Resource

Cell Reports

Inhibiting the Ca\textsuperscript{2+} Influx Induced by Human CSF

Graphical Abstract

Highlights

- Human cerebrospinal fluid (CSF) can permeabilize membranes and induce Ca\textsuperscript{2+} influx

- CSF of control individuals and those with Alzheimer’s disease show similar Ca\textsuperscript{2+} influx

- An extracellular chaperone clusterin and a nanobody Nb3 can inhibit Ca\textsuperscript{2+} influx

- Bapineuzumab reduces A\textsubscript{\beta}-aggregate-induced Ca\textsuperscript{2+} influx but is less effective in CSF

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In Brief

Drews et al. develop and implement sensitive \textit{in vitro} assays to quantitatively measure the Ca\textsuperscript{2+} influx caused by human cerebrospinal fluid. If a given chaperone, antibody, or nanobody is effective in reducing Ca\textsuperscript{2+} influx, the authors determine what concentration is needed to prevent Ca\textsuperscript{2+} influx.

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Inhibiting the Ca\textsuperscript{2+} Influx Induced by Human CSF

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SUMMARY

One potential therapeutic strategy for Alzheimer’s disease (AD) is to use antibodies that bind to small soluble protein aggregates to reduce their toxic effects. However, these therapies are rarely tested in human CSF before clinical trials because of the lack of sensitive methods that enable the measurement of aggregate-induced toxicity at low concentrations. We have developed highly sensitive single vesicle and single-cell-based assays that detect the Ca\textsuperscript{2+} influx caused by the CSF of individuals affected with AD and healthy controls, and we have found comparable effects for both types of samples. We also show that an extracellular chaperone clusterin; a nanobody specific to the amyloid-β peptide (Aβ); and bapineuzumab, a humanized monoclonal antibody raised against Aβ, could all reduce the Ca\textsuperscript{2+} influx caused by synthetic Aβ oligomers but are less effective in CSF. These assays could be used to characterize potential therapeutic agents in CSF before clinical trials.

INTRODUCTION

Protein misfolding and aggregation underlies a range of neurodegenerative diseases, including Alzheimer’s disease (AD). Monomeric amyloid-β peptide (Aβ) is released from the amyloid precursor protein (APP) by proteolysis, aggregates into a range of soluble oligomers, and, ultimately, aggregates into insoluble amyloid fibrils deposited in the brain (Chiti and Dobson, 2017; Glenner and Wong, 1984; Riek and Eisenberg, 2016). A body of evidence indicates that Aβ oligomers play a key role in the development of AD by initiating a cascade of biochemical processes (including disruption of Ca\textsuperscript{2+} homeostasis) that ultimately lead to neuronal death (Arbel-Ornath et al., 2017; Benilova et al., 2012; Chiti and Dobson, 2017; Haass and Selkoe, 2007). Picomolar concentrations of synthetic Aβ oligomers can cause Ca\textsuperscript{2+} influx into neuronal cells and astrocytes, leading to oxidative stress and caspase 3 activation, which can result in apoptosis (Narayan et al., 2014). The soluble aggregates present within human biofluids have not been studied in such detail, and indeed Aβ can be post-translationally modified and has the potential to interact with other proteins present in the brain (Luo et al., 2016). Moreover, it is unclear which of the aggregates present are most neurotoxic and most directly contribute to the onset of disease. We have in the past lacked the techniques needed to measure and quantify effectively such species present in vivo, for example, in human cerebrospinal fluid (CSF), where the concentrations are approximately 1 pM (Savage et al., 2014; Yang et al., 2015). This is an important problem since a number of therapeutic strategies are based on the use of antibodies or nanobodies to reduce the number of or the toxic effects of protein aggregates.

