

Interaction between prion protein and toxic amyloid β assemblies can be therapeutically targeted at multiple sites

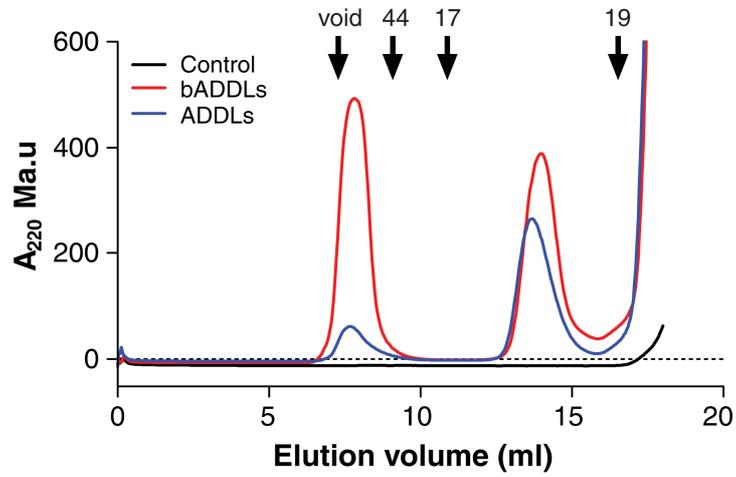
Darragh B. Freir^{1*}, Andrew J. Nicoll^{2*}, Igor Klyubin³, Silvia Panico⁴, Jessica M. Mc Donald¹, Emmanuel Risse², Emmanuel A. Asante², Mark A. Farrow², Richard B. Sessions⁵, Helen R. Saibil⁴, Anthony R. Clarke^{2,5}, Michael J. Rowan³, Dominic M. Walsh^{1#} and John Collinge^{2#}.

*These authors contributed equally.

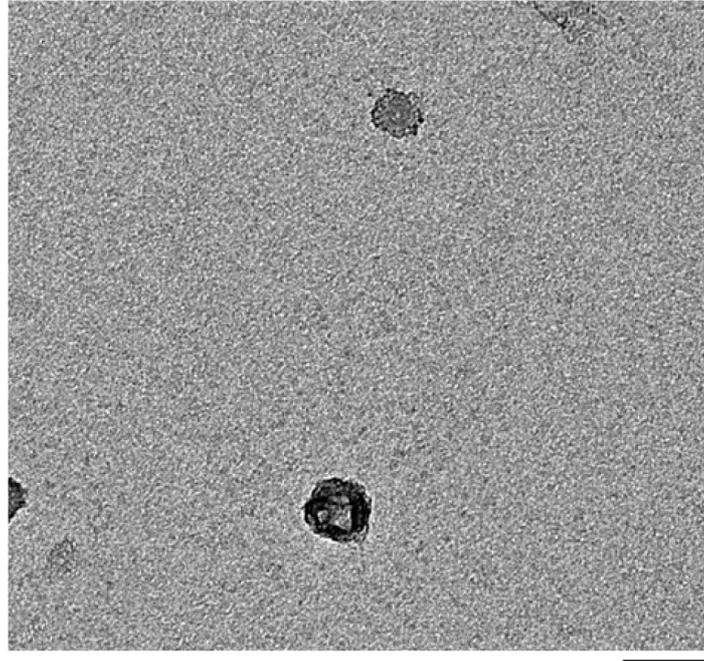
¹Laboratory for Neurodegenerative Research, School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Dublin 4, Republic of Ireland, ²Medical Research Council Prion Unit and Department of Neurodegenerative Disease, UCL Institute of Neurology, Queen Square, London WCN1 3BG, United Kingdom, ³Department of Pharmacology and Therapeutics, and Institute of Neuroscience, Biotechnology Building, Trinity College, Dublin 2, Republic of Ireland and ⁴Crystallography, Institute of Structural and Molecular Biology, Birkbeck College, Malet Street, London WC1E 7HX, UK, ⁵Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol. BS8 1TD, United Kingdom.

