD and one AD sample were incubated with glycine coated beads and analyzed by LC-MS. As expected, very few proteins, 3 from the non-AD and 2 from AD patient sample, were found to bind to the control (Supplementary Table S1). Likewise, the peptide abundance

indices (PAI) [30] verify that the binding to control is much lower compared to binding to $A\beta_{42}$ fibrils. The PAI values of three proteins bound to control are 0.08, 0.08 (±0.04) and 0.09 (±0.03) for apoE, clusterin and serum albumin, respectively; while PAI values for these proteins bound to fibril are 0.8 (±0.07), 0.4 (±0.04) and 0.37 (±0.09).

Protofibrillar and fibrillar binding partners

One major objective with this study was to identify $A\beta_{42}$ fibril-binding proteins in CSF and compare these with $A\beta_{42}$CC protofibril-binding partners [20]. We tested the same set of samples (except one AD sample that was excluded due to limited amount), maintained the same conditions for capturing assay and LC-MS characterization as for the previous study. Additionally, to verify the accuracy of the experimental conditions, two CSF samples were tested for binding to $A\beta_{42}$CC protofibrils, and analyzed by LC-MS. The result was in agreement with previous analysis. Three new proteins were identified, and the total number of identified proteins was less compared to previous results, approximately 27 proteins were identified compared to 38 proteins in the earlier study. However, it cannot be ruled out that there was some technical variability in the LC-MS analyses carried out at the two different occasions. Furthermore, the $A\beta_{42}$ fibril-binding protein list was further corrected by subtracting proteins with molecular weight below 20 and above 250 kDa. The rational for this subtraction was, for identification of $A\beta_{42}$CC protofibril-binding proteins, the pull-down fraction was loaded on to an SDS-PAGE and proteins migrating between 20-250 kDa were recovered, digested and subjected to LC-MS analysis, while in this study the whole pull-down fraction was analyzed through LC-MS. The subtracted proteins are listed in Supplementary Table S3.

We found that $A\beta_{42}$ fibrils attract more proteins (ca. 2.4-fold) than $A\beta_{42}$CC protofibrils (Fig. 1c). Thus, binding of proteins to Aβ is enhanced upon aggregation from

protofibrils to fibrils. Proteins identified to bind to $A\beta_{42}$CC protofibrils and $A\beta_{42}$ fibrils shared some similarities, approximately 20% common proteins (Supplementary Table S4). However, the list of $A\beta_{42}$ fibril-binding proteins contained a substantial number of proteins, 66% CSF proteins, that did not bind to $A\beta_{42}$CC protofibrils. Hence, we have called these $A\beta_{42}$ fibril-specific proteins (Supplementary Table S5). Some of these proteins, including agrin, extracellular matrix protein-1, neurocan and SPARC-like protein 1 have been reported to bind to $A\beta_{42}$ fibrillar aggregates [10]. Interestingly, many of the $A\beta_{42}$ fibril-specific proteins, *e.g.*, amyloid-like protein 1, dickkopf-related protein 3, major prion protein, fibulin-5, and proSAAS were identified to bind to fibrils formed by non-disease related protein Sup35 [20], indicating that these proteins have more specificity toward fibrils than protofibrils. Furthermore, a number of amyloid-related proteins, including transthyretin and prion protein were identified to bind to fibrils which were not observed for $A\beta_{42}$CC protofibrils (Supplementary Table S5 and supplementary information S1 in reference [20]).