Previous work to test potential therapeutic strategies, for example antibodies, has used aggregates derived from human sources (Walsh et al., 2002), such as those isolated from the soluble fraction of brain homogenates (Shankar et al., 2008). However, this method involves several preparative steps and may lead to soluble aggregates being formed by dissociation from any insoluble fibrils present. A small number of experiments have, however, been previously performed using human CSF without requiring any preparative steps. CSF freely exchanges with the interstitial fluid in the brain, making it a medium that is both available for analysis and contains aggregates formed within a living individual. These experiments have shown that the aggregates present in CSF can induce long-term potentiation deficit in brain slices which can be suppressed by the addition of antibodies raised against Aβ (Kiyubin et al., 2008; Walsh et al., 2002). CSF from individuals diagnosed with AD has also been shown to cause cell death, which can be reduced significantly by the addition of physiological amounts of extracellular chaperones (Yerbury and Wilson, 2010).

RESULTS

We have previously developed an extremely sensitive and high-throughput assay based on the quantitative measurement of
Ca²⁺ influx at low picomolar concentrations of oligomers that induces the diffusion time to the cell surface, leading to detectable

We have also shown that even low levels of Aβ42 oligomers can induce Ca²⁺ influx into neuronal cells by local dosing with a nanopipette (Figure 1B) (Drews et al., 2016). Local dosing reduces the diffusion time to the cell surface, leading to detectable Ca²⁺ influx at low picomolar concentrations of oligomers that is comparable to the concentration of species present in CSF. We have also recently developed a Thioflavin-T (ThT)-based imaging method to count the total number of ThT-active species in CSF using sensitive single-molecule fluorescence measurements (Horrocks et al., 2016) (Figure 1C). Herein we describe how these methods have been used in a series of experiments to characterize the protein aggregates present in the CSF of AD patients. We have also tested the effectiveness of a single-chain nanobody raised against the Aβ peptide, the extracellular chaperone clusterin, and bapineuzumab (a humanized monoclonal antibody that was recently used in an unsuccessful AD drug trial) (Blennow et al., 2012; Liu et al., 2015; Salloway et al., 2014; Vandenberghe et al., 2016) at reducing the Ca²⁺ influx induced by samples of CSF.

We first carried out experiments using the high-throughput vesicle assay or the cell assay to measure the extent of Ca²⁺ influx induced by samples of AD CSF and HC CSF. We detected no difference in the extent of Ca²⁺ influx between these two sets of samples (Figures 1D and 1E; Tables S1 and S2). A separate set of experiments also showed no significant difference in the number of ThT-active species detected in AD CSF and in HC CSF (Figure 1F; Table S3). Previous work using ELISA-based detection of the total number of aggregates in CSF also showed no clear difference between AD and HC samples (Savage et al., 2014; Yang et al., 2015), a result consistent with our findings. Together, these data suggest that comparable numbers of species in AD CSF and in HC CSF cause CA²⁺ influx.

