Structure-specific amyloid precipitation in biofluids

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The composition of soluble toxic protein aggregates formed in vivo is currently unknown in neurodegenerative diseases, due to their ultra-low concentration in human biofluids and their high degree of heterogeneity. Here we report a method to capture amyloid-containing aggregates in human biofluids in an unbiased way, a process we name amyloid precipitation. We use a structure-specific chemical dimer, a Y-shaped, bio-inspired small molecule with two capture groups, for amyloid precipitation to increase affinity. Our capture molecule for amyloid precipitation (CAP-1) consists of a derivative of Pittsburgh Compound B (dimer) to target the cross β-sheets of amyloids and a biotin moiety for surface immobilization. By coupling CAP-1 to magnetic beads, we demonstrate that we can target the amyloid structure of all protein aggregates present in human cerebrospinal fluid, isolate them for analysis and then characterize them using single-molecule fluorescence imaging and mass spectrometry. Amyloid precipitation enables unbiased determination of the molecular composition and structural features of the in vivo aggregates formed in neurodegenerative diseases.

α-Synuclein, amyloid-β (Aβ) and tau are examples of proteins that self-aggregate in cross β-sheet motifs, and are present in Lewy bodies, amyloid plaques and tau tangles, respectively. These cross β-sheets (or amyloid structures) are found in the brains of people with neurodegenerative diseases such as Parkinson’s disease (PD) and Alzheimer’s disease (AD) 2–4. Importantly, brain extracts containing misfolded Aβ from patients with AD and preformed α-synuclein fibrils induced cerebral β-amyloidosis and α-synuclein propagation, respectively, and associated pathologies in mice 4,5. However, depletion of aggregates from an AD brain suppressed in vivo seeding capability 6, reinforcing the idea that the induction of pathophysiology is likely governed by the structure and concentration of the aggregate seeds 6,7. This highlights the importance of studying the aggregated protein as opposed to its monomeric counterpart.

The exact mechanism by which protein aggregates lead to progressive loss of neuronal cells and result in subsequent pathophysiological effects like dementia and movement disorders remains poorly understood. It is known that subtle differences in amino acid content result in major structural changes that have an impact on the pathophysiology of these diseases 8–16. Also, in vitro studies have revealed that protein aggregation is a dynamic process where a wide range of aggregates with variable sizes and hydrophilicities are formed, and that the aggregates become more toxic when they acquire a cross β-sheet structure 17,18. We have previously studied the aggregation of α-synuclein in detail using super-resolution imaging and single-molecule fluorescence 15–18. Aggregation proceeds by the formation of small soluble aggregates, which undergo slow structural conversions to small oligomeric species with increased β-sheet structure over 24 hours; these oligomeric species are cytoxic to cells 16,17. Aggregates with a β-sheet structure were also shown to be more effective at membrane permeabilization, leading to increased cell death 19. Other studies showed that thioflavin T (ThT)-active aggregates of α-synuclein interact with ATP synthase and increase the probability of the opening of the permeability transition pore of mitochondria, ultimately leading to cell death 20. Thus, ThT-active aggregates of α-synuclein are toxic to cells by a number of mechanisms. ThT-active aggregates are formed early in the aggregation reaction (from 1 hour onward) together with non-ThT-active species 15. Both species are spherically symmetric and smaller than ~50 nm in size, and they are clearly distinct from high-aspect-ratio and longer fibrils formed at later times (24 h), which also have a different surface hydrophobicity. The fibrils are also much more highly ordered than the ThT-active species that form initially, as measured using fluorescence anisotropy, providing further evidence for a structural conversion 18. Overall these experiments show that oligomeric non-fibrillar ThT-active species form early in the aggregation process, with distinct properties from fibrils, and are toxic to cells by a variety of mechanisms.

These oligomeric aggregates (<200 nm) of α-synuclein, as well aggregates of Aβ and tau, are implicated in cellular cytotoxicity 20–26.
Moreover, it was recently reported that small soluble Aβ aggregates induced extensive membrane permeability, while larger β-sheet-containing aggregates were most effective at causing an inflammatory response in microglia cells\(^5\). These findings were replicated in a recent study of the aggregates present in the cerebrospinal fluid (CSF) of patients at different stages of AD\(^7\). The aggregates in the CSF of mildly cognitively impaired patients induced more membrane permeabilization, while larger β-sheet aggregates present in the CSF of AD patients were more effective at inducing inflammation\(^7\). Together these studies reinforce the idea that aggregates of different size and structure trigger different toxic mechanisms and that the relative proportion of these different aggregates changes during the development of the disease.

It is now understood that AD develops before the manifestation of clinical symptoms, so it is important to develop new diagnostic methods in readily available biofluids such as blood, urine and CSF. In particular, CSF is one of the major clearance systems and provides an accessible biofluid that can be used to assess extracellular protein aggregates. However, the protein aggregates present in CSF are at very low (subpicomolar) concentrations and they are very heterogeneous in size\(^28,29\). These two factors have significantly hindered the development of suitable tools to isolate and study protein aggregates from human biofluids. New methods are needed to isolate and characterize the low levels of aggregates present in human biofluids in order to better understand how compositional and structural differences in these aggregates impact cellular toxicity and contribute to disease pathogenesis. This is a fundamental step towards the development of effective therapeutic strategies and for early diagnosis of disease.

Until recently, protein aggregates implicated in neurodegenerative diseases have largely been characterized using capture techniques based on antibodies or aptamers\(^34,35\). However, both antibody and aptamer capture strategies have a fundamental limitation that they only target aggregates of a selected protein, as well as having other problems such as epitope accessibility on misfolded proteins, inefficient targeting if the aggregated proteins contain post-translational modifications and that the relative proportion of these different aggregates impacts cell viability and contribute to disease pathogenesis. This is a fundamental step towards the development of effective therapeutic strategies and for early diagnosis of disease.

