

# Amyloid $\beta$ -peptide directly induces spontaneous calcium transients, delayed intercellular calcium waves and gliosis in rat cortical astrocytes

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Cite this article as: Chow S-K, Yu D, MacDonald CL, Buibas M and Silva GA (2010) Amyloid  $\beta$ -peptide directly induces spontaneous calcium transients, delayed intercellular calcium waves and gliosis in rat cortical astrocytes. ASN NEURO 2(1):art:e00026.doi:10.1042/AN20090035

## ABSTRACT

The contribution of astrocytes to the pathophysiology of AD (Alzheimer's disease) and the molecular and signalling mechanisms that potentially underlie them are still very poorly understood. However, there is mounting evidence that calcium dysregulation in astrocytes may be playing a key role. Intercellular calcium waves in astrocyte networks *in vitro* can be mechanically induced after A $\beta$  (amyloid  $\beta$ -peptide) treatment, and spontaneously forming intercellular calcium waves have recently been shown *in vivo* in an APP (amyloid precursor protein)/PS1 (presenilin 1) Alzheimer's transgenic mouse model. However, spontaneous intercellular calcium transients and waves have not been observed *in vitro* in isolated astrocyte cultures in response to direct A $\beta$  stimulation in the absence of potentially confounding signalling from other cell types. Here, we show that A $\beta$  alone at relatively low concentrations is directly able to induce intracellular calcium transients and spontaneous intercellular calcium waves in isolated astrocytes in purified cultures, raising the possibility of a potential direct effect of A $\beta$  exposure on astrocytes *in vivo* in the Alzheimer's brain. Waves did not occur immediately after A $\beta$  treatment, but were delayed by many minutes before spontaneously forming, suggesting that intracellular signalling mechanisms required sufficient time to activate before intercellular effects at the network level become evident. Furthermore, the dynamics of intercellular calcium waves were heterogeneous, with distinct radial or longitudinal propagation orientations.

Lastly, we also show that changes in the expression levels of the intermediate filament proteins GFAP (glial fibrillary acidic protein) and S100B are affected by A $\beta$ -induced calcium changes differently, with GFAP being more dependent on calcium levels than S100B.

Key words: Alzheimer's disease (AD), amyloid  $\beta$ -peptide (A $\beta$ ), astrocyte network, calcium signalling, intercellular calcium wave, intermediate filament protein.

## INTRODUCTION

AD (Alzheimer's disease) is a devastating age-related neurodegenerative disorder. Neuropathological hallmarks of AD include loss of neurons, deposition of neuritic plaques and reactive astrogliosis in affected brain regions, particularly the hippocampus and neocortex (Masliah, 2008). Accumulation of A $\beta$  (amyloid  $\beta$ -peptide) fragments and sustained disruption of intracellular calcium concentration ( $[Ca^{2+}]_i$ ) homeostasis are prevailing hypotheses for the pathogenesis of AD (Hardy and Selkoe, 2002; Stutzmann et al., 2004).

The role and contribution of astrocytes to the pathophysiology of AD and the molecular and signalling mechanisms that potentially underlie them are still very poorly understood. Calcium signalling in astrocytes in the form of intracellular calcium transients and intercellular calcium waves putatively play critical roles in neuronal-astrocytic bidirectional communication (Pasti et al., 1997; Scemes and

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**Abbreviations:** A $\beta$ , amyloid  $\beta$ -peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; A.U., arbitrary units; BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); DAPI, 4',6'-diamidino-2-phenylindole; FBS, foetal bovine serum; Fluo-4AM, Fluo-4 acetoxymethylester; GFAP, glial fibrillary acidic protein; KHB, Krebs Hepes buffer; PDL, poly-D-lysine.