#To whom correspondence should be addressed:

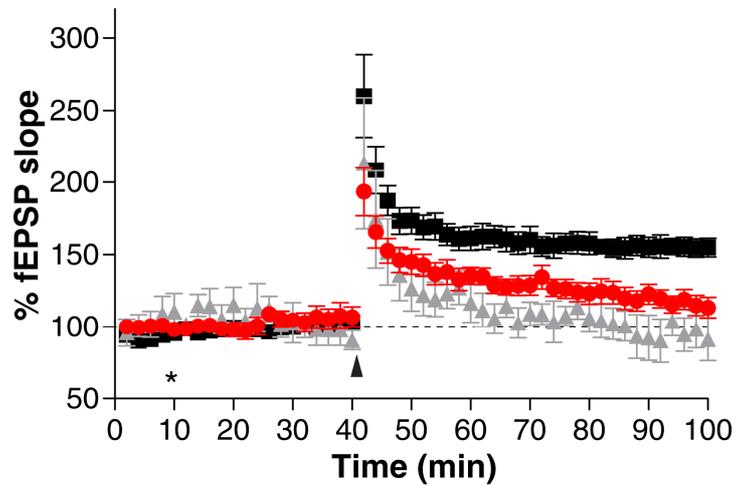
Email: dominic.walsh@ucd.ie or j.collinge@prion.ucl.ac.uk



Supplementary Figure S1. ADDLs and bADDLs (1 ml of 100 μ M stock) were chromatographed on a Superdex 75 (10/30HR) column and eluted with ACSF at a flow rate of 0.8 ml/min. Peptides were detected by absorbance at 220 nm. The ADDL/bADDL preparations produced two peaks, one eluting in the void volume after 8 ml and the other eluting after 14 ml in a manner highly similar to the freshly dissolved peptide (see Fig. 1a). ADDLs and bADDLs eluted in Ham's F12 medium produced a pattern similar to that shown above and in Fig. 1a.



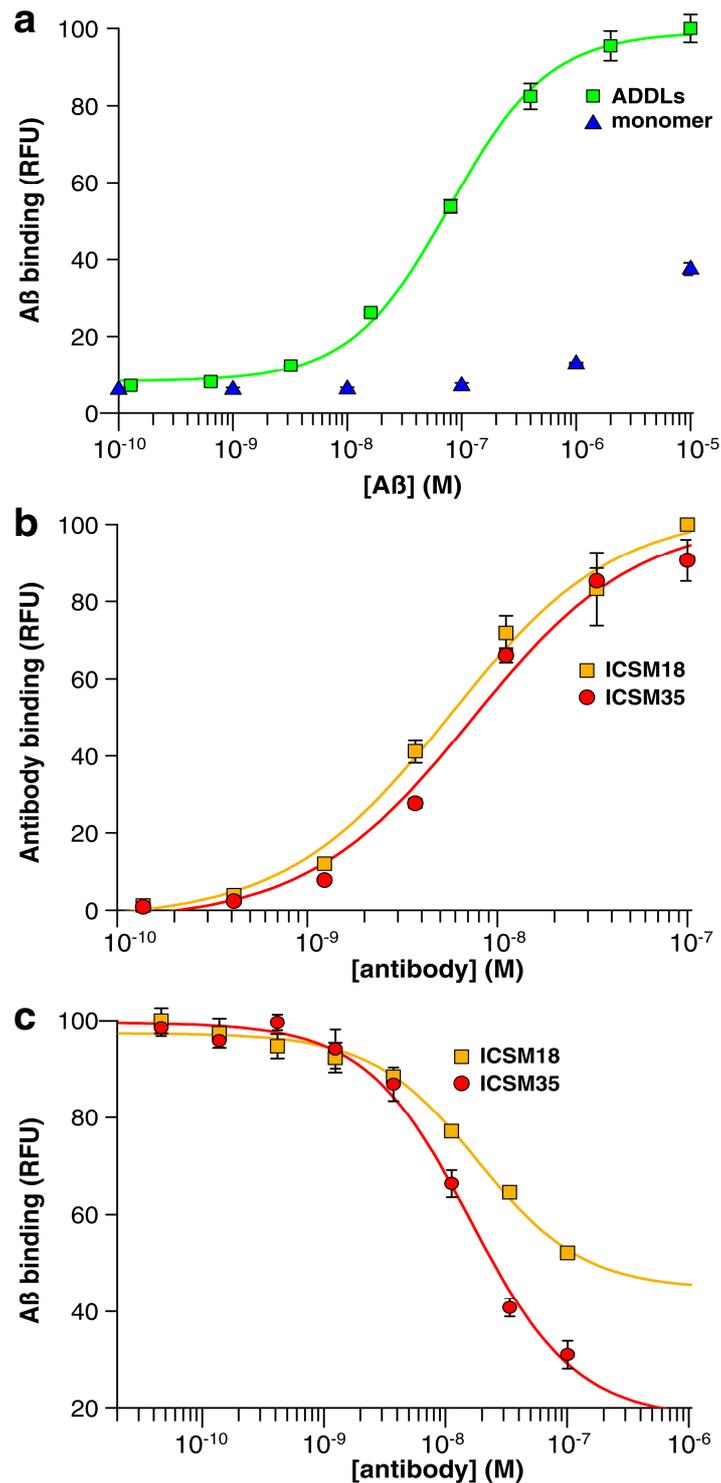
Supplementary Figure S2: Negatively stained transmission electron micrograph of buffer (Ham's F12 medium 2% DMSO). Scale bar 50 nm.