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The presence of fibrils (right panel) attached to the beads is visible by the 'hairy' appearance of the beads and highlighted in the magnified bead, and contrasts with the plain look of beads without protein (left panel). Despite the heterogeneous bead-to-fibril attachment—some beads contain many small fibrils, and other beads contain fewer but longer fibrils—there is a significant difference in the diameter (measured as the fluorescence intensity profile) between beads in the presence or absence of α-synuclein fibrils, 105 nm ($P=0.0002$), confirming the successful binding of fibrils to beads (Supplementary Fig. 17).

In Fig. 2d, we tested the efficacy of AP towards α-synuclein fibrils (purple) versus α-synuclein monomers (orange; Supplementary Fig. 18 for representative fluorescent spectra of both fractions). The difference between beads incubated with α-synuclein fibrils (100%, purple) and beads with α-synuclein monomers (33%, orange) highlights the absence of a cross β-sheet in the monomeric solution and corresponds to the fluorescence of CAP-1 alone. The fluorescence intensity for the samples, beads + CAP-1 + monomers (orange) and beads + CAP-1 + PBS (grey), is the same for both beads and supernatant, confirming that CAP-1 does not bind to monomers. The low fluorescence intensity detected for the supernatant of both samples, 11% for fibrils and 3% for monomers, reflects the presence of residual CAP-1 molecules released from the beads during the incubation and as expected is higher for the sample containing fibrils. Overall, the fluorescence increase between the supernatant (11%) and beads (100%) for the α-synuclein fibril sample demonstrates the successful pulling down (and concentration) of aggregates by the beads.

In both TIRFM (Fig. 2e) and bulk (Fig. 2d) measurements, detection of protein aggregates is based on CAP-1 intrinsic fluorescence, highlighting its ability to strongly bind (capture) aggregates.

Fig. 1 | Design and characterization of a bio-inspired structure-specific chemical dimer. a, The CAP-1 chemical structure. In purple are the amyloid binding regions, and in blue, the biotin used for surface attachment via streptavidin binding. b, Illustrative diagram highlighting the selective affinity of CAP-1 to cross β-sheets present in early stage aggregates and fibrils but not in monomers. c, TIRFM images of α-synuclein aggregation at 0 h, 8 h (red circles highlighting oligomers) and 24 h using 5 µM CAP-1 and 2.8 µM α-synuclein; $\lambda_{ex}=405$ nm. Insets show the areas in the white boxes. Scale bar = 5 µm; inset scale bar = 2 µm. d, Maximum fluorescence intensity increase of CAP-1 (20 µM) upon binding to 10 µM α-synuclein at different time points of the aggregation reaction using $\lambda_{ex}=355$ nm. Data are presented as the mean ± s.d. of $n=2$ independent experiments. One-way analysis of variance (ANOVA; $P=0.0006$) and Tukey’s post hoc comparisons (***$P<0.0007$ and not significant (NS) $P>0.05$). e, Binding affinity of CAP-1 and ThT to α-synuclein fibrils, increasing amounts of CAP-1 or ThT were added to 100 nM total monomer concentration of sonicated α-synuclein fibrils. The inset shows a zoomed-in view of the shaded area. Data are presented as the mean ± s.d. of $n=3$ or $n=2$ independent experiments for CAP-1 or ThT, respectively. The $K_d$ for ThT was obtained by fitting the experimental points to a hyperbolic curve (specific binding); $K_d$ (ThT) = 1,400 ± 132 nM. For CAP-1, a model for binding fluorescent ligands that takes account of the change in fluorescence between bound and unbound molecule was used; $K_d$ (CAP-1) = 14 ± 5 nM. For more details, see the Supplementary Methods.
and work as an optical read-out for the presence of β-sheets. We also used atomic force microscopy (AFM), an orthogonal non-optical technique, to confirm the successful binding of α-synuclein fibrils to CAP-1 beads (Supplementary Fig. 19). As shown in the three-dimensional (3D; height) image, fibrils localize preferentially close to the beads (Supplementary Fig. 19a), once more demonstrating the preference of protein aggregates to CAP-1-coated beads.

Until now, we have used mature α-synuclein fibrils (sonicated, 200 nm; non-sonicated, >1 μm) as a model of protein aggregation to test AP. However, in biological fluids such as CSF, the aggregates present are smaller. These ‘early stage’ soluble aggregates, or oligomers, have been shown to be much smaller than the optical diffraction limit (~250 nm)25 and differ in size, shape and structure from fibrils, as seen using higher resolution methods such as spectral point accumulation for imaging in nanoscale topography (sPAINT)15 and AFM27. For this reason, we used α-synuclein aggregates collected at the 8 hour time point to maximize the number of these oligomers25,26 and to validate the AP method for use in a biological context. We used electron microscopy (EM) to characterize aggregates present at 8 h (Supplementary Fig. 22) and confirmed their subdiffraction limit size (~30 nm). The results in Fig. 2c show the number of fluorescent puncta before and after AP: 6.0 × 10^4 μm^-2 and 3.6 × 10^4 μm^-2, respectively (Supplementary Fig. 21 for TIRFM images). In the presence of CAP-1, the number of protein aggregates in solution after pull-down is reduced to background levels (Fig. 2c, grey column; 4.2 × 10^4 ± 2.3 × 10^4 μm^-2). In the absence of CAP-1, there was partial removal of aggregates (Supplementary Fig. 21), suggesting unspecific binding to the beads, but this removal was negligible compared to the virtually complete depletion, 99.4%, in the presence of CAP-1. Overall, these experiments demonstrate that AP can be used to capture α-synuclein oligomers.

Next, we investigated the use of mass spectrometry (MS) to quantify the amount of α-synuclein enriched on the beads after pull-down, as MS will allow identification of molecular composition of the amyloids captured using AP. For this, we used high-resolution parallel reaction monitoring MS (PRM-MS). After AP, α-synuclein was eluted from the beads and digested using trypsin, converting full-length α-synuclein into small peptides, namely (α-synuclein peptide from residue 13 to residue 21) α-syn13-21, (α-synuclein peptide from residue 40 to residue 49) α-syn40-49, (α-synuclein peptide from residue 61 to residue 80) α-syn61-80, and (α-synuclein peptide from residue 81 to residue 96) α-syn81-96. In order to confirm the specificity of CAP-1, we compared the presence and absence of CAP-1 during the AP. In the presence of CAP-1, the amount of individual tryptic α-synuclein peptides recovered was 5 to 13 times higher, depending on the peptide, than without CAP-1 (Supplementary Fig. 23). This agrees with TIRFM results (Fig. 2c). The PRM-MS spectrum in Fig. 2f shows the relative abundance of the α-syn13-21 peptide fragment ion (y6) in the fragment ion spectrum in the presence (right) and absence (left) of CAP-1.