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Giaume, 2006; Fellin, 2009), and potentially modulate neuronal activity and survival (Parri et al., 2001; Takano et al., 2006). There is mounting evidence that  $[Ca^{2+}]_i$  dysregulation in astrocytes contributes to the pathophysiology of AD. Intracellular calcium transients associated with A $\beta$  exposure in astrocytes result in calcium-dependent glutathione depletion in neurons and the formation of reactive oxygen species in astrocytes that directly produce neuronal death (Abramov et al., 2003, 2004). Cytoplasmic calcium transient changes in presenilin-transfected astrocytes induced by pharmacological treatment with ATP or glutamate in the presence of A $\beta$  required lower concentrations of these agonists for responses to manifest themselves (Johnston et al., 2006). Intercellular calcium waves in astrocyte networks *in vitro* can be mechanically induced after A $\beta$  treatment, and they travel further and faster than comparable waves induced in the absence of A $\beta$  (Haughey and Mattson, 2003). Importantly, spontaneously forming intercellular calcium waves have recently been shown *in vivo* in the APP (amyloid precursor protein)/PS1 (presenilin 1) Alzheimer's transgenic mouse model independent of neuronal hyperactivity (Kuchibhotla et al., 2009), although the nature of the experiments did not allow testing whether A $\beta$  itself was responsible and sufficient for the observed waves or whether there was a signalling effect from other cell types (e.g. microglia). Spontaneous intercellular calcium waves have not been observed *in vitro* in isolated astrocyte cultures in response to direct A $\beta$  stimulation. The consequences of these waves on neuronal physiology or the clinical manifestation of AD are not yet known.

Changes in intracellular calcium levels have also been implicated in the regulation of gene expression in both neurons and astrocytes (Dolmetsch et al., 1998; Morita et al., 2003). Hence it is possible that changes in astrocytic calcium dynamics induced by A $\beta$  may molecularly contribute to the pathogenesis of AD. Reactive astrogliosis in AD characterized by up-regulation of GFAP (glial fibrillary acidic protein) and S100B (Peskind et al., 2001; Pamplona et al., 2005) is associated with A $\beta$  plaques and the formation of abnormal neurites that contribute to the formation of neuritic plaques and neurofibrillary tangles (Casas et al., 2004). The molecular and cellular consequences of intracellular calcium dysregulation in neurons in AD and its effects on neurotoxicity are well documented (LaFerla, 2002), but little is known regarding the molecular response of astrocyte calcium signalling to A $\beta$  exposure and its effects on the cell.

In the present study, we show that A $\beta$  alone at relatively low concentrations is directly able to induce intracellular calcium transients and spontaneous intercellular calcium waves in isolated astrocytes in purified cultures, raising the possibility of a potential direct effect of A $\beta$  exposure on astrocytes *in vivo* in the Alzheimer's brain. Waves did not occur immediately after A $\beta$  treatment, but were delayed by many minutes before spontaneously forming, suggesting that intracellular signalling mechanisms required sufficient time to activate before intercellular effects at the network level become evident.

Furthermore, the dynamics of intercellular calcium waves were heterogeneous, with distinct radial or longitudinal propagation orientations. Lastly, we also show that changes in GFAP and S100B expression levels are affected by A $\beta$ -induced calcium changes differently, with GFAP being more dependent on calcium levels than S100B, which was up-regulated after exposure to A $\beta$  independent of calcium levels.