Validation of conformation-dependent binding

We used an SPR biosensor-based assay to further validate protein-binding to $A\beta_{42}$ aggregates, and also verify $A\beta_{42}$ conformation-dependent binding of CSF proteins. For more detailed binding studies, by SPR, we selected agrin, dickkopf-related protein 3, neurocan, osteopontin and SPARC-like protein 1, since they were all found in this study to bind to $A\beta_{42}$ fibrils but not to $A\beta_{42}$CC protofibrils, and they are also close associated to AD biology (discussed below). We also tested apolipoprotein E4 (apoE4) which was found to bind to both $A\beta_{42}$CC protofibrils and $A\beta_{42}$ fibrils, thus serving as positive control. For this experiment, we immobilized $A\beta_{42}$CC protofibrils or $A\beta_{42}$ fibrils on a Biacore CM5 sensor chip using standard amine coupling chemistry. Binding of human proteins to the immobilized surface was recorded. The SPR kinetics confirmed that all tested proteins, except osteopontin,

bound well to $A\beta_{42}$ fibrils immobilized surface, but did not bind to $A\beta_{42}$CC protofibrils immobilized surface, as expected (Fig. 2 and supplementary figure S1). Although it seemed that the neurocan showed some affinity to $A\beta_{42}$CC protofibrils at high concentration (60 nM, 5.7 RU, cyan line in PF surface in Fig. 2), binding kinetics on this data set could not be determined. The experiment was repeated with higher neurocan concentration (125 to 500 nM), but no significant improvement of binding kinetics was observed (data not shown). ApoE4 (positive control) was found to bind to both $A\beta_{42}$CC protofibrils and $A\beta_{42}$ fibrils (Fig. 2, bottom panel). Data from binding to $A\beta_{42}$ fibrils (all tested proteins) and to $A\beta_{42}$CC protofibrils (apo E4) fitted well to a heterogeneous ligand-binding model with global kinetics fitting but local maximum response [28]. The equilibrium dissociation constant (K_D) for binding to $A\beta_{42}$ fibrils was determined to be K_D = 3.5 nM for agrin, K_D = 26.2 nM for dickkopf-related protein 3, K_D = 11.7 nM for neurocan; and K_D = 6.2 nM for the SPARC-like protein 1. The positive control, apoE4, bound to $A\beta_{42}$CC protofibrils and $A\beta_{42}$ fibrils with a K_D of 5.7 nM and 0.3 nM, respectively. The association and dissociation rates and the equilibrium dissociation constant of all tested proteins are found in Supplementary Table S6.

Gene ontology annotation

Gene ontology (GO) analysis was performed to annotate and compare annotated categories of protofibril- and fibril-binding proteins. The annotation was performed using the PANTHER classification system (http://pantherdb.org/, database version 12.0, released 2017-07-10) [31].

Almost half of the proteins (46.6%) that were identified as protofibril binders were classified as proteins with catalytic activity, and about one-third of the proteins (30.9%) were categorized as protein with binding properties. In contrast, the major portion (49%) of the fibril-binding proteins were annotated as proteins with binding properties, and a quarter of

the proteins (24%) were classified as proteins with catalytic activity (Fig. 3a). The other notable difference was that 12.9% of the fibril-binding proteins were related to structural proteins, while this proportion was only 7.4% for protofibril-bound proteins. A small proportion (2%) of the fibril-binding proteins was annotated as being involved in signal transduction, but this functional group was not seen in protofibril-binding proteins.

As shown in Fig. 3b, more than half of the proteins identified to bind to both protofibrils and fibrils were annotated as extracellular region proteins (44.6%) and extracellular matrix proteins (11.9%). The brain extracellular components are annotated to be involved in networking or have a structural and functional role [32]. The other half of the identified proteins was annotated as macromolecular complex proteins (9.2%), plasma membrane (8.7%), organelle (11.5%) and intracellular associated proteins (12.6%). Notably, 2% of the fibril-binding proteins were annotated to be located in nerve synapses but the protofibril-binding proteins were not presented in the synapses.