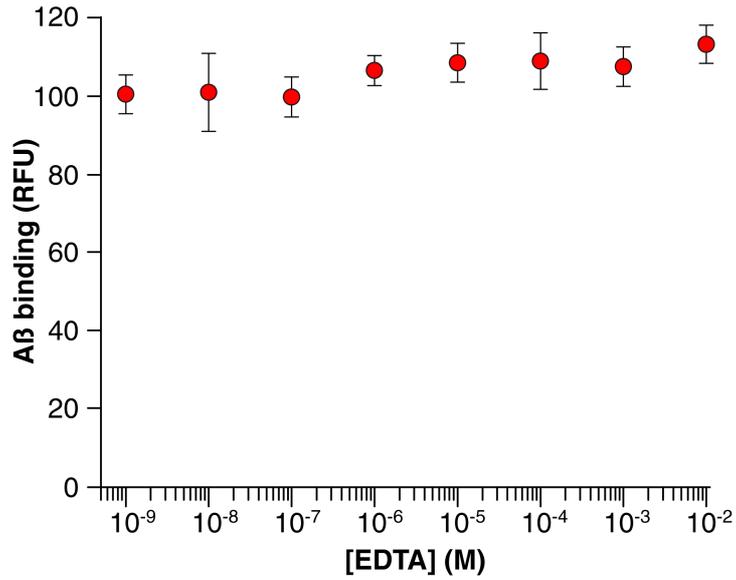
Supplementary Figure S3: ADDLs and bADDLs potently inhibit hippocampal LTP.

Treatment of C57BL/6J mice with 500 nM bADDLs (\blacktriangle $94 \pm 13\%$, $n=5$) and ADDLs (\bullet $115 \pm 7\%$, $n=6$) produced a significant depression of LTP when compared to controls (\blacksquare $154 \pm 6\%$, $n=10$, $p<0.01$, one-way ANOVA). With bADDLs tending to cause a greater depression than ADDLs. This trend may relate to the fact that bADDL preparations tended to have larger amounts of high molecular weight A β species than did ADDLs (Fig 1b and Supplementary Fig. S1). All values are stated as mean \pm SEM of baseline values prior to LTP induction.