## MATERIALS AND METHODS

### Astrocyte cell culture

All experiments were carried out in accordance with approved protocols from the Institutional Animal Care and Use Committee of the University of California (San Diego, CA, U.S.A.). Primary cortical cultures were prepared from postnatal day 1 Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, U.S.A.) as previously described (Haughey and Mattson, 2003). Briefly, 1-day-old Sprague-Dawley rat pups were sacrificed by anaesthesia overdose. Their brains were removed and the cerebral cortices dissected in sterile KHB (Krebs Hepes buffer; 10 mM Hepes, 4.2 mM NaHCO<sub>3</sub>, 10 mM dextrose, 1.18 mM MgSO<sub>4</sub>·2H<sub>2</sub>O, 1.18 mM KH<sub>2</sub>PO<sub>4</sub>, 4.69 mM KCl, 118 mM NaCl and 1.29 mM CaCl<sub>2</sub>; pH 7.3). Cells were mechanically dissociated in KHB through a 70  $\mu$ m cell strainer (BD Biosciences, San Jose, CA, U.S.A.) and cultured in Modified Eagle's Medium (Cellgro/Fisher, Herndon, VA, U.S.A.) with 10% (v/v) heat-inactivated horse serum (Omega Scientific, Tarzana, CA, U.S.A.), 1% penicillin-streptomycin (Gibco/Invitrogen, Carlsbad, CA, U.S.A.) and 1% Glut-Max (Gibco/Invitrogen). Before trypsinizing the cell cultures, they were hand shaken for 5 min, resulting in the detachment of contaminating cell types such as oligodendrocytes and microglia sitting on top of the astroglial monolayer. Manual shaking resulted in microglia-free cultures similar to plates purified by shaking in an orbital shaker for at least 12 h. For both approaches, we tested for microglia using immunocytochemistry and saw no significant differences (results not shown). Astroglial cultures were trypsinized and subcultured once to obtain highly enriched astroglial cultures (>95% astrocytes) (Saura, 2007). Culture medium was changed twice per week, and all cultures were used within 2 passages. Astrocytes were trypsinized and plated on to PDL (poly-D-lysine)-coated 35 mm glass bottom dishes (MakTek, Ashland, MA, U.S.A.) at a density of  $1 \times 10^5$  cells per well. Cells were allowed to grow at 37 °C and 5% CO<sub>2</sub>/95% atmospheric air to confluency (~90%) prior to performing calcium imaging studies and immunocytochemistry.

### Calcium imaging

Cortical astrocyte cultures of ~90% confluency were washed twice with KHB and incubated with 8  $\mu$ M Fluo-4AM (Fluo-4 acetoxymethyl ester; Molecular Probes/Invitrogen, Carlsbad,

CA, U.S.A.) in KHB for 40 min at room temperature (25°C). Excess dye was removed by washing twice with KHB, and an additional incubation of 20 min at room temperature was performed to equilibrate intracellular dye concentration. Under specific experimental conditions, cells were pretreated for 30 min in 10  $\mu$ M thapsigargin (T9033; Sigma) or 10  $\mu$ M BAPTA/AM [1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); A1076; Sigma]. Calcium-free KHB with 1.5 mM BAPTA (A4926; Sigma) was also used in certain experiments. Typically, 5  $\mu$ M of A $\beta$  fragment 1–40 (A1075; Sigma), an amyloid fragment known to be neurotoxic *in vivo* and *in vitro* (Emre et al., 1992; Kowall et al., 1992), was used in the experiment, and the time point of A $\beta$  dosing was regarded as zero time. We did nine intercellular calcium experiments, with 8–10 randomly selected fields of view observed for each, during which real-time movies of 4.5 min were taken of primary astrocyte cultures at three time points (0, 15 and 30 min) under five conditions (control, A $\beta$ , A $\beta$ +thapsigargin, A $\beta$ +BAPTA/AM and A $\beta$ +BAPTA). In each experiment, 5  $\mu$ M of A $\beta$  was bath applied and 4.5 min movies were recorded at randomly chosen fields for 1.5 h and seven time points (0, 15, 30, 45, 60, 75 and 90 min post-administration of A $\beta$ ). All movies were acquired at 5 Hz using a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics, Hamamatsu City, Japan). Visualization of Ca<sup>2+</sup> indicator dye fluorescence was done using a 488 nm (FITC) filter on an Olympus IX81 inverted fluorescence confocal microscope (Olympus Optical, Tokyo, Japan) using a  $\times$ 20 UPlanFLN objective (NA=0.5; Olympus Optical). Images were acquired with custom-written LabVIEW (National Instruments, Austin, TX, U.S.A.) software developed in our laboratory. Similar experiments were performed with the non-bioactive control of A $\beta$  1–40, where we used a reverse 40–1 A $\beta$  (A2326; Sigma). Images were subsequently processed in ImageJ (<http://rsbweb.nih.gov/ij/>) and Matlab (Mathworks, Natick, MA, U.S.A.).