To identify which species present in the CSF might be responsible for the Ca²⁺ influx, we added clusterin to AD CSF and to HC CSF (Figures 2 and S1). Clusterin is an extracellular chaperone known to bind misfolded and aggregated proteins at sub-stoichiometric concentrations compared to the monomer concentration (Yerbury et al., 2007). Additionally, multiple clusterin molecules were found to bind to each Aβ oligomer (Narayan et al., 2011, 2012). Moreover, in previous work, we determined that 100 pM clusterin halved the Ca²⁺ influx induced by recombinant Aβ42 oligomers (Flagmeier et al., 2017). This result shows that clusterin has a very high affinity for oligomers of Aβ and, in our experiments with CSF, we used clusterin concentrations more than 100 times greater than 100 pM. Clusterin was very effective at suppressing the Ca²⁺ influx induced by CSF as measured using the vesicle assay (Figures 2A and S1A) and cell
not high enough to be totally effective at reducing Ca\(^{2+}\) influx. In a control experiment we showed that the addition of Nb3 to A\(\beta\) oligomers in the absence of CSF and that approximately 18 nM Nb3 was needed to halve the influx of Ca\(^{2+}\) ions in the vesicle assay (Figures S3A and S3B). Using the vesicle assay we found that 150 nM bapineuzumab caused a relative decrease of 70% in the Ca\(^{2+}\) influx (Figure 3A), and that 1 \(\mu\)M bapineuzumab reduced the Ca\(^{2+}\) influx completely (Figure S3C). Bapineuzumab also prevented Ca\(^{2+}\) influx in the cell assay (Figures 3B and 3D). We then tested 150 nM bapineuzumab on samples of AD CSF and of HC CSF. Addition of the antibody did not detectably alter the number of ThT-active species (Figure S2), indicating that the addition of bapineuzumab does not significantly alter the number of \(\beta\)-sheet containing species within the timescale of our experiments (Table S3). In the vesicle assay, using an antibody at a concentration of 150 nM, we observed an insignificant relative decrease in the Ca\(^{2+}\) influx induced by AD CSF and HC CSF of \(\sim\)12% (Figures 3B and S3D). A 10-fold higher concentration of bapineuzumab, 1 \(\mu\)M, caused a relative decrease in Ca\(^{2+}\) influx of \(\sim\)75% (absolute decrease 18%) (Figures 3C and S3E). However, we observed no reduction in Ca\(^{2+}\) influx in the cellular assay at either antibody concentration (Figures 3E and 3F). In the cell assay, this higher antibody concentration is presumably still insufficient to prevent the interaction with the cell membrane, possibly because the aggregates have a higher affinity for the cell membrane than for the membrane of a vesicle. Overall, the results of the single vesicle experiments show that higher bapineuzumab concentrations are needed to reduce the extent of Ca\(^{2+}\) influx caused by CSF samples compared to the concentration needed to counteract the effect of synthetic A\(\beta\)42 oligomers in buffer solution. This reduced effectiveness of bapineuzumab in the inhibition of CSF-induced Ca\(^{2+}\) influx can have a number of causes including (1) molecular crowding and non-specific binding to a multitude of components present in CSF, which might reduce the effective interaction between the species causing Ca\(^{2+}\) influx and the antibody; (2) the morphology of the species present that are responsible for Ca\(^{2+}\) influx in CSF might be different from the species in the experiments with purified A\(\beta\); (3) there might be post-translationally modified species of A\(\beta\) present in CSF in which the binding epitope of bapineuzumab is absent or inaccessible to the antibody. A combination of these effects could reduce the binding of the antibody to the aggregates, and as a consequence bapineuzumab is less effective at reducing the Ca\(^{2+}\) influx induced by CSF.

Figure 2. Assessing the Ability of Chaperones and Nanobodies to Counteract the Influx of Ca\(^{2+}\) Ions Resulting from the Addition of Aliquots of Human CSF
(A and B) Inhibition of the influx of Ca\(^{2+}\) ions into individual vesicles caused by AD CSF by clusterin (150 nM) (A) and the nanobody Nb3 (150 nM) (B). (C and D) Inhibition of the influx of Ca\(^{2+}\) ions into astrocytes caused by human CSF of individuals suffering from AD by clusterin (25 nM) (C) and the nanobody Nb3 (150 nM) (D). The statistical data for the experiments are summarized in Tables S1 and S2. Error bars, SEM.