**AP followed by PRM-MS of α-synuclein spiked in human CSF.** AP is an unbiased method to capture amyloid protein from solution, allowing subsequent MS identification of proteins present in such aggregates45,46. As CSF is a complex biofluid made of more than two thousand different proteins47, we first determined the sensitivity of CAP-1 beads to capturing known amounts of α-synuclein spiked in CSF.

Increasing amounts of either purified α-synuclein monomers (t = 0 hours) or an α-synuclein mixture of monomers (>95%) and oligomers (<5% (ref. 16); t = 8 hours) were spiked in control CSF (Fig. 3a for the outline of the experiment and Supplementary Fig. 25 for TIRFM representative images). After AP, the beads were trypsin-digested and analysed by PRM-MS. In Fig. 3b the amount of α-syn13-21 peptide recovered as a function of the initial α-synuclein concentration spiked is shown. Naturally occurring α-synuclein oligomers present in CSF were undetectable (Supplementary Tables 3–8 for list of proteins pulled down). For concentrations equal to and below 1 nM, monomers were not detected, while in 1 nM of mixed species, 28 femtomoles (28 pM) of α-syn13-21, captured were detected (Supplementary Fig. 24 for other peptides). For α-synuclein concentrations higher than 1 nM, the increase in α-syn13-21 detected is linear and about three times higher for the mixed species sample than for the monomers (Fig. 3b). CAP-1 beads captured 0.6% of total α-synuclein monomers (orange) spiked in CSF and 2.3% of the total α-synuclein mixture (monomers >95% and oligomers <5%, purple). This means that almost no monomers in solution are captured, while approximately 50% (2.3% out of <5%) of the oligomers added to CSF are captured. For this reason, the threefold change in the total amount of α-syn13-21 recovered (Fig. 3b) represents a large difference in capture affinity between the monomer, which is present at high concentration, and the low concentration of aggregated α-synuclein. This result confirms the specificity of AP in capturing protein aggregates compared to the monomers in complex biofluids such as CSF.

The proteins captured using CAP-1 beads should be enriched in amyloid-prone proteins or contain proteins in the CSF that bind amyloid proteins37. Using PASTA 2.0 (ref. 48) and RFAmyloid49, two highly cited web servers for the prediction of protein aggregation from sequence, we observed an increase in the total number of amyloid-prone proteins when using CAP-1 compared to unmodified beads (Supplementary Table 9). As expected, in the presence of CAP-1, there is an increase in the total β-strand content of captured proteins (25–26%) compared to not using the capture molecule (19%), providing further computational evidence.

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**Fig. 2** | **AP using CAP-1.** a, Schematic of a magnetic bead (Dynabead) coated with streptavidin conjugated with CAP-1 via a biotin moiety. b, Outline of the AP method. Functionalized beads with CAP-1 are incubated with an amyloid-containing solution. After incubation, beads bound to protein aggregates are isolated using a magnetic separator. Both fractions, the depleted fraction (SN, supernatant) and enriched fraction (beads), can be analysed by bulk fluorescence and TIRFM. c, TIRFM of Dynabeads MyOne Streptavidin C1 beads conjugated with CAP-1 and in the presence (right panel) or absence (left panel) of 10 μM α-synuclein (α5) fibrils, using λem = 405 nm. α-Synuclein fibrils can be seen attached to the beads, creating a ‘hairy’ bead look (right panel inset), or, in other cases, having a single, long, thick spike. In the absence of protein aggregates, the beads have a plain look (left panel inset). Scale bars = 3 μm. d, Bulk fluorescence intensity (normalized) of beads (B) and supernatant (SN) after AP using 10 μM α-synuclein, with sonicated fibrils (5 days incubation), monomers and PBS only; λex = 355 nm and maximum λem = 440 nm. SN represents the supernatant or ‘depleted’ fraction, and B the ‘beads’ fraction. The horizontal green area highlights that the fluorescence of beads + CAP-1+ monomers is the same as beads + CAP-1 without protein, supporting the idea that CAP-1 does not bind to monomers; that is, the fluorescence measured is due to CAP-1 alone. Data are presented as the mean ± s.d. of n = 2 independent experiments (each value corresponds to mean of n = 3 replicates), and differences between groups were analysed using the unpaired two-tailed Student’s t-test; *P = 0.0345, **P = 0.0062 and ***P = 0.0004. e, Depletion of α-synuclein oligomers (time point 8 h of α-synuclein aggregation reaction) by AP and quantification of aggregates left in the supernatant (depleted fraction). Plotted is the fluorescent puncta counts (x10^5 μm^-2) for the sample before and after AP using TIRFM. AP captures approximately 100% of oligomers in solution. Data are presented as the mean ± s.d. of n = 27 fields of view per sample for one representative experiment (Supplementary Fig. 21 for TIRFM images). f, Schematic depicting AP followed by on-bead digestion. The lower panels show the α-syn13-21 peptide fragment ion (y6 to y8) PRM spectrum in the presence (right) and absence (left) of CAP-1, recovered after AP from a solution containing 1 nM total α-synuclein (<50 pM oligomers).
evidence of the ability of CAP-1 to select β-sheet-containing proteins (Supplementary Tables 1 and 2).