Identification of brain-enriched proteins

The Human Protein Atlas (HPA) database [33] (database version 16) was utilized to search for brain-enriched proteins among the $A\beta_{42}$CC protofibril and $A\beta_{42}$ fibril binding proteins. The brain tissue-enriched proteins (n=415, at least five-fold higher mRNA levels in a particular tissue as compared to all other tissues) database was downloaded from the HPA website (https://www.proteinatlas.org). Based on HPA tissue-enriched proteins database, a total of 10 brain-enriched proteins were identified among the $A\beta_{42}$ fibril-binding proteins (Fig. 4a). On the contrary, the $A\beta_{42}$CC protofibril-binding proteins did not represent any proteins enriched in the brain. Interestingly, the brain-enriched proteins were found to be more abundant in AD compared to non-AD samples (Fig. 4b), according to peptide abundance indices [30].

Novel proteins

We identified several proteins that readily bound to $A\beta_{42}$ fibrils from at least 2 AD samples, but no proteins bound from non-AD samples, and vice versa. Proteins that were identified in a number of non-AD samples, and only in one or two AD samples were categorized as 'abundant in non-AD CSF' and the opposite identification pattern was categorized as 'abundant in AD CSF' (Fig. 5). Neurexins (including neurexin-1, neurexin-2, neurexin-2 beta and neurexin-3), glypican-1, plexin-B2 and glutamate receptor 4 were found to bind to $A\beta_{42}$ fibrils from AD samples only. There were also examples of proteins like calreticulin, neurofilament heavy polypeptide and protein AMBP that were only identified to bind to $A\beta_{42}$ fibrils from non-AD samples.

Agrin and decorin are extracellular matrix protein that belongs to the proteoglycan family. These proteins were found to be abundant in AD CSF (agrin was identified in 4 and decorin was identified in 5 out of 6 samples) than in non-AD CSF (agrin was identified in 1 and decorin was identified in 2 out of 5 samples). Another interesting protein in the AD abundant protein list was growth arrest-specific protein 6 (Gas6), this protein has neurotrophic and neuroinflammatory functions [34]. Neurexins are transmembrane proteins, expressed at the presynaptic side of the neuron. Neurexins seemed to be more abundant in AD than non-AD CSF (Fig. 5). However, proteins identified to readily bind to $A\beta_{42}$ fibrils from AD CSF were correlated with Aβ and Alzheimer's disease.

Of non-AD abundant proteins, calreticulin was identified to bind to fibrils from three (out of six) non-AD samples, and from none of five AD samples (Fig. 5). Complement-related proteins, *e.g.*, complement C1r subcomponent, complement factor H and complement factor H-related protein 1 were identified in at least five out of six non-AD samples, whilst these proteins were identified in only one or two (complement factor H was identified in two samples) out of five AD samples. Protein AMBP (alpha-1-microglobulin/bikunin, an

abundant serum glycoprotein) was identified in four non-AD samples but was absent in the AD samples, which corroborates earlier data [35].

DISCUSSION

A frequently discussed hypothesis is that the interaction of Aβ with certain molecular partners may contribute to the development of AD [5-7, 29]. In this work, we have identified and compared $A\beta_{42}$ fibril-binding proteins in CSF samples from AD vs. non-AD patients using a pull-down assay coupled to mass spectrometry. A couple of hundred proteins from CSF were identified to bind to $A\beta_{42}$ fibrils. Then, we compared $A\beta_{42}$ fibril-binding proteins with the protein bound to $A\beta_{42}$CC protofibrils (a mimic of wild-type protofibrils), which were recently identified by us [20]. Protein binding was further validated using an SPR-based biosensor assay. Several studies have been performed to identify Aβ-interacting partners in serum [11] and Aβ precursor protein (AβPP)-interacting partners in brain extract [18], and some of the proteins identified in our study have been reported to bind Aβ or AβPP previously, *e.g.*, agrin [36, 37], glypican-1 [38], apoE, apoJ, and serum amyloid P [11]. However, these studies were either performed in buffers or included only a few biologically relevant samples whereas our study was performed on many CSF samples. We choose to work with CSF since this body fluid is the most similar to the interstitial fluid where Aβ aggregates. Moreover, CSF offers an environment that is close to the brain environment thus the best body fluid to study brain proteins. Furthermore, CSF $A\beta_{42}$ level reflect the amyloid load in AD brain accurately [39]. Brain tissue extracts could potentially provide additional interaction partners to Aβ aggregates, which should be an interesting topic for future studies.

