SUPPLEMENTARY INFORMATION

METHODS

Aβ40 and Aβ42 peptide preparation and characterization

Monomeric solutions of HiLyteFluor488 and HiLyteFluor647-labeled Aβ40 or Aβ42 (Anaspec, San Jose, CA) were prepared by dissolving the lyophilized peptides in SSPE buffer (150 mM NaCl, 10 mM $Na_2H_2PO_4$ x H_2O, 10 mM Na_2EDTA, adjusted to pH 12 using NaOH) followed by sonication over ice for 30 min (Bandelin Sonorex, Berlin, Germany) and subsequently flash freezing into 5 μL aliquots[1]. Prior to each of the incubations, aliquots of each peptide were diluted into SSPE buffer (pH adjusted to 7.4 using HCl) to the desired concentration and placed under conditions for aggregation (e.g. 37 °C, agitation). The concentration of each labeled peptide was measured before mixing the two different fluorophore-labeled samples using cTCCD as previously described [1].

For each experiment Aβ monomers (Aβ40 at 20 µM and Aβ42 at 10 µM) were incubated in SSPE buffer (defined above) at 37 °C with agitation (200 rpm on a rotary shaker). After 1 h of aggregation, the samples were placed at 4 °C and used within 10 h of preparation. Monomeric solutions were kept frozen at -80 °C until use. For experiments with clusterin, the chaperone was added at a 1:1 molar ratio to Aβ (unless otherwise stated) and incubated for 30 min at 25 °C.

For each preparation of Aβ40 or Aβ42, the number and size distributionS of oligomers were determined using the single molecule cTCCD method. The instrumentation and methodology required for this characterization have been described in detail previously [1].

Preparation and labeling of human clusterin

Clusterin was extracted from human serum from Wollongong Hospital (Wollongong, NSW, Australia), as described previously (2). Labeling of clusterin was carried using lysine conjugation of succinimidyl ester-functionalized AlexaFluor647 (Molecular Probes, Grand Island, NY) using previously described protocols (1).

Cell cultures

Mixed cultures of neurons and glial cells were prepared as described previously with modifications, from Sprague-Dawley rat pups 2-4 days post-partum (UCL breeding colony)(3). Experimental procedures were performed in full compliance with the United Kingdom Animal (Scientific Procedures) Act of 1986. The hippocampus and cortex were removed and placed in ice-cold PBS (Ca^{2+}, Mg^{2+}-free, Invitrogen, Paisley, UK). The tissue was then minced and trypsinized (0.25% for 5 min at $37^{o}C$), triturated and plated on poly-D-lysine-coated coverslips, and cultured in Neurobasal A medium (Invitrogen, Paisley, UK) supplemented with B-27 (Invitrogen) and 2 mM L-glutamine. Cultures were maintained at $37^{o}C$ in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was in each case replaced twice a week and maintained for 12-15 days before experimental use to ensure expression of glutamate and other receptors. Neurons were easily distinguishable from glia using microscopy: they appeared phase bright, had smooth rounded somata and distinct processes, and lay just above the focal plane of the glial layer.

Measurements of $[Ca^{2+}]_c$ and ROS

For measurements of $[Ca^{2+}]_c$, cells were loaded for 30 min at room temperature with 5 μM fura-2 AM and 0.005% pluronic acid in a HEPES-buffered salt solution (HBSS) containing 156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25mM KH_2PO_4, 2mM $CaCl_2$, 10mM glucose and 10mM

HEPES; the pH of each solution was adjusted to 7.35 with NaOH, and the fluorescence of 488 nM-excitable fura-2 was measured as a function of time.

For measurement of ROS production, dihydroethidium (2 μM HEt) was added into the solutions during the experiments. No pre-incubation ('loading') was used for HEt to limit the intracellular accumulation of oxidized products. Measurements monitored the ratio of two fluorescent wavelengths, representing the oxidized and non-oxidized form, as a function of time (see Microscopy) (4-6).