### Quantification of Ca<sup>2+</sup> transients

Quantification of Ca<sup>2+</sup> transients was performed using methods similar to those described previously (Yu et al., 2009). Briefly, using ImageJ [an NIH (National Institutes of Health; Bethesda, MD, U.S.A.)-funded open source JAVA-based morphometric application], individual cell fluorescence intensity versus time was extracted. The fluorescence intensity of Fluo-4AM is assumed to be proportional to calcium concentration. The data were run through a low pass filter and a running average filter of 10 frames implemented in Matlab. In order to identify individual [Ca<sup>2+</sup>]<sub>i</sub> transients, we identified periods of sustained increase in fluorescence intensity for each centroid. A first-derivative filter was used to distinguish significant and sustained increases in calcium (i.e. 10 or more consecutive frames or 2 s at 5 Hz, with positive derivative values). Cells with sustained calcium increases were considered to have transient responses. The percentage of active cells was the ratio of the number of cells

that displayed calcium transients to the total number of cells in the field of view, while the average transient frequency (in Hz) was the ratio of the total number of transients to the number of active cells acquired per movie. For the observed calcium waves, the average speed of calcium wave propagation was calculated by plotting the radius from the centre of the wave against activation time of the cell involved and fitting the slope of the line through the scatter plot. All calculations and graphs were done using Matlab and SigmaPlot (Systat, San Jose, CA, U.S.A.), and the results were expressed as means  $\pm$  S.E.M. In all the cases, consistent imaging settings were used across experimental conditions and individual experiments (e.g. frame rates, exposure times etc.).

### GFAP and S100B immunocytochemistry

Cultures grown on PDL-coated dishes were fixed with 4% (w/v) paraformaldehyde in PBS for 15 min. Cells were permeabilized in 0.2% (v/v) Triton X-100 and incubated for 2 h with mouse monoclonal anti-S100B antibody (S2657; Sigma) or mouse anti-glia fibrillary acidic protein (anti-GFAP; 556330; BD Biosciences), diluted 1:100 and 1:400 respectively with 10% (v/v) FBS (foetal bovine serum). Goat anti-mouse IgG (whole antibody)-FITC (F0257; Sigma) was diluted 1:100 in PBS with 5% FBS and applied for 1 h. Cells were mounted with ProLong Gold antifade reagent counterstained with DAPI (4',6-diamidino-2-phenylindole; Molecular Probes/Invitrogen). Images were acquired using the same hardware as described previously and a  $\times$ 40 LUCPlanFLN objective (NA=0.6; Olympus Optical), with filters for FITC, TRITC (tetramethylrhodamine  $\beta$ -isothiocyanate) and DAPI using the same customized LabVIEW software used for calcium imaging.