A substantial number of proteins was identified to bind to $A\beta_{42}$ fibrils in CSF samples, and the number of interaction partners is much larger (ca. 2.4-fold) compared to $A\beta_{42}$CC protofibril-binding proteins. One can think of several factors that could play critical role for binding more proteins to $A\beta_{42}$ fibrils and for the difference in protein binding profiles of $A\beta_{42}$ fibrils compared to $A\beta_{42}$CC protofibrils (see below). Such biophysical determinant

differences could be the surface charge potential, tertiary structure of the binding surface, surface modification such as N- or O-glycosylation of the target protein, or pure structural sterical differences between the bound proteins. However, a possible explanation for the greater number of proteins identified as fibril binders could be that the protein ligand could have access to more binding sites onto the long fibrils surface compared to protofibrils that are much smaller in length and might be more compactly oriented onto the Dynabeads. Likewise, the protofibril and fibril could represent completely different binding surfaces. Indeed, distinct sets of protein were identified for both types of aggregates which is in agreements with earlier data [10]. The structural differences between *in vitro* protofibril and fibril are well characterized. However, a recent study showed that fibrils isolated from two different AD brain are structurally and pathologically different [40], and such *in vivo* structurally different fibrils could potentially have distinct set of binding partners which might also reflect on disease progression. Furthermore, protofibrils and fibrils could potentially also recognize different molecular surface on closely related proteins or peptides. For instance, complement C1q subcomponent subunit A, B, and C were identified to bind to protofibril but not to fibril, while their associate complement C1r and C1s subcomponent did not bind to protofibril but fibril. A set of proteins is identified as specific for fibrils, and some of these have previously been found to bind to non-disease related Sup35 fibril [20], which has a similar structure to the Aβ fibril [41], suggesting that the distinct set of fibril-binding proteins may be due to conformation-specific interaction. Furthermore, our SPR data also suggests that the protein binding to Aβ is directed by the conformation of the Aβ aggregates. However, further structural studies of Aβ aggregates and other binding proteins is required to get insight into the structural basis of the binding.

The gene ontology annotation [31] of the protofibril- and fibril-binding proteins revealed that they form distinct functional classes. $A\beta_{42}$CC protofibrils trigger interaction

networks with enzymes (47% protofibril-binding proteins were annotated to possess catalytic activity), whereas $A\beta_{42}$ fibrils are more likely to bind proteins such as lipid, nucleic acid and calcium ion binding (49% fibril-binding proteins were annotated to binding activity). However, this picture might not be entirely accurate due to the notable differences between the number of protofibril- and fibril-binding partners (relatively small number of proteins was identified for protofibrils compared to fibrils). The GO terms cellular component analysis showed that more than half of the Aβ aggregate-binding proteins are extracellular region and matrix proteins. In the brain, the extracellular components play important roles in networking, structure, and function, and the distribution of extracellular component in the brain is region-dependent [32]. Moreover, the extracellular proteins have been reported to be more abundant in CSF compared to serum [42]. Thus, it is not unexpected that Aβ would bind to a large degree of extracellular proteins in CSF, which is frequently in communication with the extracellular space in the brain [35].

We explored differences in expression profiles of protofibrillar and fibrillar interaction partners in CSF. Ten brain-enriched proteins were identified as selective interaction partners to Aβ fibrils and some of them, *e.g.*, neurosecretory protein VGF, have previously been described as candidate biomarkers for AD [43].