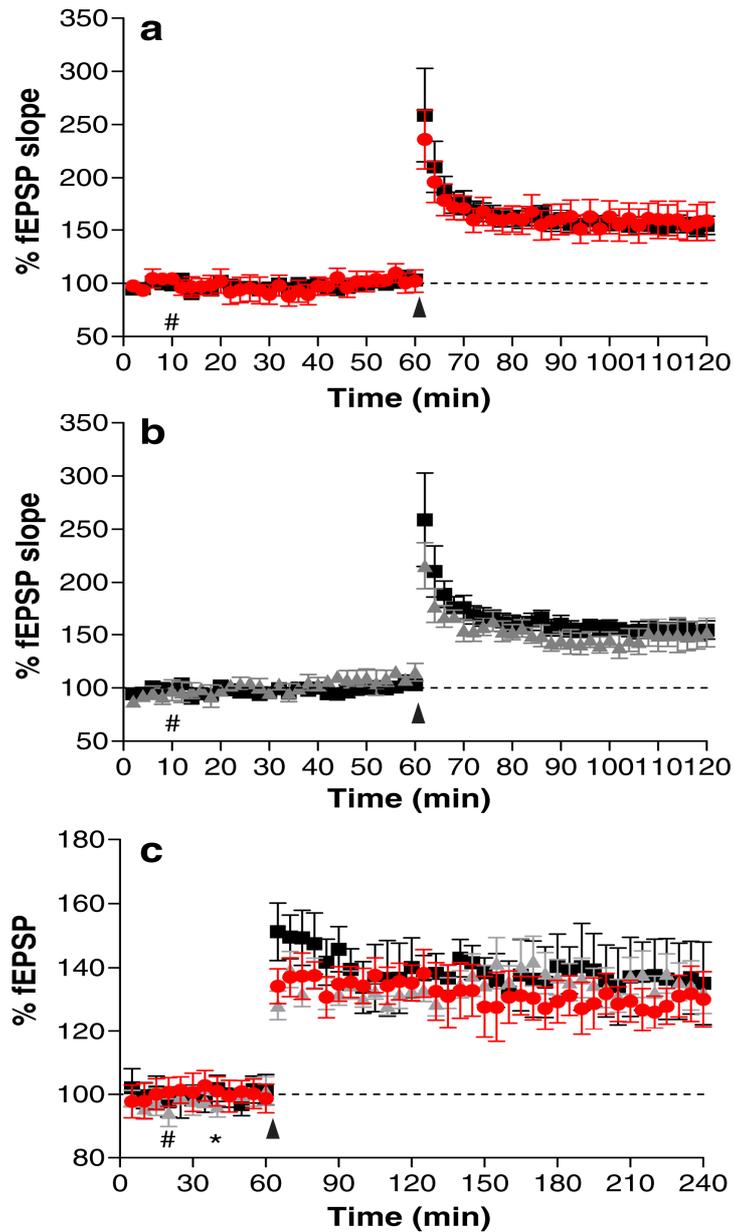
* denotes perfusion of ADDLs/bADDLs, arrow denotes theta-burst stimulation.



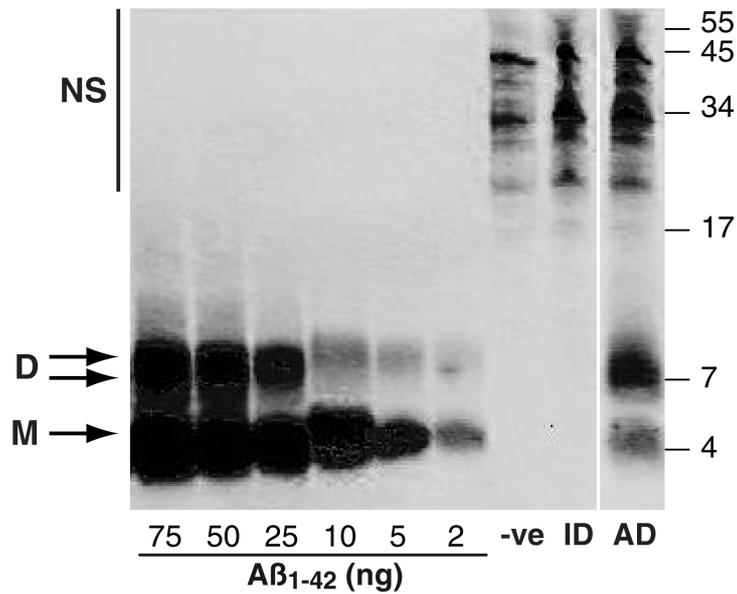
Supplementary Figure S4: The mode of antibody-induced inhibition of ADDL binding to huPrP₂₃₋₂₃₁. **a**, Relative binding of monomeric (\blacktriangle) and oligomeric (\blacksquare) A β_{1-42} to surface-bound huPrP₂₃₋₂₃₁ detected using 6E10 and DELFIA[®] Eu-N1 anti-mouse antibodies. **b**, binding of ICSM-18 (\blacksquare) and ICSM-35 (\bullet) to surface-bound huPrP₂₃₋₂₃₁ detected using 6E10 and DELFIA[®] Eu-N1 anti-mouse antibodies. **c**, Inhibition of bADDL binding to surface-bound huPrP₂₃₋₂₃₁ by ICSM-18 (\blacksquare) and ICSM-35 (\bullet) and detected using DELFIA[®] Eu-N1 streptavidin. Error bars show standard deviations and are the average of at least three replicates.