We then tested the effectiveness of a single-chain nanobody Nb3, raised against the A\(\beta\) peptide, at reducing CSF-induced Ca\(^{2+}\) influx. Nb3 binds to the epitope 17–28 of the A\(\beta\) peptide with a measured dissociation constant for the monomeric protein of 13 nM. We have previously shown using the vesicle assay that Nb3 reduces the Ca\(^{2+}\) influx induced by recombinant A\(\beta\)42 oligomers in the absence of CSF and that approximately 18 nM Nb3 was needed to halve the influx (Flagmeier et al., 2017). We also found that the addition of Nb3 to A\(\beta\)42 oligomers significantly reduced the Ca\(^{2+}\) influx in the cell assays ( Drews et al., 2016). In these experiments, we pre-incubated the AD or HC CSFs with 150 nM Nb3 for 15 min and then performed experiments using our vesicle and cell assays (Figures 2B, 2D, and S1B). These experiments show that Nb3 is able to reduce the extent of Ca\(^{2+}\) influx induced by samples of AD CSF and HC CSF. In a control experiment we showed that the addition of a GFP antibody caused no significant reduction in Ca\(^{2+}\) influx (Figure S1C). Moreover, the addition of the Nb3 nanobody did not detectably alter the number of ThT-active species (Figure S2), indicating that the addition of the nanobody does not lead to the disaggregation of the aggregates within the timescale of our experiments, approximately 15 min. The observation that Nb3 can reduce the Ca\(^{2+}\) influx caused by A\(\beta\) oligomers and also by CSF both in the vesicle and in the cellular assays suggests that at least a portion of the influx-inducing species present in CSF contains the amino acids 17–28 of the A\(\beta\) sequence. Presumably, Nb3 binds to the species that are potent at inducing Ca\(^{2+}\) influx and thereby reduces or prevents their interaction with the membranes of vesicles or cells.

Next, we probed the efficacy of bapineuzumab, which is a bivalent antibody that binds soluble A\(\beta\) monomer and oligomers at the N terminus (Miles et al., 2013). Using recombinant A\(\beta\)42 oligomers, we found that \~90 nM bapineuzumab was required to halve the influx of Ca\(^{2+}\) ions in the vesicle assay (Figures S3A and S3B). Using the vesicle assay we found that 150 nM bapineuzumab caused a relative decrease of 70% in the Ca\(^{2+}\) influx (Figure 3A), and that 1 \(\mu\)M bapineuzumab reduced the Ca\(^{2+}\) influx completely (Figure S3C). Bapineuzumab also prevented Ca\(^{2+}\) influx in the cell assay (Figures 3B and 3D). We then tested 150 nM bapineuzumab on samples of AD CSF and of HC CSF. Addition of the antibody did not detectably alter the number of ThT-active species (Figure S2), indicating that the addition of bapineuzumab does not significantly alter the number of \(\beta\)-sheet containing species within the timescale of our experiments (Table S3).
**DISCUSSION**

The mechanisms by which amyloid aggregates cause toxic effects and neuronal cell death remain a topic that is under investigation. One commonly proposed mechanism is that aggregates permeabilize membranes, leading to altered calcium homeostasis and, ultimately, to cell death. However, aggregates can also act by a range of other mechanisms such as by causing oxidative stress, neuroinflammation, or synaptic dysfunction. It is therefore extremely important to develop and apply techniques that explore the significance of particular molecular processes that are likely to contribute to the development of AD in humans.

The experiments described above show that human CSF can induce Ca²⁺ influx in our assays using both single vesicles and astrocytes. In addition, our results show that Nb3 and bapineuzumab, both of which were designed to bind to the Aβ peptide, can both inhibit this Ca²⁺ influx. Interestingly, we detected no significant difference in the number of ThT-active species (an indication of β-sheet containing aggregates) or between Ca²⁺ influx induced by aliquots of AD CSF and HC CSF. These results are in agreement with the results of ELISA measurements, using the same antibody to capture and detect aggregates (Yang et al., 2015). This result suggests that the total number of the aggregates by itself is not the critical factor in the onset of AD, but that other factors such as the nature of the aggregates and their effects are also important.

Our results, however, suggest that the other components present in CSF interact with clusterin, Nb3 and bapineuzumab, making them less available to bind the oligomers, or to compete for binding to the aggregates or that the oligomers present are different in their structure or composition from the synthetic peptide.

**EXPERIMENTAL PROCEDURES**

**Aβ42 Aggregation**

For the cell assays, Hilyte Fluor 647 Aβ42 (Cambridge Bioscience LDT) was aggregated as described previously (Drews et al., 2016).