In all experiments, we identified the neurons initially with bright field imaging and during the experiments by calcium imaging. Neurons were easily distinguishable from glia: they appeared phase bright, had smooth rounded somata and distinct processes, and lay just above the focal plane of the glial layer. Cells were imaged for up to 30 minutes following the addition of Aβ and large fields of cells containing between 100 and 200 cells were imaged at a time at an image acquisition rate of 10 s^{-1}.

Caspase-3 activation assay

For measurements of caspase-3 activation, cells were loaded for 15 min at room temperature with 10 μM NucView 488 caspase-3 substrate (Biotium, Hayward, CA) in HBSS. NucView 488 is a member of a novel class of enzyme substrates for real-time detection of caspase-3 activity in live cells. The substrate can rapidly cross cell membranes to enter the cytoplasm, where it is cleaved by caspase-3 to release the high-affinity DNA dye, which fluoresces when excited by a 488 nm laser. The released DNA dye migrates to the cell nucleus. Therefore, cells having undergone caspase-3 activation are distinguishable from all other cells by visualizing their

bright-green nuclei. The NucView 488 substrate was used according to the manufacturer's specifications.

Microscopy

All microscopy protocols used for monitoring intracellular Ca^{2+} and ROS have been described previously (5, 6). Fluorescence measurements were obtained on an epi-fluorescence inverted microscope equipped with a ×20 (0.5 NA) fluorite objective. $[Ca^{2+}]_c$ was monitored in single cells using excitation light provided by a xenon arc lamp, the beam passing through a monochromator centered sequentially at 340 and 380 nm (Cairn Research, Kent, UK). The emitted fluorescence was reflected through a 515 nm long-pass filter to a cooled CCD camera (Retiga, QImaging, Canada). All imaging data for Ca^{2+} and ROS experiments were collected every 15-30 s and analyzed using software from Andor (Belfast, UK). Each coverslip on which cells were cultured was placed in an AttoFluor Cell Culture chamber (Molecular Probes, Grand Island, NY) in 300 μL of Hank's Balanced Salt Solution (HBSS). Solutions containing Aβ and clusterin were added for specific experiments to the HBSS surrounding the cells. The fura-2 data were not calibrated in terms of $[Ca^{2+}]_c$ because of the difficulty in deconvoluting the strength of the signal from the amount of dye taken up by each cell. The fura-2 ratio is defined as the ratio of fluorescence emission upon excitation at 340 nm (Ca^{2+}-bound form) to emission upon excitation at 380 nm (unbound fluorophore). For HEt measurements a 543 nm excitation and 560 nm long pass filter were used to quantify oxidized HEt and excitation at 355 nm and measurement at 405-470 nm was used to detect non-oxidized HEt. HEt ratio is defined as the ratio of fluorescence emission upon excitation at 543 nm to emission upon excitation at 355 nm. All the data shown were obtained from at least 5 coverslips and 2-3 different cell preparations.

Confocal images were obtained using a Zeiss (Oberkochen, Germany) 710 confocal laser scanning microscope and a 40x oil immersion objective. A 488 nm argon laser was used to excite NucView 488 and the resulting fluorescence was measured using a bandpass filter from 510 and 560 nm. Images were acquired at 10 frames s^{-1} for 30 minutes.