Several proteins identified in our study may have potential for AD biomarkers. We found that some proteins were more prone to bind $A\beta_{42}$ fibrils from AD samples than from non-AD samples. Agrin, an extracellular matrix heparin sulphate proteoglycan expressed in neurons in different brain areas [36], is one such protein that was identified as particularly abundant in AD samples. The protein is often reported to be present in senile plaque and also reported to accelerate Aβ fibril formation [37]. Our finding resonates well with an earlier study showing increased levels of agrin in seven samples from AD patients compared with non-AD controls (n=12) [44]. Moreover, a recent study showed that agrin concentration

correlates with the age of the AD patient [45], a result that is also corroborated by our findings; we identified agrin in four samples, where the patient age was 59-75 years, whilst the age of the patient in which agrin was not found was 54 year. Furthermore, our kinetics data showed that agrin has high affinity (K_D= 0.3 nM) to $A\beta_{42}$ fibrils. Like agrin, decorin was also found abundant in AD CSF samples. Notably, both of these proteins belong to the proteoglycan family. Evidence suggests that decorin is colocalized with Aβ in a transgenic mice model of AD [46], and in brains of patients diagnosed with AD [47]. Neurexins were also found to be more abundant in CSF samples from AD patients than non-AD. They have been reported to bind AβPP, and more interestingly, processing of neurexin *e.g.*, neurexin-3β isoform is similar to AβPP processing by α- and γ-secretases [48]. Gas6 was also found abundant in AD samples. Recently, Sainaghi and co-workers [34] measured an increased Gas6 concentration in CSF samples from AD patients (n= 63) compared to samples from non-AD controls (n=67). They also suggested that upregulation of CSF Gas6 might be a defensive response against AD progression. Proteins that are more readily bind to $A\beta_{42}$ fibrils from non-AD CSF samples, thus abundant in non-AD CSF include calreticulin, complement factor H, and protein AMBP. Calreticulin is a major calcium-binding protein found in smooth muscle sarcoplasmic reticulum and non-muscle endoplasmic reticulum. The protein has been identified as an AβPP-interacting partner and binds to the γ-secretase cleavage site within AβPP which leads to reduced level $A\beta_{42}$ production in cell culture [49]. Moreover, reduced levels of calreticulin were measured in serum of patients with AD [50], and may thus be negatively correlated with AD, which potentially could help explain the abundance of calreticulin in non-AD samples.

Aβ is one of the major players in the pathogenesis of AD, but the pathways it activates to initiate neurodegeneration remain elusive. In this study, we present Aβ-binding protein partners in CSF from AD and non-AD patients. A comparison between protofibrillar

and fibrillar partners was also carried out. Our results demonstrate that protofibrillar and fibrillar Aβ interact with a broad range of CSF proteins, and that the binding profile is conformation-dependent since distinct protein sets were identified for each type of aggregate. The identified proteins also present distinct functionality when comparing protofibrillar and fibrillar Aβ-interacting partners. Taken together, our results pinpoint a number of Aβ-interacting partners that should be included in future studies on biomarkers as well as in studies addressing mechanisms associated with Aβ toxicity.

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Prof. Härd is a shareholder and serves on the Board of Directors of Alzinova AB, Gothenburg, Sweden. Prof. Zetterberg is a founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg, and has served at scientific advisory board panels for Roche Diagnostics, Eli Lilly and Wave.

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TABLE

Table 1. Demographic information of CSF samples.

	non-AD	AD
n	6	5
Age (mean ±SD)	71 (±7.1) [a]	63 (±9.6)
Gender M/F	1/3 [b]	2/3
CSF Tau (mean ±SD, ng/L)	263 (± 146)	789 (± 104)
CSF $A\beta_{1-42}$ (mean ±SD, ng/L)	809 (± 254)	468 (± 152)
CSF Phospho tau (mean ±SD, ng/L)	40 (± 18)	95 (± 12)