To evaluate the efficiency of AP in removing toxic amyloid species from CSF, we used a sensitive membrane permeability assay, developed previously (Fig. 3c for outline of the experiment). CSF is diluted in a solution containing Ca²⁺ ions and then added to liposomes containing a Ca²⁺-dependent dye. If CSF contains amyloids/oligomers that cause membrane permeability, Ca²⁺ ions enters the liposome, resulting in increased fluorescence. The increase in signal when the concentration of Ca²⁺ ions equals the bath concentration is determined at the end of the experiment by adding ionomycin. This corresponds to 100% Ca²⁺ ion entry and means that the measurement is quantitative with a scale from 0–100%. In Fig. 3d, the average Ca²⁺ influxes for CSF before AP (purple), CSF after AP (‘depleted’ fraction; white) and CSF after AP without CAP-1 (white with purple dots) using the same CSF as in Fig. 3b. AP removed most of the CSF proteins responsible for Ca²⁺ influx, reducing membrane permeability from 27% to 6%. There is some depletion in the absence of CAP-1 due to non-specific binding to the beads. Having established that AP is able to remove amyloid proteins from control CSF (Fig. 3b–d), we then decided to use CSF from PD patients in a separate set of experiments (Fig. 3e,f). TIRFM images showed a substantial decrease in the number of ThT-active proteins from control CSF (Fig. 3b–d), we then decided to use CSF from PD patients in a separate set of experiments (Fig. 3e,f). TIRFM images showed a substantial decrease in the number of ThT-active proteins from control CSF (Fig. 3b–d), we then decided to use CSF from PD patients in a separate set of experiments (Fig. 3e,f). TIRFM images showed a substantial decrease in the number of ThT-active proteins from control CSF (Fig. 3b–d), we then decided to use CSF from PD patients in a separate set of experiments (Fig. 3e,f).
**Fig. 3 | AP of CSF spiked with recombinant α-synuclein oligomers.** a, Schematic of the experiment. Known amounts of recombinant α-synuclein monomers or a mixture of oligomers plus monomers (<95%) are spiked into control CSF. b, Quantification of α-syn_{13–21} peptide recovered from on-bead digestion after AP using PRM-MS. Results were plotted as amount of α-syn_{13–21} peptide recovered in femtomoles (fmol) as a function of the initial α-synuclein concentration used for AP in picomolarity (pM), including both the α-synuclein monomers and the α-synuclein mixture (oligomers + monomers). Shaded area illustrates noise level and dashed box highlights the lowest concentration at which oligomers are detected. Data are presented as the mean ± s.d. of n = 3 independent experiments. c, Schematic of the membrane permeabilization essay. d, Average Ca^{2+} influx in control CSF (CSF used in b) before and after AP, and after AP in the absence of CAP-1. Data are presented as the mean ± s.d. of n > 10 fields of view per sample for one representative experiment. e, Average Ca^{2+} influx of PD CSF sample before and after AP in the presence of CAP-1, and after AP in the absence of CAP-1. Data are presented as the mean ± s.d. of n = 5 (CSF from five different patients), and each point corresponds to the mean of n > 10 fields of view per sample for one representative experiment. One-way ANOVA (P < 0.0001) and Tukey’s post hoc comparison (***, P < 0.0001, ****P = 0.0002 and NS P > 0.05). f, Example of TIRFM image of PD CSF sample before (left) and after (right) AP. λ_{ex} = 405 nm and ThT = 5 μM. Scale bars = 10 μm.

**Conclusions**

Protein aggregates have been known to be implicated in neurodegenerative diseases for more than three decades\(^5\). Yet, despite much progress, there are still important technological limitations in isolating and characterizing the intermediate small species that are formed during the development of disease. This is due to the low abundance, small size and heterogeneity in conformation and composition of the aggregates\(^5\).\(^6\).\(^7\).\(^8\).\(^9\).\(^10\). Traditional immunocapture or immune recognition approaches have improved in being able to target misfolded or aggregated proteins but are not capable of distinguishing between aggregates of different structures that may have very different properties and toxicities\(^5\).\(^6\).\(^7\).\(^8\).\(^9\).\(^10\). The goal of this work is to design an unbiased method to capture and characterize all the aggregates with a cross β-sheet structure that are present in human biofluids to determine their composition.

In this study, we presented the synthesis and characterization of a structure-specific chemical dimer designed to capture from solution the protein aggregates associated with neurodegeneration. This molecule has been specifically developed to bind and isolate a target molecule based on secondary structure (the presence of β-sheets) using chemical head groups that form the basis of PET ligands\(^5\).\(^6\).\(^7\). Previous studies have made use of dimerized ligands (protein/peptide\(^5\).\(^6\)) as a way to improve binding affinity to a particular target molecule. To the best of our knowledge, this is the first study to exploit the increased affinity of dimerized ligands in order to enable isolation/precipitation of the target species based on its structure rather than its protein composition.

The CAP-1 structure was successfully designed and then demonstrated to bind and isolate aggregates with amyloid structure (fibrils of Aβ_{40}, tau and α-synuclein), but crucially not monomers, using synthetic aggregates. Furthermore, the K_D values for CAP-1 binding Aβ_{40} and α-synuclein fibrils were comparable, which suggests that there will be no selective bias in capturing aggregates of different proteins. Importantly, we have also demonstrated successful AP of early aggregates of α-synuclein formed after 8 hours of aggregation, which are predominantly oligomers (Fig. 2c). Our data show that most of the oligomers are selectively removed by CAP-1 but not by the beads alone (Supplementary Fig. 21). This is strong evidence that CAP-1 captures oligomers of α-synuclein as well as fibrils.

However, it is also critical to demonstrate translational relevance. In vivo, the complexity of the biofluids that surround the central nervous system and the brain tissue itself makes the detection of small amyloids a major challenge\(^5\).\(^6\). We demonstrated that the sensitive detection of amyloid-containing aggregates can be performed...
in CSF by amyloid pull-down using CAP-1, followed by bead digestion and detection by MS. We observed an increased number of amyloid-prone proteins when using CAP-1 compared to plain beads and an increase in the total content of the β-strand, highlighting the strength of our AP method in enriching β-sheet-containing proteins (Supplementary Tables 1 and 2).