Electrophysiology

All the protocols used are as described previously (7). Acute hippocampal slices were prepared from 26 to 32 day-old male Wistar rats. Experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986. Animals were sacrificed by dislocation of the neck followed by decapitation. The brains were rapidly removed and placed in ice-cold artificial CSF (aCSF) containing (in mM): 124 NaCl, 3 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, 10 D-glucose (bubbled with 95 % O_2 / 5 % CO_2). Transverse hippocampal slices (400 µm thick) were prepared using a McIllwain tissue chopper (Mickle Laboratory Engineering, Gomshall, UK). Hippocampal slices were stored in aCSF (between 20 – 25 °C) for 1 – 2 h before transferring to the recording chamber, in which they were submerged in aCSF (30 °C) flowing at 2 ml / min. Extracellular field potentials were recorded in the CA1 region of the hippocampus using glass electrodes containing 3 M NaCl. Stimulating electrodes were placed in the Subiculum and CA2 (Schaffer Collateral pathway) of the hippocampus. Stimuli (constant voltage) were delivered alternately to the two electrodes (each electrode at 0.016 Hz). LTP was induced by two trains of tetanic stimuli (each 100 Hz, 1 s; repeated after a 30 s interval). The slopes of the evoked field potential responses were measured and expressed relative to the normalized preconditioning baseline.

Statistical methods

All statistical analysis was performed using both Origin 8 (OriginLab) and Prism 6.00 (GraphPad, La Jolla, CA). Non-parametric tests were performed to avoid assumptions of normality.

Supplementary results

Clusterin binds Aβ42 oligomers

cTCCD was used to identify and characterize oligomers formed from mixtures of Aβ42 monomers tagged with either the HiLyteFluor488 or the HiLyteFluor647 fluorophore. In a similar experiment, run concurrently, cTCCD was used to determine the number of oligomer:clusterin complexes in the solution by incubating a solution of Aβ42 tagged with a HiLyteFluor488 fluorophore, with an equimolar quantity of clusterin tagged with an AlexaFluor647 fluorophore. This has been confirmed previously for Aβ40[1]. The number of oligomers from the first experiment was, within experimental error, the same as the number of clusterin-associated Aβ species in the second, indicating that effectively all of the oligomers are bound to clusterin (Figure S3A).

Mechanism of oligomer entry

In order to investigate further the mechanism underlying the Aβ-induced $[Ca^{2+}]_c$ transients, the analogous experiments to those discussed above at 25 °C were performed at lower temperatures (~4-10 °C) to reduce the efficacy of ATP-dependent active processes including cellular uptake of

extracellular species (8). In these experiments, we observed no change in the amplitude or the frequency of the $[Ca^{2+}]_c$ transients relative to those observed in the experiments performed at higher temperatures suggesting that the $[Ca^{2+}]_c$ transients and the downstream effects do not depend significantly on active processes such as exocytosis and endocytosis (Figure S1H).

Single molecule tracking on the cell membrane

Possible mechanisms by which oligomers induce Ca^{2+} transients in astrocytes include creating defects in the cell membrane or activating cell-surface ion transporters(9, 10). Both of these mechanisms involve interactions of Aβ oligomers with the cell membrane. In order to investigate whether or not pre-incubating the Aβ oligomers with clusterin inhibited the interactions between the oligomers and astrocytic membranes, we used a single-molecule imaging technique that allows us to visualize individual Aβ species on the cell surface(11). We incubated primary astrocytes with a mixture of Aβ42 monomers and oligomers labeled with a HiLyteFluor647 fluorophore and, using this approach, we observed that these Aβ42 species were bound to the cell surface in the absence of clusterin at a low surface density (approximately one monomer or oligomer was found for every 5 μm^2 of surface). When the Aβ42 oligomers were incubated with clusterin prior to adding them to the astrocytes, the number of Aβ42 species observed on the surfaces of the astrocytes was reduced by a factor of approximately two (Figure S3B). This result suggests that incubation of Aβ oligomers with clusterin prevents them from interacting detrimentally with the cell surface. Taken together, the aforementioned experiments show that picomolar concentrations of Aβ40 and of Aβ42 oligomers act on the cell membranes of astrocytes giving rise to subsequent Ca^{2+} influx. The presence of clusterin, however, can

suppress this effect by binding the oligomers, thereby preventing the initial interaction between the Aβ oligomers and the cell membranes.