## RESULTS

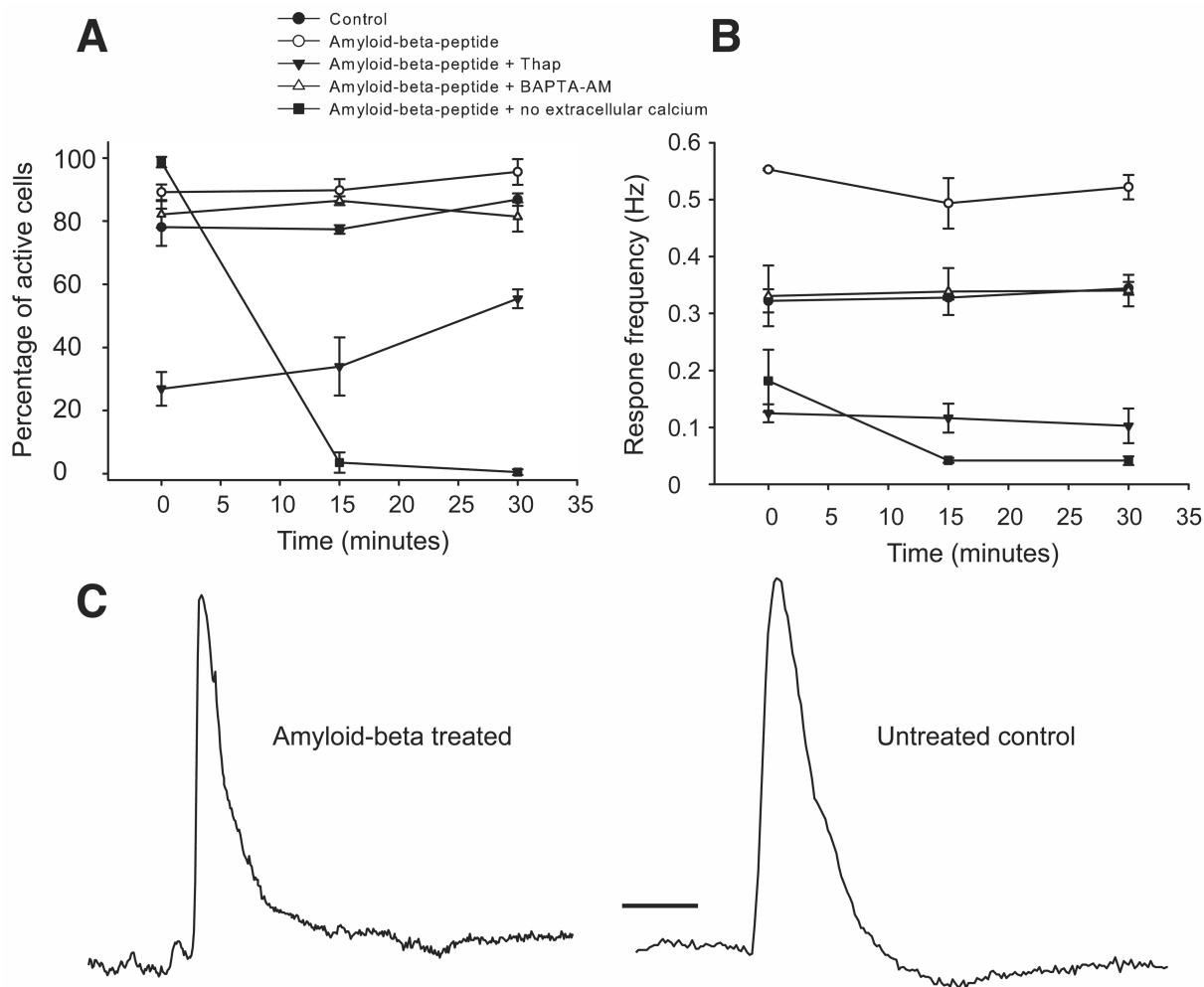
### A $\beta$ increases the frequency of intracellular calcium transients and the number of activated astrocytes

In cultured cortical astrocytes, 5  $\mu$ M of A $\beta$  1–42 is sufficient to alter evoked intercellular calcium waves and to result in increased amplitude, velocity and travelling distance compared with untreated controls (Haughey and Mattson, 2003). Dosing levels of A $\beta$  1–40, 25–35 and 1–42 at 10  $\mu$ M have been reported to increase the expression of GFAP and S100B in cultured astrocytes (Meske et al., 1998). In both studies, astrocytic toxicity from A $\beta$  at these concentrations was not observed. We began by doing a number of experiments using a range of A $\beta$  1–40 up to a concentration of 10  $\mu$ M, and did not observe differences in the results compared with the use of 5  $\mu$ M (results not shown). Given the previous work by these

other investigators and our own preliminary experiments, we used 5  $\mu$ M as the working concentration of A $\beta$  in all our subsequent experiments.