Supplementary Figure S5: bADDL binding to huPrP₂₃₋₂₃₁ in the presence of different concentrations of EDTA. This demonstrates that the observed interaction between PrP and A β is not caused by copper coordination unless the dissociation constant is tighter than 10⁻²² M. Binding is shown relative to binding in the absence of EDTA. Error bars show standard deviations and are the average of at least three replicates.



Supplementary Figure S6: When administered alone neither ICSM-35 nor ICSM-18 alters LTP **a**, TB conditioning stimulation in C57Bl/6J mice treated with 2 μ g/ml ICSM-35 (●) induced LTP ($157 \pm 17\%$, $n=4$) that was similar in magnitude to LTP induced in controls (■) ($153 \pm 7\%$, $n=6$). **b**, Similarly, TB in mice treated with 2 μ g/ml ICSM-18 (▲) induced LTP ($150 \pm 13\%$, $n=3$) that was not significantly different from LTP induced in controls (■) ($153 \pm 7\%$, $n=6$). **c**, Injection of ICSM-18 (●) or an IgG1 isotype control antibody (▲) i.c.v. (#, both 30 μ g in 10 μ l) 30 min before injection of vehicle (*, 5 μ l i.c.v.) did not significantly affect HFS-induced LTP in the anaesthetised rat ($131 \pm 9\%$ and $133 \pm 7\%$, respectively; $p>0.05$ compared with animals that received two (■) vehicle injections, $136 \pm 12\%$, $n=3$ per group, Mann-Whitney U test). All values are stated as mean \pm SEM of baseline values prior to LTP induction. Arrow denotes the time of application of conditioning stimulation.



Supplementary Figure S7: Characterisation of AD brain extract used for *in vivo* electrophysiology. IP/Western blot analysis revealed the presence of abundant A β monomer and SDS-stable dimer in the TBS extract of a brain from an 80 year old female diagnosed with Alzheimer's disease (AD) and the complete absence of A β in an immunodepleted (ID) sample of the same brain. NS indicates non-specific immunoreactive bands detected when Tris-buffered saline alone was immunoprecipitated (TBS). Molecular weight markers are on the left. M and D denote A β monomer and SDS-stable dimer. -ve refers to TBS control.

Supplementary Methods.

Production of ADDL/bADDL Preparations. bA β_{1-42} or A β_{1-42} (~1.25 mg) was weighed into a screw-cap 1.7 ml eppendorf tube, dissolved in ice-cold hexafluoro-2-propanol (HFIP) to a concentration of 1 mM, sonicated for 10 min, the tube sealed and left to stand at room temperature for 1 h. The solution was then transferred to a 2 ml glass vial and the HFIP evaporated under a stream of dry air/N₂ to produce a clear film. The peptide film was dissolved in anhydrous DMSO with vigorous vortexing for 10 min to produce a 5 mM solution and then diluted to 100 μ M in phenol red-free Ham's F12 medium (Promocell GmbH, Heidelberg, Germany) and vortexed for 15 s. Equal volumes of sample were then transferred to two separate sealed glass vials and incubated at room temperature for 16 h. Monomeric A β_{1-42} was produced by dissolving A β_{1-42} peptide to 100 μ M in 10 mM NaOH (pH 11) for 1 hour and monomeric status was confirmed by size-exclusion chromatography. Finally, samples were centrifuged at 14,200 x g for 15 min to remove any large aggregates and the upper 90% for each solution collected, used immediately, or snap frozen in liquid N₂ and stored at -80°C.

Preparation of human AD brain samples. All procedures for use of human tissue in this research were approved by a multicentre Research Ethics committee. Three brains were used for this study; one from a 78 year old women with a history of dementia and confirmed Alzheimer's disease pathology (from Asterand, Detroit, MI), a second from an 80 year old female (Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology) with clinical and pathological diagnoses of AD and the other from a cognitively intact 68 year old female (Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology). Samples of frozen posterior temporal cortex were thawed on ice and gray