Need for clusterin preincubation

The incubation of Aβ40 and Aβ42 oligomers with clusterin, prior to adding them to cells, inhibited the appearance of the oligomer-induced $[Ca^{2+}]_c$ transients in astrocytes (Figure 6A). Incubating the cells with clusterin (500 nM) for 15 min before exposing them to oligomeric Aβ42 resulted in only partial inhibition of the Aβ42-induced $[Ca^{2+}]_c$ transients ($62\pm4\%$ of astrocytes responded when pre-incubated with clusterin compared to $89\pm5.7\%$ of astrocytes without an initial incubation of the cells with clusterin, $p<0.005$). This result suggests that a minimum time is required for the interaction of Aβ oligomers and clusterin in order to enable effective suppression of the former from initiating increases in $[Ca^{2+}]_c$; indeed, such a necessity for pre-incubation to allow time for binding between chaperones and oligomers has also been observed for a number of other systems (12). The protective effect of clusterin, therefore, appears not to be mediated directly by the effects of clusterin on the cells but by its interaction with Aβ oligomers.

References

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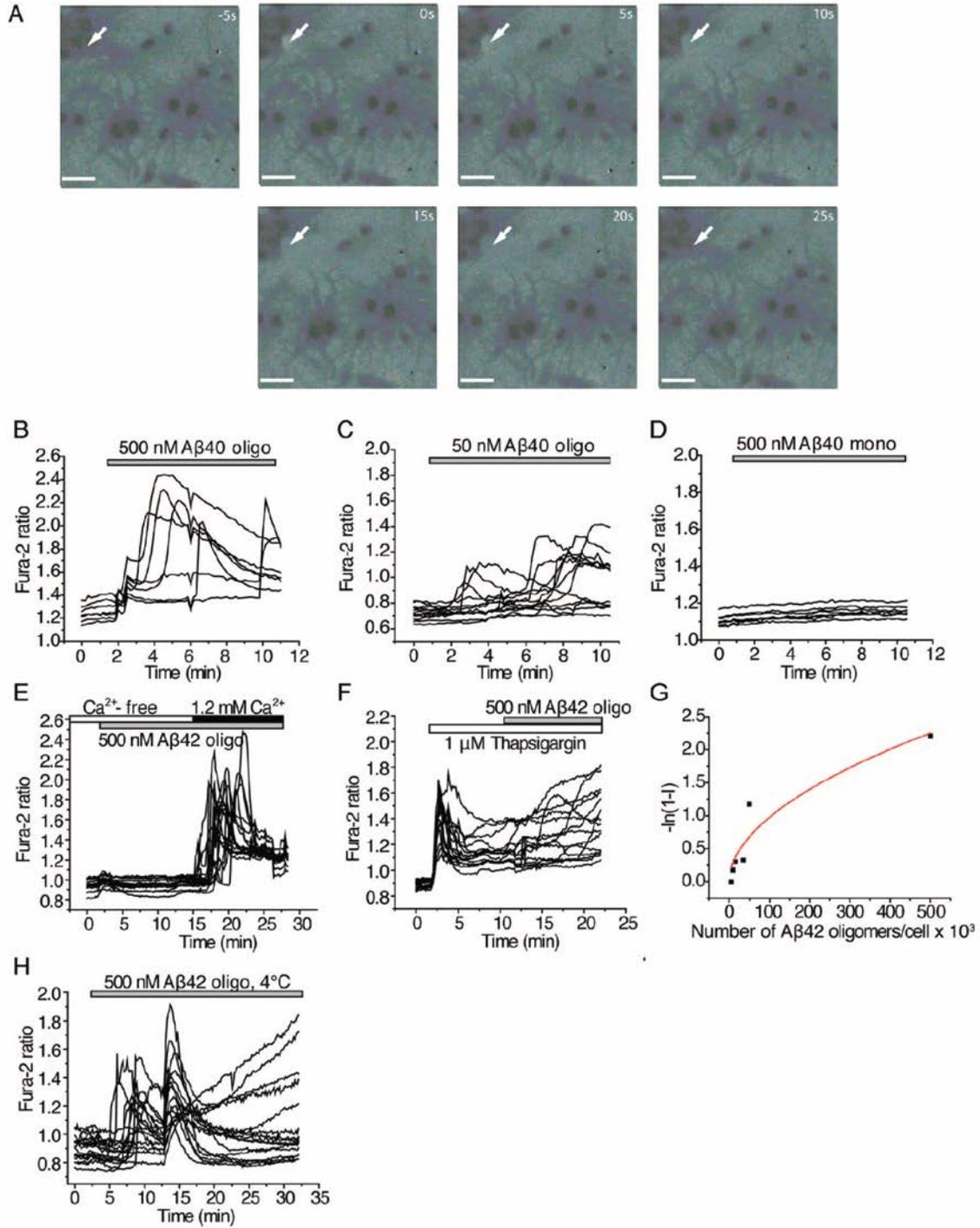**FIGURES AND FIGURE LEGENDS**