[a] Calculated from four samples
[b] Two samples without recorded gender

FIGURES

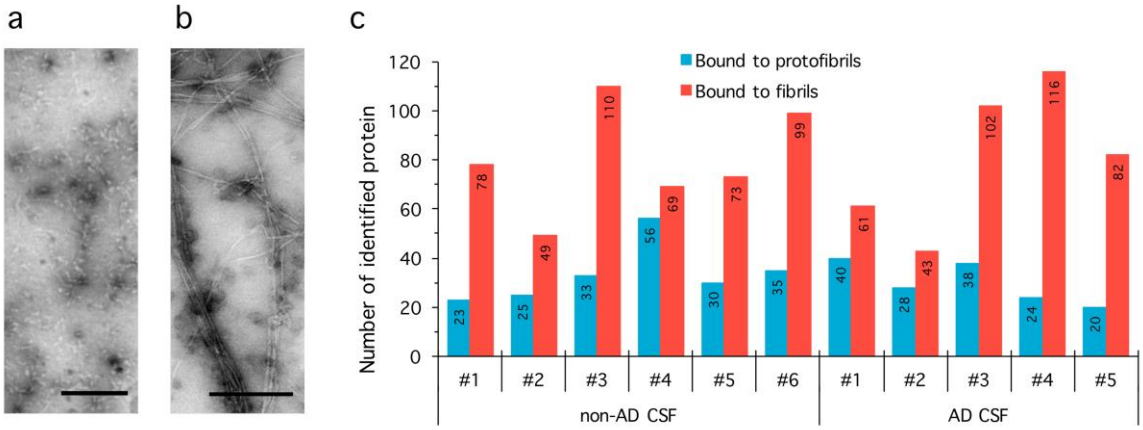

Fig. 1. TEM micrograph showing the assembly of $A\beta_{42}$CC protofibrils (**a**) and $A\beta_{42}$ fibrils (**b**). The scale bar is 200 nm. (**c**) A comparison of the number of proteins identified to bind to $A\beta_{42}$CC protofibrils with proteins identified to bind to $A\beta_{42}$ fibrils in individual CSF samples. More proteins are pulled down by $A\beta_{42}$ fibrils compared to $A\beta_{42}$CC protofibrils.