An important question to consider is whether CAP-1 will capture toxic aggregates. Our previous work showed that the oligomers formed after a structural conversion to a more protease-K-resistant structure are cytotoxic to neurons\(^1\), and the formation of β-sheet-active species has been shown to lead to increased membrane permeabilization\(^2\). These species were also shown to be ThT active\(^3\) and hence should be captured by CAP-1. We also previously showed that monomers of Aβ or α-synuclein cannot cause membrane permeabilization but aggregates can\(^4\), and hence it is likely that only protein aggregates (and not monomers) present in CSF cause membrane permeabilization. Since our experiments using CSF showed that CAP-1 captures the species responsible for membrane permeabilization and calcium ion entry, then, included in the species captured by CAP-1 should be the toxic aggregates present in CSF responsible for disrupting calcium ion homeostasis in vivo. This data therefore show that CAP-1 captures toxic aggregates from CSF. However, we cannot rule out that there will be non-ThT-active species that are also toxic and will not be captured by CAP-1, but these species appear not to be the dominant toxic species present in CSF as measured by the membrane permeabilization assay.

This new capability for unbiased detection and capture of amyloids, coupled with a MS approach to provide the molecular composition, is a powerful combination because it has the potential to define the amyloids present in the brain, CSF and other biofluids. The ability of disease-causing aggregates (containing β-sheets) to cause membrane permeabilization has been previously correlated with their cytotoxic potential.\(^5\). Importantly, in AP, the beads contain negligible monomers and, since it is possible to remove the captured aggregates from the beads, this approach allows further characterization of the human-derived aggregates and cytotoxicity experiments to be performed (Supplementary Fig. 20). Further improvements to the design of the capture molecule are possible by optimizing the linker length and the head groups and by synthesizing multimeric molecules to further improve the sensitivity and selectivity of AP.\(^6\). This may further increase the affinity of the dimer of CAP-1 over the monomeric version. There are also important advantages to using the AP method in terms of stability and resistance to degradation compared to conventional antibodies.

One particular advantage of this approach is the unbiased selection of amyloid conformations of proteins, regardless of their molecular identity. The appearance of misfolded and aggregated proteins is likely important in the early stages of disease. Developing complementary methods that do not require protein-specific approaches, but rather structural approaches, may identify the important biomarker in any disease. This is particularly true as the structural conformation is the biggest determinant of toxicity in disease, and therefore this approach may select for the pathogenic biomarker of disease.

AP may ultimately lead to early diagnostic tools. The capability to detect β-sheet aggregates present in an unbiased way will allow us to determine which protein aggregates change during the development of AD or PD and hence develop new diagnostic methods for early disease detection. Several studies show that aggregates of Aβ, α-synuclein and tau are present in human biological fluids such as the CSF and serum of patients affected with AD (Aβ and tau) and PD (α-synuclein)\(^10^\)–\(^22^\). Since the aggregation and deposition of cross-β-sheet-rich protein aggregates start in the central nervous system from 5 to 15 years before clinical manifestations of disease, detection of these β-sheet-rich aggregates holds the promise of developing a long-awaited diagnosis of AD and PD at the clinically asymptomatic stage, as well as predicting disease progression and monitoring the effects of potential drugs.

This method will also yield insights into disease mechanisms: it provides fundamental information about the role of protein aggregation in human disease, allowing one to study how protein homeostasis is disrupted in humans and whether this is due to the aggregation of a specific protein such as α-synuclein in PD, or the more general aggregation of several aggregation-prone proteins such as Aβ, tau, α-synuclein and TAR DNA binding protein 43 (TDP43). It is also not known how the relative amounts of these different aggregates will change during the development of different neurodegenerative diseases. In the latter case, differences in the relative amounts of these different proteins in biofluids would define the different diseases. AP may also reveal if protein aggregates play a fundamental role in human physiology, since they are present from a young age, or form only as a result of ageing. Addressing all these questions is only possible using an unbiased method that detects all β-sheet aggregates.

In conclusion, we have successfully developed a molecule inspired by the trimeric shape of an antibody to target all aggregates containing cross β-sheet motifs present in complex biofluids. CAP-1 has two binding sites to improve avidity and a third moiety to enable surface immobilization and therefore capture of aggregates based on their structure, but not on their protein composition. This simple and versatile method allows the identification of molecular components of aggregates, using MS. Overall, this structure-based approach will pave the way to understanding the exact molecular species responsible for neurodegeneration in humans and consequently hasten development of simple and robust early diagnosis methods.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41557-022-00976-3.

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Methods
This research complies with all relevant ethical regulations and was approved by the Ethics Committee at University of Gothenburg (EPN 140811).

Synthesis of the N-biotinylated bis-benzothiazole CAP-1. Bis-mesylate. To a solution of diol (1.47 g, 5.0 mmol, 1.0 equiv.) in anhydrous CH2Cl2 (10 ml) was added Et3N (2.53 ml, 18.0 mmol, 3.6 equiv.) at 0 °C. A solution of methanesulfonyl chloride (1.01 ml, 13.0 mmol, 2.6 equiv.) in CH2Cl2 (5 ml) was then added dropwise. The reaction was allowed to warm to room temperature and stirred for an additional 18 h. Aqueous hydrochloric acid (1 N, 100 ml) was added slowly and the reaction mixture extracted with CH2Cl2 (4 x 60 ml). The combined organic layers were washed with saturated sodium bicarbonate solution (50 ml) and brine (60 ml), dried over Na2SO4, and concentrated under reduced pressure to give the bis-mesylate A (1.57 g, 3.30 mmol, 70%) as a colourless oil. Boc-protected bis-benzothiazole. Sodium hydride (60% in oil, 19 mg, 0.44 mmol, 1.0 equiv.) was added; it was then heated to 80 °C for 18 h. After cooling to room temperature, H2O (15 ml) was added with vigorous stirring. The resulting precipitate was collected by filtration and washed with water (10 ml) and ethanol (10 ml) to give the Boc-protected bis-benzothiazole B (64 mg, 0.08 mmol, 40%) as a white solid. The melting point was 122–124 °C.