Figure S1

Figure S1. Effects of Aβ42 on cytosolic calcium levels.

(A) Images taken of primary hippocampal cultures displaying calcium transients upon addition of Aβ42 oligomers (500 nM total peptide containing 19.5 nM oligomers). The cell indicated with the arrow shows a clear spike in intracellular calcium upon the addition of Aβ oligomers (at time 0 s). Scale bars are 10 μm.

(B) The cytosolic Ca^{2+} concentration (as quantified by the fura-2 ratio) as a function of time in astrocytes to which 500 nM of total Aβ40 (containing 1.5 nM oligomer) has been added. Each line represents the intracellular calcium within a single cell. 118 astrocytes were examined.

(C) The cytosolic Ca^{2+} concentration (as quantified by the fura-2 ratio) is shown as a function of time in astrocytes to which 50 nM (total peptide) Aβ40 (containing 150 pM oligomers) has been added. Each line represents the intracellular calcium within a single cell. 211 astrocytes were examined.

(D) The cytosolic Ca^{2+} concentration (as quantified by the fura-2 ratio) is shown as a function of time in astrocytes to which 500 nM of monomeric Aβ40 were added. Each line represents the intracellular calcium within a single cell. 57 astrocytes were examined.

(E) The cytosolic Ca^{2+} concentration (as quantified by the fura-2 ratio) is shown as a function of time in astrocytes to which 500 nM of total Aβ42 (containing 19.5 nM oligomer) has been added. The experiment was performed in a medium with Ca^{2+} omitted from the solution (including 0.5 mM EGTA to chelate any endogenously released calcium). 15 minutes following the addition of Aβ42, 1.2 mM Ca^{2+} was added to the medium surrounding the cells. 99 astrocytes and 78 neurons were examined.

(F) The cytosolic Ca^{2+} concentration (as quantified by the fura-2 ratio) is shown as a function of time in astrocytes to which 500 nM of total Aβ42 (containing 19.5 nM oligomer) has been added following the active depletion of intracellular endoplasmic reticulum Ca^{2+} using thapsigargin. 122 astrocytes and 89 neurons were examined.

(G) The data from 2E, re-plotted and fitted to the dose-response relationship $-ln(1-I)=p(x)^k$ where I represents the fraction of total astrocytes displaying cytosolic Ca^{2+} transients, while x represents the number of Aβ42 oligomers per cell. This function was fitted by varying p and k using Origin 8.0 (OriginLab).