matter dissected for use, chopped into small pieces with a razor blade and then homogenised in 5 volumes of ice-cold 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl (TBS) with 25 strokes of a Dounce homogeniser (Fisher, Ottawa, Canada). To isolate water-soluble A β , free from membrane-bound or plaque-associated material, homogenates were centrifuged at 91,000 g and 4 °C in a TLA 55 rotor (Beckman Coulter, Fullerton, CA) for 78 min and the supernatant removed and used. In order to eliminate low molecular weight bioactive molecules and drugs, homogenates were dialysed at 4 °C using slide-a-lyzer dialysis cassettes with 2 kDa molecular weight cut-offs (Fisher, Dublin, Republic of Ireland) against a total volume of 5 l of TBS (with 2 changes) over a 48 h period. The dialysate was then aliquoted into 1 ml lots and either stored at -80 °C pending use or used directly to measure the amount and form of A β present. For the latter, 0.8 ml of dialysate was immunoprecipitated with AW7³⁹ at a dilution of 1:80 and analysed by western blotting using a combination of the C-terminal monoclonal antibodies, 2G3 and 21F12 each at a concentration of 2 μ g/ml (Dr. Peter Seubert, Elan Pharmaceuticals, San Francisco, CA). Immunoreactive bands were visualised using a fluorochrome-coupled secondary antibody (Rockland, Gilbertsville, PA) and quantified by comparison to synthetic A β standards using a Licor Odyssey imaging system (Licor Biosciences, Lincoln, NE, USA).

***In vitro* electrophysiology.** Male, two to four month old FVB/N (Harlan, Wyton, UK) or PrP null mice (MRC Prion Unit) were used to study the effects of bADDLs and A β -containing extracts of human brain. In addition, two to three month old C57BL/6J mice (Charles River, Margate, UK) were used to examine the effects of bADDLs/ADDLs and the anti-PrP antibodies, ICSM-18 and ICSM-35. In all cases, mice were anaesthetised with isoflurane/O₂ and decapitated. The brain was rapidly removed and immersed in ice-cold sucrose-based

artificial cerebrospinal fluid (sACSF) containing 87 mM NaCl, 2.5 mM KCl, 7 mM MgSO₄, 0.5 mM CaCl₂, 25 mM NaHCO₃, 25 mM Glucose, 1.25 mM NaH₂PO₄ and 75 mM Sucrose. Parasagittal sections (350 μ m) were prepared on a Leica VT1000S vibratome using stainless steel razor blades (Campden, Loughborough, UK). Slices were immediately transferred to a holding chamber (BSC-PC, Warner Instruments, Hamden, CT) containing ACSF : 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 26.2 mM NaHCO₃, 11 mM Glucose and 1.25 mM NaH₂PO₄. Circulating ACSF was continuously bubbled with a mixture of 95% O₂ and 5% CO₂ and slices allowed to recover for at least 90 min at room temperature.

Extracellular recordings were performed as described previously (O'Nuallain et al. 2010). Briefly, slices were submerged in a recording chamber and perfused with oxygenated ACSF at a rate of 2-3 ml/min and the perfusate warmed to 30°C using an inline heating tube (HPT-2A, ALA Scientific Instruments, Westbury, NY). A stainless steel microelectrode (FHC, Bowdoin, USA) was used to stimulate Schaffer collateral fibres, and extracellular field EPSPs (fEPSPs) were recorded from stratum radiatum of CA1 using a glass microelectrode. fEPSPs were recorded using a Multiclamp 700B amplifier in tandem with a Digidata 1440A digitiser (Axon Instruments). Data were collected using pClamp 10 software and analysed using Clampfit 10.2 (Molecular Devices). For all experiments, test stimuli were given once every 30 s (0.033Hz), and the stimulus intensity was set to give a baseline fEPSP of 40-50% of the maximal response. A stable baseline was recorded for at least 20 min prior to application of ADDLs/antibody. In experiments using bADDLs/ADDLs (stock solutions were diluted 1:200 into ACSF to produce nominal concentrations of 500 nM based on the starting weight of A β ₁₋₄₂ monomer) or TBS extracts of human brain (1 ml of extract diluted into 20 ml ACSF) the sample was added to the perfusate 30 min prior to induction of LTP. Where a

combination of ADDLs and anti-PrP antibodies were used, the antibody was added to the perfusate 20 min prior to the ADDLS. LTP was induced by theta burst stimulation (TB, 10 bursts of 4 stimuli at 100 Hz, with an interburst interval of 200 ms) given at baseline intensity. The ACSF was recycled using peristaltic pumps (101U/R, Watson-Marlow, UK) ensuring that the ADDLs, brain samples and/or antibodies were present for the duration of the experiment. LTP is expressed as the mean \pm SEM % of baseline fEPSP slope. Statistical comparisons used ANOVA with post hoc Tukey-Kramer test. All experiments were interleaved with respect to genotype. In addition, vehicle and ADDL/bADDL/human brain derived A β experiments were performed on the same day ensuring each animal was its own control while alternating treatments daily to avoid any temporal bias.