**Aβ Nanobody and Clusterin**

Nb3 is an Aβ-specific nanobody isolated from a llama and was prepared as described previously (Drews et al., 2016). Clusterin was obtained as previously described (Drews et al., 2016; Wilson and Easterbrook-Smith, 1992).

**Bapineuzumab Equivalent Antibody**

The bapineuzumab equivalent antibody was prepared as described in its U.S. patent (US 7179892 B2) in 25 mM histidine, 7% sucrose, and 0.02% polysorbat 80 (pH 6.0) at 48 mg/mL. Endotoxin levels were < 0.005 (EU/mg).
Single Aggregate Visualization through Enhancement Imaging

All CSF samples were imaged with the single aggregate visualization through enhancement (SAVE) method as previously described (Horrocks et al., 2016). In short, a ThT stock solution was prepared in DMSO, diluted into PBS, and filtered (0.22-μm filter, Whatman) with the stock solution prepared daily. Borsilicate glass coverslips were cleaned in an argon plasma cleaner (PDC-002, Harrick Plasma) and coated with poly-(L)-lysine (PLL) for at least 1 hr. The PLL-coated surfaces were washed with PBS before the sample was applied. CSF samples were diluted 10-fold into PBS with a final concentration of 5 μM ThT. Each sample was incubated on the coverslip for 10 min prior to imaging to ensure fixation of the species on the surface. The samples were imaged using a home-built total internal reflection fluorescence (TIRF) microscope. ThT was excited with a 405-nm laser (Oxius LaserBoxx, LBX-405-100-CIR-PP) aligned to the optical axis of a 1.49 NA TIRF objective (APON60XO TIRF, Olympus, product number N2709400). Imaging was performed in TIRF mode on an inverted Olympus IX-71 microscope with an automated stage (Prior Scientific). The fluorescence signal was recorded on an EMCCD camera (Evolve 512, Photometrics) operating in frame transfer mode (EMGain of 11.5 e–/ADU and 250 ADU/photon) after being separated from the excitation light by a dichroic (D01-405/488/532/635, Semrock) and a filter (BLP01-488R-25, Semrock). Each pixel was 206 nm in length. For each dataset, 3 x 3 image grids were measured from three different areas of the coverslip with set grid distances to prevent user bias. Images were recorded at 50-ms exposure and 100 frames each field of view in the blue channel (ThT emission). Data analysis was performed as previously described (Horrocks et al., 2016) using ImageJ software, averaging all 100 frames and using the “Find Maxima.” The noise tolerance for all measurements was set to 1,000 fluorescent counts. The number of total events was then divided by the image area to give the average number of aggregates per micrometer squared.

CSF Samples

Control CSF samples were collected by lumbar puncture from 6 cognitively normal individuals (aged 49–68 years) and 6 individuals with an AD diagnosis (aged 51–68 years). A standardized protocol for the collection and storage of CSF was followed. In short, lumbar puncture in the L3/L4 interspace was performed between 9 a.m. and 12 a.m. to collect 15 mL of CSF in sterile polypropylene tubes. The samples were de-identified, spun at 3,000 rpm for 10 min, formed between 9 a.m. and 12 a.m. to collect 15 mL of CSF in sterile polypropylene tubes (aged 51–68 years). A standardized protocol for the collection and storage of CSF was adopted from the literature (Horrocks et al., 2016) using ImageJ software, averaging all 100 frames and using the “Find Maxima.” The noise tolerance for all measurements was set to 1,000 fluorescent counts. The number of total events was then divided by the image area to give the average number of aggregates per micrometer squared.