(H) The cytosolic Ca^{2+} concentration (as quantified by the fura-2 ratio) is shown as a function of time in astrocytes to which 500 nM of total Aβ42 (containing 19.5 nM oligomer) has been added. The entire experiment was performed with cells kept at a temperature of 4 °C. Each line represents the intracellular calcium within a single cell. 58 astrocytes were examined.
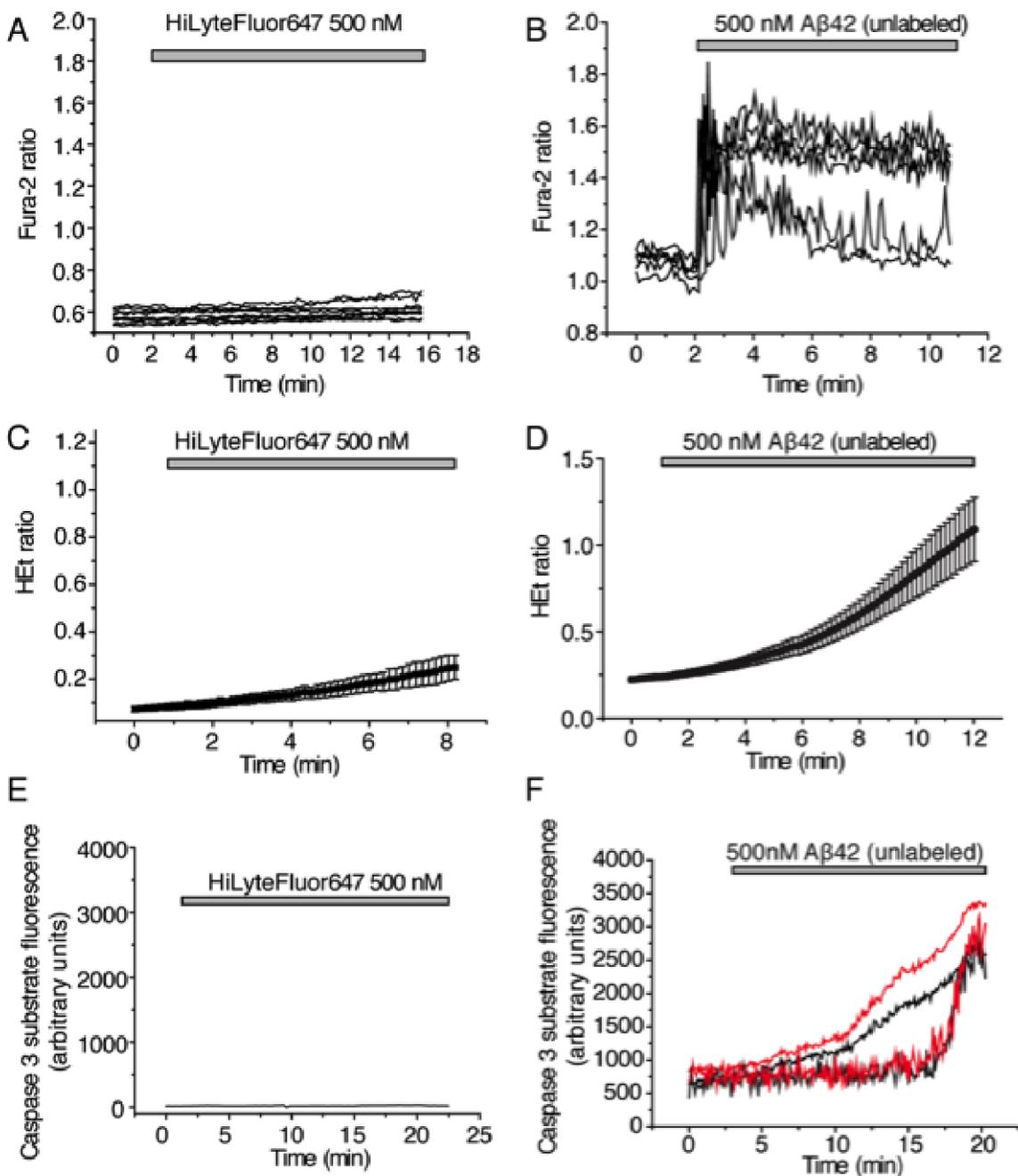
Figure S2

Figure S2. Effect of unconjugated HiLyteFluor fluorophores and unlabeled Aβ on intracellular calcium levels, rates of ROS production, and caspase-3 activation.