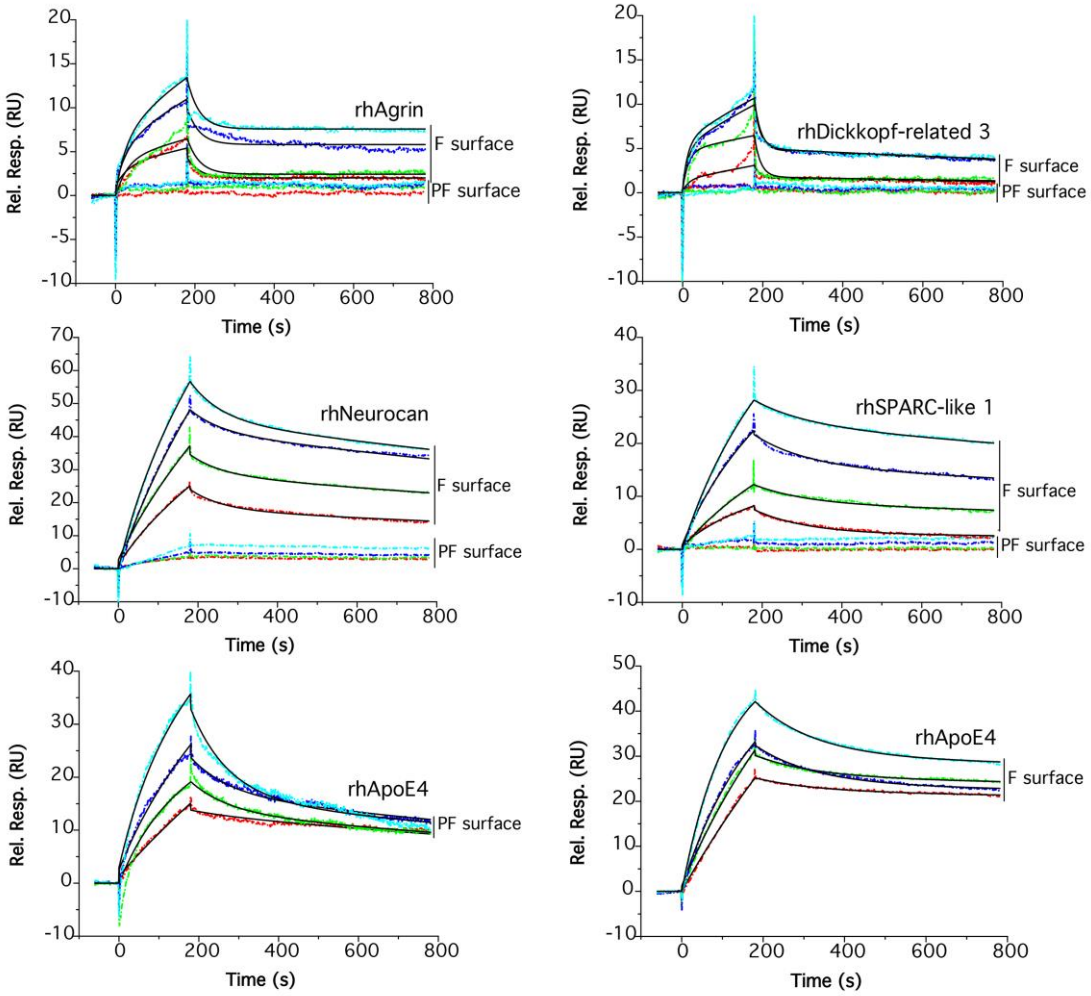

Fig. 2. Representative Biacore sensorgrams showing interactions of recombinant human proteins with $A\beta_{42}$ fibril- and $A\beta_{42}$CC protofibril-immobilized surfaces (F and PF surfaces, respectively). The protein concentrations used are 10 (red), 20 (green), 40 (blue) and 60 (cyan) nM, respectively. The dashed lines represent experimental data. Data collected from the interaction with the two surfaces were fitted to a heterogeneous ligand model. The fitted data are shown with solid black line. Three independent experiments were performed in each case.

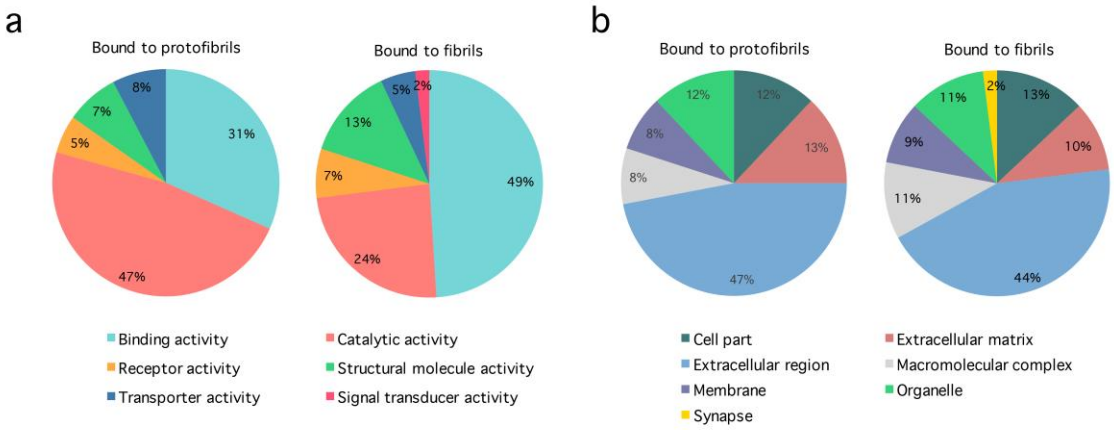

Fig. 3. Pie graphs representing the gene ontology terms molecular function (**a**) and cellular component (**b**) annotation of proteins bound to $A\beta_{42}$CC protofibrils and $A\beta_{42}$ fibrils in CSF.