NHS-bis-benzothiazole. Boc-protected bis-benzothiazole B (64 mg 0.08 mmol, 1 equiv.) was added to a solution of HCl (4 M in MeOH, 3 ml) at room temperature. After 1 h, aqueous NaOH (3 M, 10 ml) and EtOAc (10 ml) were added and the layers separated. The aqueous layer was further extracted with EtOAc (2 × 10 ml), and the combined organic layers dried over Na2SO4 and concentrated to give the NHS bis-benzothiazole C (55 mg, 80 mmol, 99%) as a light yellow solid. The melting point was 168–170 °C.

N-Biotinylated bis-benzothiazole. To a solution of biotin (49 mg, 0.2 mmol, 1 equiv.) in anhydrous DMF (2 ml) was added i-Pr2NEt (452 l, 0.26 mmol, 1.3 equiv.) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 153 mg, 0.26 mmol, 1.3 equiv). After 30 min, the amine C (140 mg, 0.2 mmol, 1 equiv.) and i-Pr2NEt (350 l, 1.0 equiv.) in anhydrous DMF (1 ml) were added dropwise. After 18 h, saturated NH4Cl (10 ml) was added and the mixture extracted with EtOAc (3 x 5 ml). The combined organic layers were washed with brine (10 ml), dried over Na2SO4, and concentrated under reduced pressure. The residue was purified by column chromatography (SiO2; methanol/CH2Cl2, 1:8) to give the N-biotinylated bis-benzothiazole CAP-1, or D (56 mg, 0.06 mmol, 30%) as a light yellow solid. The melting point was 120–122 °C.

Aggregation of α-synuclein. Monomeric wild-type α-synuclein was purified from Echschitrich coli as previously described. Prior to use, α-synuclein aliquots were ultracentrifuged at 350,000 x g for 1 h at 4 °C using a TL120.2 rotor (Beckman) in an Optima TLX Ultracentrifuge (Beckman) to remove possible seed contaminants. Two-thirds of the total volume in the tube was used as the supernatant fraction (mononers only) and removed with minimal perturbation to avoid reaggregation. Sonicated α-synuclein-1-y1- etyrypyrrolidinophosphonium hexafluorophosphate (PyBOP; 135 mg, 0.26 mmol, 1.3 equiv). After 30 min, the amine C (140 mg, 0.2 mmol, 1 equiv.) and i-Pr2NEt (350 l, 1.0 equiv.) in anhydrous DMF (1 ml) were added dropwise. After 18 h, saturated NH4Cl (10 ml) was added and the mixture extracted with EtOAc (3 x 5 ml). The combined organic layers were washed with brine (10 ml), dried over Na2SO4, and concentrated under reduced pressure. The residue was purified by column chromatography (SiO2; methanol/CH2Cl2, 1:8) to give the N-biotinylated bis-benzothiazole CAP-1, or D (56 mg, 0.06 mmol, 30%) as a light yellow solid. The melting point was 120–122 °C.

Preparation and photophysical characterization of CAP-1. A CAP-1 1 mM stock solution was prepared in DMSO solvent, divided into 20 μl aliquots and stored at 20 °C for 2–3 min. The aliquots were used once to terminate and thaw cycles. The photophysical properties of CAP-1 were determined using a Varian Cary Eclipse fluorescence spectrophotometer. Experimental settings used were λem = 355 nm (5–10 nm bandwidth) and λex = 370–600 nm (10–10 nm bandwidth). Ultraviolet-visible absorption and fluorescence (both excitation and emission) spectral characterization of CAP-1 (20 μM) were carried out in both PBS and Tris (25 mM) supplemented with NaCl (100 mM, pH 7.4). To test the CAP-1 solubility in PBS (CAP-1tiss), different dilutions were prepared between 0 mM and 200 mM, and the emission spectrum was recorded using λem = 355 nm. Data points were plotted as [CAP-1]tiss versus maximum fluorescence intensity. The linear relationship between concentration and fluorescence intensity (R2 = 0.98; R, coefficient of multiple correlation) strongly suggests that CAP-1 obeys the Beer–Lambert law and is therefore completely soluble in the range used.

Detection of bead-bound α-synuclein. After AP, both ‘beads’ (diluted 1:32 in PBS) and the ‘depleted’ fraction were added to a 96-well flat-bottom plate with a clear bottom (Corning 3881) for the bulk fluorescence measurement. The plate was placed in a plate reader (CLARIOstar, BMG Labtech) and the fluorescence intensity (bottom reading) was measured straight away at room temperature using the following settings: end-point mode, excitation filter 440 nm and bandwidth
10 nm, emission filter 480 nm and bandwidth 10 nm; or spectrum mode, excitation at 355 nm and emission from 380 to 600 nm.

Preparation of slides for single-molecule measurements. Borosilicate glass coverslips (20 × 20 mm; VWR International, product number 631-0122) were cleaned using an argon plasma cleaner (PDC-002, Harrick Plasma) for 1 h to remove impurities and contaminants and create a hydrophilic surface. Frame-seal slide chambers (9 × 9 mm²; Biorad, product number SLF-0601) were affixed to the glass, and 50 µl of poly-L-lysine (molecular weight, 70,000–150,000; Sigma-Aldrich, product number P4707-50 ML) was added to the coverslip on the inside of the chamber and incubated for 30 min before being washed with filtered PBS buffer (Whatman Anatop 25; 0.02 µm). Each batch of coverslips was tested for fluorescent artefacts (that is, false positives) by imaging 5 µM ThT. ThT stock solution was prepared as described elsewhere. ThT working solution (50–100 µM) was filtered (Whatman Anatop 25) prior to use; and concentration was determined using ε 1242 = 36,000 M⁻¹ cm⁻¹.