***In vivo* electrophysiology.** *In vivo* studies on urethane (1.5 gm/kg *i.p.*) anaesthetised male Adult Wistar rats (250-300 g) were approved by Trinity College Dublin's ethical review committee and by the Department of Health, Republic of Ireland. Electrodes were made and implanted as described previously⁴⁰. Briefly, twisted-wire bipolar electrodes were constructed from Teflon-coated tungsten wires (62.5 μ m inner core diameter, 75 μ m external diameter). Single pathway recordings of fEPSPs were made from the stratum radiatum in the CA1 area of the right hippocampal hemisphere in response to stimulation of the ipsilateral Schaffer collateral - commissural pathway. Electrode implantation sites were identified using stereotaxic coordinates relative to bregma, with the recording site located 3.4 mm posterior to bregma and 2.5 mm right of midline, and the stimulating electrode located 4.2 mm posterior to bregma and 3.8 right of midline. The optimal depth of the wire electrodes in the stratum radiatum of the CA1 region of the dorsal hippocampus was determined using electrophysiological criteria and verified post-mortem. Test fEPSPs were

evoked at a frequency of 0.033 Hz and at a stimulation intensity adjusted to elicit a fEPSP amplitude of 50% of maximum. The high frequency stimulation (HFS) protocol for inducing LTP consisted of 10 bursts of 20 stimuli with an inter-stimulus interval of 5 ms (200 Hz), and an inter-burst interval of 2 s. The intensity was increased to give an EPSP of 75% of maximum amplitude during the HFS. To inject samples, a stainless-steel guide cannula (22 gauge, 0.7 mm outer diameter, 13 mm length) was implanted above the right lateral ventricle (1 mm lateral to the midline and 4 mm below the surface of the dura) just prior to electrode implantation. Animals received two intracerebroventricular (i.c.v.) injections via an internal cannula (28 gauge, 0.36 mm outer diameter). The first injection contained water vehicle, ICSM-18 or mouse IgG1 isotype control antibody (MAB002, R&D Systems, Minneapolis) in a 10 μ l volume. The second injection (5 μ l), which contained either water vehicle or human brain TBS extract, was administered 30 min later, 15 min before the HFS. The experimenter was blinded regarding treatment group in the experiment directly comparing ICSM-18 and control antibody. Verification of the placement of the cannula was performed post-mortem by checking the spread of i.c.v. injected ink dye. LTP is expressed as the mean \pm SEM % baseline field EPSP amplitude recorded over at least a 30 minute baseline period. Similar results were obtained when the EPSP slope was measured. Statistical comparisons used ANOVA with post hoc Tukey test, paired and unpaired Student t-tests or Mann Whitney U-test, as appropriate.

DELFA. 100 μ l of 1 μ M human PrP (10 mM sodium carbonate, pH 9.6) was bound to medium binding 96-well white plates (Greiner) overnight at 4 $^{\circ}$ C, washed with 3 x 300 μ l of PBS (0.05% Tween-20), blocked with 300 μ l 2% BSA in PBS at 37 $^{\circ}$ C for 2 h and washed with 3 x 300 μ l of PBS (0.05% Tween-20). If required, 100 μ l of antibody was then incubated in