a

Gene	Acc#	Protein name
APLP1	P51693	Amyloid-like protein 1
BCAN	Q96GW7	Brevican core protein
GRIA4	P48058	Glutamate receptor 4
NRXN1	Q9ULB1	Neurexin-1
NRXN2	Q9P2S2	Neurexin-2
NCAN	O14594	Neurocan core protein
NPTXR	O95502	Neuronal pentraxin receptor
NPTX1	Q15818	Neuronal pentraxin-1
VGF	O15240	Neurosecretory protein VGF
SPOCK3	Q8WXD2	Secretogranin-3

b

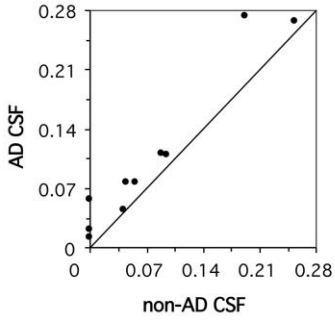

Fig. 4. (**a**) CSF proteins identified to bind to fibrils represent a set of brain-enriched proteins. (**b**) Peptide abundance indices of the brain-enriched proteins showed that the proteins are more abundant in AD compared to non-AD samples.

Protein name	Accession	non-AD CSF						AD CSF					non-AD (6)/ AD(5)
		#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	
Abundant in AD CSF													
Agrin	O00468						✔	✔		✔	✔	✔	1/4
Decorin	P07585			✔			✔	✔	✔	✔	✔	✔	2/5
Glutamate receptor 4	P48058									✔	✔		0/2
Glypican-1	P35052									✔	✔		0/2
Growth arrest-specific protein 6	Q14393	✔						✔		✔	✔		1/3
Neurexin-1	Q9ULB1									✔	✔		0/2
Neurexin-2	Q9P2S2									✔	✔		0/2
Neurexin-2-beta	P58401									✔	✔		0/2
Neurexin-3	Q9Y4C0								✔	✔	✔		0/3
Neuronal pentraxin-1	Q15818			✔		✔		✔		✔	✔	✔	2/4
Plexin-B2	O15031									✔	✔		0/2
Testican-3	Q9BQ16						✔		✔	✔	✔	✔	1/4
Abundant in non-AD CSF													
Apolipoprotein L1	O14791	✔		✔			✔			✔			3/1
Calreticulin	P27797	✔		✔			✔						3/0
Collagen alpha-1(I) chain	P02452	✔		✔	✔	✔	✔				✔	✔	5/2
Complement C1r subcomponent	P00736	✔	✔	✔	✔	✔					✔		5/1
Complement factor H	P08603	✔	✔	✔	✔	✔	✔				✔	✔	6/2
Complement factor H-related protein 1	Q03591	✔		✔	✔	✔	✔				✔		5/1
EGF-containing fibulin-like extracellular matrix protein 2	O95967		✔	✔	✔	✔				✔			4/1
Insulin-like growth factor-binding protein 2	P18065	✔	✔	✔	✔	✔	✔		✔		✔	✔	6/3
Insulin-like growth factor-binding protein 7	Q16270			✔	✔	✔	✔				✔	✔	4/2
Neurofilament heavy polypeptide	P12036	✔		✔			✔						3/0
Nucleobindin-1	Q02818			✔	✔	✔	✔				✔	✔	4/2
Protein AMBP	P02760	✔		✔		✔	✔						4/0

Fig. 5. A subset of CSF proteins that were found more prone to bind $A\beta_{42}$ fibrils either from AD or non-AD samples. Proteins that were identified in a number of AD CSF samples and only in one or in two non-AD CSF samples are shown as abundant in AD CSF and the opposite identification pattern are indicated as abundant in non-AD CSF.