Single Vesicle Assay

Preparation of Cal-520-Filled Vesicles. Vesicles with a mean diameter of 200 nm were prepared by freeze-and thaw cycles combined with extrusion as previously described (Flagmeier et al., 2017). Brieﬂy, chloroform stocks of 16:0-18:1 PC (10 mg/mL) and 18:1-12:0 biotin PC (1 mg/mL) were mixed such that the ratio of lipid to biotinylated lipid was 100:1 before the chloroform was removed under vacuum overnight. The lipid mixture was then hydrated in HEPES buffer (50 mM [pH 6.5]) with 100 μM Cal-520. Five freeze-and-thaw cycles were performed using a water bath and dry ice. The lipid solution was extruded 10 times through a membrane with a size cutoff of 200 nm. Size exclusion chromatography was performed using a Superdex 200 to remove free dye from the surrounding solution. Finally, the size of the vesicles was determined using a Zetasizer (Zetasizer Nano ZSP, Malvern Instruments).

Vesicle Immobilization on a PE-Glylated Glass Surface. Glass coverslips were cleaned by sonication in 2% (v/v) Hellmanex III in water for 10 min followed by sonication twice in water and twice in methanol for 10 min each (Flagmeier et al., 2017). Then the coverslips were dried under a nitrogen stream for 5 min and plasma cleaned in an argon plasma cleaner (PDC-002, Harrick Plasma) for 30 min. The sample chambers were made by affixing Frame-See incubation chambers onto the glass slides. For homogeneous surface treatment, 50 μL of a mixture of 100:1 PLL-g-PEG and PLL-g-PEG biotin (both 1 g/L) in HEPES buffer (50 mM, pH 6.5) was added to the coverslip inside of the chamber and incubated for 30 min. The surface was washed three times with filtered HEPES buffer. Then, a solution of NeutrAvidin (50 μL of 0.1 mg/mL in MilliQ) was added and incubated for 15 min, and washed 3 times. Finally, 50 μL of the solution containing vesicles was added to the coverslip and incubated for 30 min before washing carefully at least 5 times with reaction buffer. Total Internal Reflection Fluorescence Microscope for Single Vesicle Experiment. The measurements of Ca2+ influx into individual vesicles was performed using a home-built total internal reflection fluorescence microscope (TIRFM) based on an inverted Olympus IX-71 microscope. A 488-nm laser (Topica, iBeam smart, 200 mW, Germany) beam was expanded and focused on the back focal plane of the 60×, 1.49 NA oil-immersion objective lens (APON60XO TIRF, Olympus, N2709400) to excite the Cal-520 dye incorporated into the vesicle. The emerging fluorescence was collected by the same objective. The emission beam was separated from the excitation beam by a dichroic (D01-405/488/561/635, Semrock), passed through a set of filters (BLP01-488R, Semrock and FF01-520/44-25, Semrock), and imaged onto an air-cooled EMCCD camera (Photometrics Evolve, EVO-512-M:FW-16:AC-110). Excitation power density was fixed to ~10 W/cm2 for 50 frames with a scan speed of 20 Hz and bit depth of 16 bits.

Measurement of the Change in Fluorescence Intensity in Individual Vesicles. Hundreds of single vesicles tethered to coverslips via biotin-neutrAvidin linkage were incubated with 50 μL of HEPES buffer (50 mM [pH 6.5]) and imaged as previously described (Flagmeier et al., 2017). Just before the imaging, the HEPES buffer was replaced with 50 μL of Ca2+ containing buffer solution Lebovitz’s L-15 (L15). 16 different fields of view were recorded under three different conditions (background; in the presence of CSF or a solution containing reagents; in the presence of CSF or a solution containing recombinant protein aggregates; and after the addition of ionomycin, respectively). The distance between each field of view was set to 100 μm and was automated (bean-shell script, Micromanager) to avoid any user bias. First, images were acquired in the presence of only L15 buffer, which we denote as blank (Fblank). For each field of view, 50 images were taken with an exposure time of 50 ms. Thereafter, a solution of a sample was added and incubated for 10 min before images of the exact same fields of view were recorded (Fsample). Next, 10 μL of a solution of ionomycin was added to the coverslip and incubated for 5 min, and, subsequently, images of the vesicles were acquired in the same fields of view.