Compared with untreated controls, A $\beta$ -treated cultures had a significantly larger percentage of cells that displayed intracellular calcium transients ( $92 \pm 3.5\%$  versus  $81 \pm 5.3\%$ ,  $P < 0.05$ ; Figure 1A). Furthermore, active cells under A $\beta$ -treated conditions that showed transient responses had on average a higher frequency of responses relative to active cells in controls ( $0.05 \pm 0.003$  Hz versus  $0.033 \pm 0.001$  Hz,  $P < 0.001$ ; Figure 1B). In all cases, we measured baseline levels of calcium transient response frequencies before exposure to A $\beta$  for 90 s, and with results statistically similar to untreated A $\beta$  controls. The quality and signal-to-noise ratio of measured transient responses for both experimental and control conditions were comparable (Figure 1C). A $\beta$ -induced increases were significantly reduced by the perturbation of

intracellular and extracellular calcium sources, suggesting that the observed changes were directly due to calcium-dependent mechanisms. Pretreatment with thapsigargin, which raises cytosolic calcium levels by preventing the pumping of calcium back into the endoplasmic reticulum, diminished both the number of active cells and the transient response frequency increase evoked by A $\beta$  ( $39 \pm 14\%$  in thapsigargin-treated cells versus  $92 \pm 3.5\%$  for A $\beta$ -treated cells alone,  $P < 0.01$ ; and  $0.011 \pm 0.001$  Hz in thapsigargin-treated cells versus  $0.05 \pm 0.003$  Hz for A $\beta$ -treated cells alone,  $P < 0.001$ ). BAPTA/AM, which is a calcium chelator, completely abolished the increase in response frequency induced by A $\beta$  ( $0.033$  Hz in A $\beta$ -BAPTA/AM versus  $0.033$  Hz in controls), whereas removal of extracellular  $Ca^{2+}$  ( $[Ca^{2+}]_e$ ) caused a 2-fold decrease in the increased frequency response. In addition, a lack of  $[Ca^{2+}]_e$  sharply reduced the number of active cells that displayed calcium transients at 15 min after



**Figure 1** Calcium transient responses to A $\beta$ -treated and control primary cortical astrocyte cultures for different experimental conditions (A) The fraction of activated responding cells for the population of each experimental condition expressed as a percentage. (B) Average calcium transient response frequency per activated cell. (C) Representative examples of measured calcium transient responses in individual astrocytes for both A $\beta$ -treated and untreated control conditions. Scale bar, 5 s. Thap, thapsigargin.

A $\beta$  dosing from  $99 \pm 1.6\%$  to  $1.5 \pm 0.9\%$  ( $P < 0.001$ ), suggesting an important role for the influx of extracellular  $\text{Ca}^{2+}$  on the effects of A $\beta$ . Control experiments using the biologically inactive reverse 40–1 A $\beta$  did not show any changes in the frequency of calcium transient responses in individual cells or the fraction of active cells relative to untreated controls (results not shown). The effects of A $\beta$  on calcium transients lasted for at least 45 min after dosing. We did not use data beyond 45 min in order to avoid potential confounding effects due to unrelated changes in the cultures, such as decreasing cell viability with time, even though we routinely confirmed good cell viability after the 45 min experimental window by using a calcein/ethidium live/dead cell cytotoxicity assay.