TIRFM imaging. Imaging was performed using a home-built TIRFM microscope as reported previously. Briefly, this imaging mode restricts the detectable axial fluorescence to ~200 nm. Beads were conjugated to a glass cover-slip interface. For imaging of recombinant α-synuclein or CSF in the presence of ThT or CAP-1, the output from a laser operating at 405 nm (Oxxius Laserboxx, product number LBX-405-100-CIR-PP) was aligned and directed parallel to the optical axis at the edge of a x60 Plan Apo TIRF (Nikon Corporation; numerical aperture, 1.45; oil objective) mounted on an Eclipse E600-U microscope (Nikon Corporation) with a Perfect Focus unit. Fluorescence was collected by the same objective and was separated from the returning total internal reflection beam by a dichroic mirror (Semrock, D01R-405/488/561/635) and passed through an appropriate filter (Semrock, FF01-480/40-25 or FF01-434/17-25 for ThT or CAP-1, respectively). The images were recorded on an electron-multiplying charge-coupled device camera (Evolve 512, Photometrics) operated in frame-transfer mode (electron-multiplying gain of 6.5 electrons per analog-to-digital unit and 250 analog-to-digital units per photon). Each pixel was 241 nm in size. For each dataset, 4 × 4 image grids were measured in at least three different regions of the coverslip. The distance between the nine images measured in each grid was set to 350 µm and was automated (bean-shell script, Micromanager) to prevent user bias. Images were recorded at 50 ms exposure time for 100 frames with 405 nm illumination (150–200 W cm⁻²).

Recombinant α-synuclein and CSF were diluted in filtered PBS (Whatman Anatop 25; 0.02 µm) and mixed with ThT or CAP-1 for a final imaging volume of 50 µl. The ThT and CAP-1 imaging concentrations were 5 µM, while the α-synuclein concentration changed between experiments (1 µM for sonicated fibrils, 2.8 µM for control fibrils), with a minimum concentration of 1 µM. Fluorescence was collected by the same objective and was separated from the returning total internal reflection beam by a dichroic mirror (Semrock, D01R-405/488/561/635) and passed through an appropriate filter (Semrock, FF01-480/40-25 or FF01-434/17-25 for ThT or CAP-1, respectively). The images were recorded on an electron-multiplying charge-coupled device camera (Evolve 512, Photometrics) operated in frame-transfer mode (electron-multiplying gain of 6.5 electrons per analog-to-digital unit and 250 analog-to-digital units per photon). Each pixel was 241 nm in size. For each dataset, 4 × 4 image grids were measured in at least three different regions of the coverslip. The distance between the nine images measured in each grid was set to 350 µm and was automated (bean-shell script, Micromanager) to prevent user bias. Images were recorded at 50 ms exposure time for 100 frames with 405 nm illumination (150–200 W cm⁻²).

AP of α-synuclein spiked in CSF followed by on-bead digestion. The CSF sample aliquots used were de-identified leftover aliquots from clinical routine analyses, following a procedure approved by the Ethics Committee at University of Gothenburg (EPN 612). AP was carried out as described above, except that 50 µl of beads per sample were used instead of 30 µl. After conjugation with CAP-1 and washing, the beads were resuspended in a solution containing 600 µl CSF and 400 µl PBS. α-Synuclein was spiked into the CSF, adding either monomer alone or a mixture containing monomers and oligomers (previously characterized using TIRFM). Concentrations spiked were 1 µM, 100 µM, 1 nM, 10 nM and 100 nM, and samples were prepared in triplicate. The in-house precipitation method for CSF samples was performed according to Bhattacharjee et al. (2019) with minor modifications. Briefly, after overnight incubation at 4 °C, the KingFisher magnetic particle processor (Thermo Fisher Scientific) was used to wash and resuspend the beads. The beads were first extracted, washed two times with PBS, washed one time with 1 ml of 50 mM ammonium bicarbonate (NH₄HCO₃, pH 8.0; Sigma-Aldrich) and finally resuspended in 100 µl of 50 mM NH₄HCO₃ for on-bead digestion. For on-bead digestion, 10 µl of 100 nM 1,4-dithiothreitol in NH₄HCO₃, was added to the solution, vortexed and incubated for 30 min at 60 °C, and then cooled to room temperature for 15 min. Afterward, 10 µl of 10 mM iodoacetamide in NH₄HCO₃, was added, vortexed and incubated for 30 min at 25 °C in darkness. Finally, 10 µl trypsin (5 ng/µl in NH₄HCO₃), was added, vortexed and incubated at 37 °C overnight with shaking at 400 r.p.m. The reaction was stopped by the addition of 10 µl 10% formic acid. Finally, samples were centrifuged at 16,910 g for 10 min at 4 °C, and the supernatant was collected in a different vial. The magnetic beads were washed with 50 µl NH₄HCO₃, and then centrifuged again, and the supernatant was collected in the same vial as before. The collected supernatants were dried by SpeedVac.

Liquid chromatography with tandem MS of α-synuclein. High-resolution PRM analyses were performed on a quadrupole–orbitrap mass spectrometer (Q-Exactive, Thermo Fisher Scientific) coupled to an Ultimate 3000 chromatography system (Thermo Fisher Scientific). Mobile phases were 0.1% aqueous formic acid (v/v; A) and 0.1% formic acid in 84% acetonitrile (ACN) in water (v/v; B). The mixture of heavy-isotope-labelled peptide standards of α-synuclein (Heavy Peptide FasTrack 1 standards, Thermo Fisher Scientific) were spiked in 20% ACN formic acid (follow 10 µl of 100 nM 1,4-dithiothreitol in ACN, was added to a solution of 100 nM α-synuclein, α-synuclein, α-synuclein, and α-synuclein (10 pmol µl⁻¹ each). Then the dried samples after pull-down and on-bead digestion were dissolved in 20 µl of a mixture of heavy-isotope-labelled peptides for 1 h and then transferred to liquid chromatography vials for analysis. Samples were loaded directly onto a HephsersGold-518 column (length, 100 mm; inner diameter, 2 mm; particle size, 1.9 µm; Thermo Fisher Scientific) with 0.1% aqueous formic acid at 300 µl min⁻¹. After 2 min of loading, the peptides were eluted off the column using the following linear gradient steps: 0 min, 0% B; 4 min, 17% B; 16 min, 35% B; 17.5 min, 100% B; 20 min, 0% B. The global MS parameters were as follows: positive ion mode, spray voltage, 3.5 kV; vaporizer temperature, +350 °C; sheath gas pressure, 40 psi; auxiliary gas pressure, 25 psi; capillary temperature, +350 °C; and collision gas pressure, 1.9 mtorr. The instrument was set to acquire scheduled pairs or triplets of PRM scans and subsequent all-ion fragmentation scans, allowing simultaneous detection of both the α-synuclein peptide and the corresponding peptide standard.