Data Analysis and Quantification of the Extent of Ca2+ Influx. The recorded images were analyzed using Fiji to determine the fluorescence intensity of each spot under the three different conditions, namely background (Fbackground), in the presence of the sample (Fsample), and after the addition of ionomycin (Fionomycin). The relative influx of Ca2+ into an individual vesicle due to aggregates of the Aβ42 peptide was then determined using the following equation:

\[
\text{Ca}^{2+}\text{ influx} = \frac{F_{\text{sample}} - F_{\text{blank}}}{F_{\text{ionomycin}} - F_{\text{blank}}}
\]

(Equation 1)
Single Vesicle Assay Using CSF. For our single vesicle analysis, we took 15 μL of CSF and diluted it two times in the coverslip with 15 μL L15 buffer and incubated for 10 min. Then the change in fluorescence intensity was measured as described above.

Single Vesicle Assay Using CSF with Antibodies or Nanobodies. Indicated concentrations of nanobodies and antibodies were added to samples of CSF and incubated for 15 min before we performed measurements of the change in fluorescence intensity in single vesicles as described above. The dilutions were performed in a manner such that the CSF sample incubated with the vesicles was diluted by a factor of two to allow direct comparisons of the measurements in the absence and presence of antibodies. Comparisons between the samples were made with a two-sample t test using Origin9.

Preparation and Purification of Recombinant Aβ42. The recombinant Aβ42 (M1-42) peptide (MDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVTV, here called Aβ42), was used for the vesicle assay experiments and prepared as described previously (Flagmeier et al., 2017; Hellstrand et al., 2010). Briefly, the purification involved sonication of the cells, ion exchange chromatography, and size-exclusion chromatography. Purified samples were aliquoted, lyophilized, and stored at −80°C. For each experiment, solutions of monomeric recombinant Aβ42 were prepared by dissolving the lyophilized Aβ42 peptide in 6 M GuHCl and then purifying the protein using a Superdex 75 10/300 GL. The center of the elution peak was collected, and the peptide concentration was determined from the absorbance of the integrated peak area using ε280 = 1,490 L mol⁻¹ cm⁻¹.

Aggregation Conditions for Recombinant Aβ42. Aliquots of monomeric Aβ42 were diluted with buffer to a concentration of 2 μM in low-binding Eppendorf tubes on ice before individual samples were then pipetted into multiple wells of a 96-well half-area plate. Then the plate was incubated under quiescent conditions at 37°C, and aliquots for measurements of Ca²⁺ influx were taken at a time point corresponding to the end of the lag phase (approximately 70 min) as previously described (Flagmeier et al., 2017).

Measurement of the Ca²⁺ Influx Caused by Aβ42. Aliquots were taken from an aggregation reaction of recombinant Aβ42 corresponding to the end of the lag phase. To measure the change of the fluorescence intensity in the single vesicle assay, we diluted the sample to 5 nM with L15 buffer as described previously.

Single Vesicle Assay Using Aβ42 with Antibodies or Nanobodies. Indicated concentrations of nanobodies and antibodies were added to an aliquot taken from an aggregation reaction of recombinant Aβ42 and incubated for 15 min. The change in the fluorescence intensity was measured as described above.

SUPPLEMENTAL INFORMATION
Supplemental Information includes three figures and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.057.

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AUTHOR CONTRIBUTIONS
A.D. and W.-H.C. performed the cellular assay; S.D. and P.F. carried out the single vesicle assay; D.C.W. and D.R.W. performed the SAVE imaging; and D.C.W. and M.R. carried out immuno-depletion. C.V. and S.M. provided the Nb3 nanobody, and R.W.P., C.F.S., N.C.F., J.M.S., H.Z., and S.G. provided the CSF samples. D.K. conceived and supervised the project with the help of C.M.D. All the authors wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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