In each of the following experiments, at least 50 cells were examined.

(A) The cytosolic Ca^{2+} concentration (as quantified by the fura-2 ratio) as a function of time in astrocytes to which 500 nM unconjugated HiLyteFluor647 has been added. Each line represents a single astrocyte.

(B) The cytosolic Ca^{2+} concentration (as quantified by the fura-2 ratio) as a function of time in astrocytes to which 500 nM unlabeled Aβ42 (containing, presumably, 19.5 nM oligomers) has been added. Each line represents a single astrocyte.

(C) The HEt ratio as a function of time upon the addition of 500 nM unconjugated HiLyteFluor647. Values are presented as mean ± SEM of three or more trials.

(D) The HEt ratio as a function of time upon the addition 500 nM unlabeled Aβ42 (containing, presumably, 19.5 nM oligomers). Values are presented as mean ± SEM of three or more trials.

(E) The fluorescence of the NucView488 caspase-3 substrate as a function of time following treatment of primary hippocampal cultures containing both neurons and astrocytes with 500 nM of unconjugated HiLyteFluor647. Black lines represent astrocytes and red, neurons. In order to present equal y-scales, the number of traces on the plot is not apparent but ~5 traces are plotted.

(F) The fluorescence of the NucView488 caspase-3 substrate as a function of time following treatment of primary hippocampal cultures containing both neurons and astrocytes with 500 nM of unlabeled Aβ42 (containing, presumably, 19.5 nM oligomers). Black lines represent astrocytes and red, neurons.
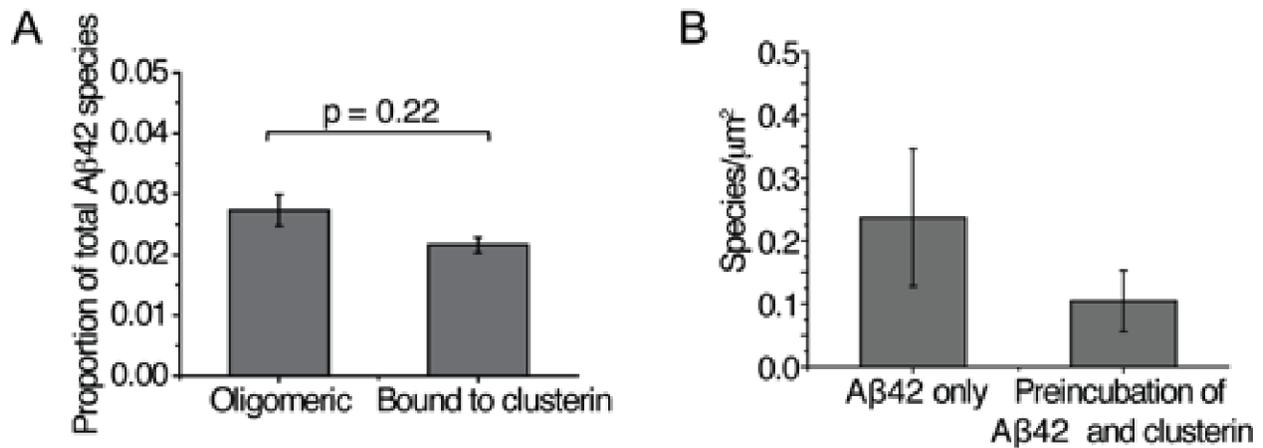
Figure S3

Figure S3. Clusterin binds Aβ oligomers and prevents their adherence to astrocytic membranes.

(A) The fraction of Aβ42 in oligomers and bound to clusterin in preparations used for the experiments described in this paper (values are mean ± S.D., n=3).

(B) Surface density of Aβ42 species bound to the surface of astrocytes in the absence and presence of clusterin as determined by total internal reflection microscopy (TIRF). The cells were incubated with 50 nM total peptide (Aβ42) and 89 cells were examined.