### A $\beta$ induces spontaneous time-delayed intercellular calcium waves in astrocytes

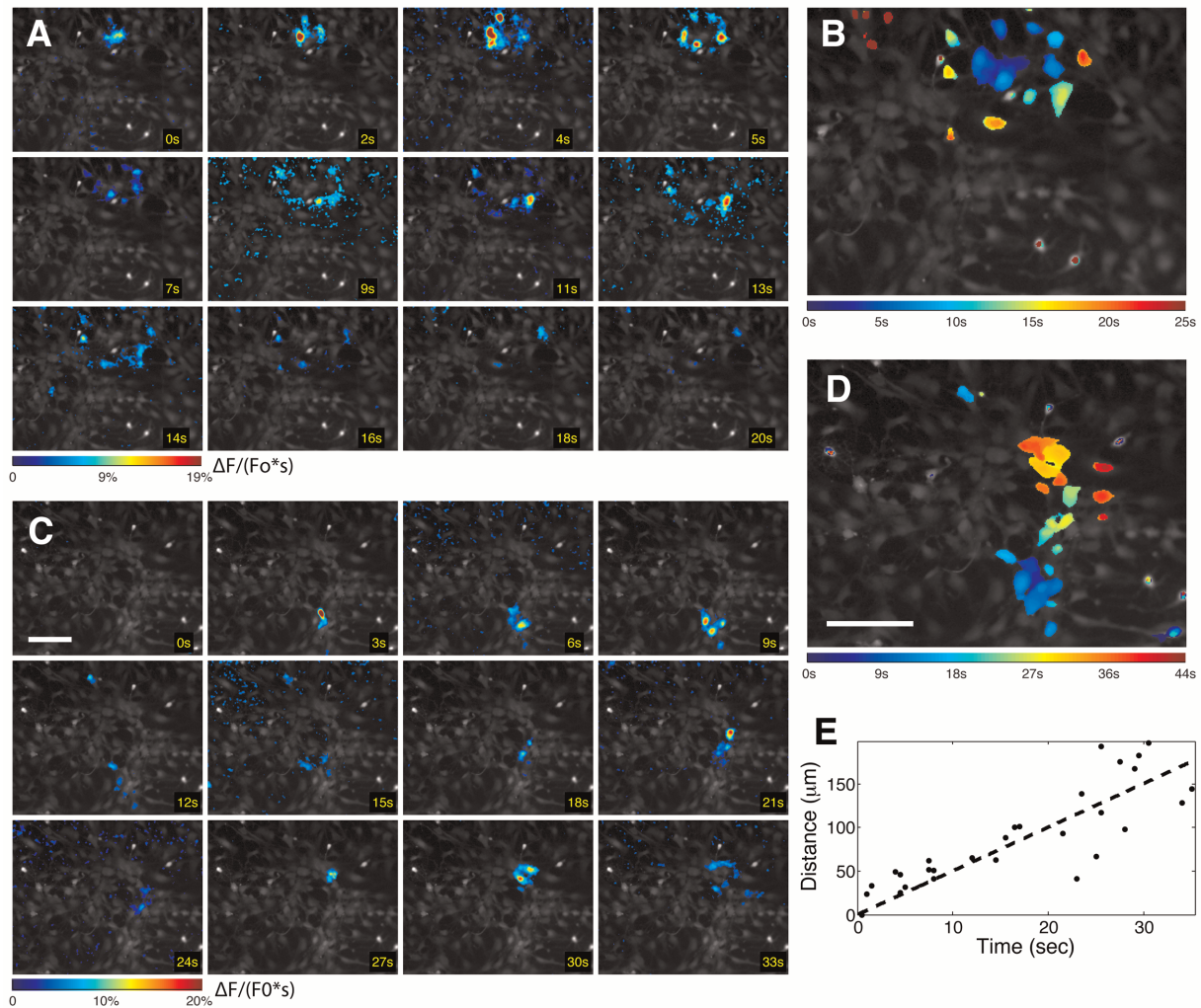
Previous work has shown that mechanically induced intercellular calcium waves in astrocyte cultures pretreated with A $\beta$  travel longer distances and at greater speeds than induced waves in control cultures not treated with A $\beta$  (Haughey and Mattson, 2003). *In vivo* in the APP/SP1 transgenic mouse model of AD, spontaneous waves in astrocyte networks have been seen (Kuchibhotla et al., 2009). We examined whether A $\beta$  by itself is sufficient to directly induce spontaneous waves in culture in isolated astrocytes. We bath applied A $\beta$  and recorded movies of randomly chosen fields of view in 15 min intervals for 90 min after A $\beta$  treatment. In contrast with untreated control cultures, which never showed any responses, all of the 8–10 randomly chosen fields in eight of the nine A $\beta$ -treated independent experiments displayed spontaneous time-delayed waves. Interestingly, two distinct patterns of calcium waves were observed: seven experiments displayed waves that propagated radially from an initial focal locus (Figures 2A and 2B), consistent with patterns of induced waves reported in most studies (Cornell-Bell et al., 1990; Newman and Zahs, 1997; Arcuino et al., 2002; Schipke et al., 2002), whereas one experiment exhibited waves that propagated linearly (Figures 2C and 2D). This is similar to *in vivo* waves seen in the cerebellum (Hoogland et al., 2009). The distances travelled by radial and linear waves were  $75 \pm 21$  and  $80.5 \pm 11.3$   $\mu\text{m}$  respectively, with speeds of  $7.8 \pm 2.6$  and  $4.66 \pm 0.48$   $\mu\text{m/s}$  respectively (Figure 2E). Both types of waves occurred not earlier than 10 min and no later than 75 min after A $\beta$  dosing. The significant delay between the application of A $\beta$  and the initiation of waves suggests that metabotropic signalling mechanisms may underlie the initiation of wave events in astrocyte networks, and excludes the possibility that they were the result of experimental artefacts (e.g. mechanical disruption of cells during bath application of A $\beta$ ). We also checked for and confirmed good cell viability after the 90 min recording period. Interestingly, some cells in the path of the wave front were not activated and did not display intracellular calcium transients, resulting in spatially heterogeneous activation patterns. This is consistent with previous results from our laboratory (Macdonald et al., 2008).