Membrane permeability assay. Details of the membrane permeability assay method have been described previously. Studies have shown that a single-vesicle assay can be used to measure the toxicity of the β-sheet-rich protein aggregates present in CSF²⁹ or a complex biological mixture³⁰. See the Supplementary Information for details.

Data analysis. Microscopy images were analysed using Imagemag and Matlab. GraphPad Prism 9 was used for statistical analysis, plotting and curve fitting. Statistical analysis was performed using the unpaired two-tailed Student’s t-test to analyse differences between two groups, or a one-way ANOVA and Tukey’s post hoc comparison to analyse differences among three or more groups. Differences were considered to be statistically significant if P < 0.05. To determine the number of fluorescent puncta in each image, an average of the entire stack was generated and used to detect each protein aggregate using the Find Maxima function in ImageJ (with a threshold value of 180, Fig. 2c). To compare proteins pulled down from the control (A) and PD (B) CSF in the presence (1) and absence (2) of CAP-1, we used a custom Matlab code. From the original database search (Swiss-Prot), we identified the most enriched new dataset (A1, A2, B1, B2). Following two criteria: proteins were present in at least three replicates and had at least two unique peptides. Then we compared list A1 with A2 and B1 with B2. Supplementary Table 3 shows the results. A list of common proteins is in Supplementary Table 3 (A1, A2) and Supplementary Table 6 (B1, B2), while a list of exclusive proteins is in Supplementary Table 4 (A1, A2) and Supplementary Table 5 (A2, Supplementary Table 7 (B1 and Supplementary Table 8 (B2). Finally, we used PASTA 2.0 (ref. 32), Supplementary Tables 1–8 and RAFAmyloid²⁴ (Supplementary Table 9), two bioinformatic tools, to predict the percentage of α-helix and of β-strands, and the amyloid formation, respectively, from the protein sequence analysis.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data supporting the findings of this study are available within the paper and its Supplementary Information. The data are also available from the corresponding

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authors on reasonable request. We used the Swiss-Prot database to identify the proteins present in the MS samples. Source data are provided with this paper.

**Code availability**
The custom Matlab code used for analysis of the proteins is available on GitHub: https://github.com/TheLeeLab/Nature-Chemisty-2022-Structure-specific-amyloid-precipitation-in-biofluids.

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**Author contributions**
S.F.L. and T.N.S. designed the CAP-1, and D.T.D. and C.M.P. synthesized the CAP-1. M.R. performed all the experiments with CAP-1 alongside with J.A.V. for the imaging experiments and analysis, I.B. for the pull-down essay, D.E. for the binding affinity experiments and J.E.L. for the bead imaging. A.P. and A.R.C. helped with imaging analysis, and K.K. prepared the Aβ42 and tau aggregates. S.D. performed the liposome assays, and F.S.R. performed the AFM measurements. H.Z. directed the MS studies, and M.R., P.B. and A.B. designed and performed the experiments and data analysis. S.F.L., S.G. and D.K. directed the research. M.R. wrote the first manuscript of the paper, and all authors contributed to the discussion and final manuscript.

**Competing interests**
H.Z. has served at scientific advisory boards for Roche Diagnostics, Wave, Samumed and CogRx; has given lectures in symposia sponsored by Alzecure and Biogen; and is a cofounder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg (outside of the submitted work). The remaining authors declare no competing interests.

**Additional information**
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Correspondence and requests for materials should be addressed to T. N. Snaddon, S. Gandhi, S. F. Lee or D. Klenerman.
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☐  For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐  For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐  Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Micromanager 1.3, Cary Eclipse Fluorescence Spectrophotometer (Agilent), plate reader CLARIOstar (BMG Labtech), Quadrupole–orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific) coupled to an Ultimate 3000 chromatography system (Thermo Fisher Scientific) and JPK nanowizard2 system (AFM), TESCAN MIRA3 FEG-SEM (SEM), Duetsa Spectrofluorometer (Horiba)

Data analysis

ImageJ 1.52, Matlab 2016b, GraphPad Prism 9, Xcalibur software version 2.2 SP1.48 (Thermo Fisher Scientific), Pinpoint 1.3.0 (ThermoFisher Scientific), PASTA 2.0 and RAmyloid

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data supporting the findings of this study are available within the paper and its Supplementary Information and are also available from the corresponding authors on reasonable request. We used Swiss-Prot database to identify the proteins presents in the mass spectrometry samples.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
For CSF experiments, sample size was limited by the amount of CSF and the number of CSF patients available. For all other experiments, the sample size was determined based on previously published studies using similar experimental settings and techniques.

**Data exclusions**
No samples were excluded

**Replication**
Fig 1d - n=2 independent experiments. Fig 1e - n=3 independent experiments for CAP-1 and n=2 for THT. Fig 2c - Representative images from two independent experiments. For each sample a minimum of 16 fields of view were taken. Fig 2d - n=2 independent experiments and each value corresponds to mean of n=3 replicates. Fig 2e - n=27 fields of view per sample, from one representative experiment and Fig 3b - n=3 independent experiments

Fig 3d - n>10 fields of view per sample for one representative experiment.

Fig 3e - n=5 (CSF from 5 different patients) and each value corresponds to the mean of n>10 fields of view per sample for one representative experiment. All attempts at replication were successful.

**Randomization**
Patient samples (Fig 3e) were randomized. For all others experiments randomization was not performed due to the low number of sample size and conditions tested.

**Blinding**
The membrane permeability assay (Fig. 3d and e) was carried out as a blind experiment, in all other experiments blinding was not possible as the same investigators were simultaneously preparing and running the samples.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| x Antibodies                    | x ChIP-seq |
| x Eukaryotic cell lines         | x Flow cytometry |
| x Palaeontology and archaeology | x MRI-based neuroimaging |
| x Animals and other organisms   |         |
| x Human research participants   |         |
| x Clinical data                 |         |
| x Dual use research of concern  |         |