PBS (0.05% Tween-20) for 1 hour and washed with 3 x 300 μ l of PBS (0.05% Tween-20). 100 μ l of different preparations of A β ₁₋₄₂ were incubated in PBS (0.05% Tween-20, 0.1% BSA) for 1 hour and washed with 3 x 300 μ l of PBS (0.1% Tween-20). A β was detected by 100 μ l of 1 μ g/ml 6E10 in PBS (0.05% Tween-20) for 1 hour, washed with 3 x 300 μ l of PBS (0.05% Tween-20), incubated for 30 min with 300 ng/ml of DELFIA[®] Eu-N1 anti-mouse antibody in DELFIA assay buffer, washed with 3 x 300 μ l of PBS (0.05% Tween-20) before enhancing with 100 μ l of DELFIA[®] Enhancement Solution. Biotinylated A β was detected by 100 μ l of 50 pM DELFIA[®] Eu-N1 Streptavidin (DELFIA assay buffer) and washed with 3 x 300 μ l of PBS (0.05% Tween-20) before enhancing with 100 μ l of DELFIA[®] Enhancement Solution. For PrP antibody-binding experiments ICSM antibodies were incubated for 30 min with 300 ng/ml of DELFIA[®] Eu-N1 anti-mouse antibody in DELFIA assay buffer, washed with 3 x 300 μ l of PBS (0.05% Tween-20) before enhancing with 100 μ l of DELFIA[®] Enhancement Solution. Binding of antibodies to PrP was detected by incubated for 30 min with 300 ng/ml of DELFIA[®] Eu-N1 anti-mouse antibody in DELFIA assay buffer, washed with 3 x 300 μ l of PBS (0.05% Tween-20) before enhancing with 100 μ l of DELFIA[®] Enhancement Solution. Plates were scanned for time-resolved fluorescence intensity of the europium probe (λ_{ex} = 320 nm, λ_{em} = 615 nm) using a Perkin Elmer EnVision plate reader. Binding Constants were calculated using a 1-site Langmuir isotherm and were tested for tight binding characteristics using the equation $y = \frac{F_{min} + (((L + E_o + K_d) - (((L + E_o + K_d)^2 - 4 * E_o * L)^{0.5}) / (2 * E_o)) * (F_{max} - F_{min}))}{2 * E_o}$ where F_{min} = minimum fluorescence, F_{max} = maximum fluorescence, E_o = the total concentration of binding sites, K_d = the dissociation constant and L = total A β concentration. This ensures a true K_d can be measured and that direct saturation of the protein surface as soon as stoichiometric quantities of peptide are added has not occurred.

Molecular modelling. A model of the full ISCM-18 antibody complex with human PrP^C₁₁₉₋₂₃₁ was constructed as follows. Two copies of the corresponding PrP-Fab complex (2W9E) were superimposed onto on the Fab domains of a full human IgG antibody structure (1HZH). The human Fab domains were removed and the loops between the 1HZH-Fc and 2W9E-Fab domains rebuilt.

The ADDL particle was constructed by first building a full atomic model of A β ₁₋₄₂ as an extended β strand with uncharged side-chains and termini. This molecule was subjected to molecular dynamics simulation at 1000 K for 0.1 ns and structures saved every 1 ps. The chain collapsed into a globular structure after 25 ps of simulation and the 57 most compact structures were chosen from the last 75 ps of the simulation. The maximum spatial extent of these selected molecules was measured (in all three dimensions) and found to be just below 3.0 nm. These 57 molecules were placed at random in the 57 vacant sites of spherical array made by removing the appropriate 8 x 8.5 corner sites (i.e. 7 per corner and 3 shared with adjacent corners) of a 5 x 5 x 5 cubic array with a 3.0 nm lattice spacing. The protonation state of the 57 A β ₁₋₄₂ molecules was reset to correspond to pH 7, water molecules were added in a 3.5 Å layer around each polypeptide and the complex relaxed 2000 steps of energy minimisation. The complex was subjected to 0.1 ns of molecular dynamics at 300 K resulting in a roughly spherical, hydrated ADDL model with a diameter around 10 nm (and a corresponding protein density of 0.82 g l⁻¹).

The N-terminus of the PrP^C molecule, taken from the Fab crystal structure complex (2W9E), was extended back to residue 95 as an unstructured polypeptide. Copies of this molecule were manually docked to the surface of the ADDL particle in an annulus around the

"equator" to illustrate how lateral interactions between such surface-bound prion molecules might occlude helix-1 and so compromise ISCM-18 binding. Molecular graphics and model building was performed using InsightII (2005) and energy calculations using Discover 2.98 (Accelrys).

Supplementary References.

39. McDonald, J. M. *et al.* The presence of sodium dodecyl sulphate-stable A beta dimers is strongly associated with Alzheimer-type dementia. *Brain* **133**, 1328-1341 (2010).
40. Klyubin, I. *et al.* Amyloid beta protein dimer-containing human CSF disrupts synaptic plasticity: prevention by systemic passive immunization. *J. Neurosci.* **28**, 4231-4237 (2008).