It is possible that the absence of observed waves in the one experiment could have been due to chance if they occurred outside our randomly chosen fields of view or during intervals between successive recordings, since we did not selectively induce them in any way. Control experiments using the reverse 40–1 A $\beta$  never showed spontaneous calcium waves, similar to untreated controls (results not shown).

### A $\beta$ induces changes in S100B and GFAP expression in astrocytes by 12 h

Finally, we also looked at the relationship between A $\beta$ -induced intracellular  $\text{Ca}^{2+}$  transients and reactive gliosis by measuring GFAP and S100B expression levels after A $\beta$  dosing using immunocytochemistry (Figure 3). Elevated levels of S100B and GFAP have been shown to occur in astrocytes associated with neuritic AD plaques (Pike et al., 1994; Simpson et al., 2008). Two previous studies have looked at changes in the immunocytochemical expression of GFAP, S100B, vimentin, as well as other putative markers of reactive gliosis (Salinero et al., 1997; Meske et al., 1998), although the nature of the analyses and experimental preparations make a comparison of those studies to our own results difficult: in the former study, the authors claimed that GFAP and S100B were up-regulated at 72 h after exposure to a number of A $\beta$  sequences, whereas the latter study claimed that GFAP and vimentin but not S100B were up-regulated in a subpopulation of cultured cells at 24 h after exposure to the 25–35 peptide. However, both studies used astrocytes that had been maintained in culture for very long periods of time prior to experiments, 21 and 14 days respectively, and in both cases they based their results on qualitative observations from micrographs without any quantification or image analysis. Interestingly, Meske and colleagues did not observe any changes in calcium signalling (Meske et al., 1998).

Images of 10 randomly chosen fields ( $\times 40$ ) were taken at three time points after A $\beta$  dosing (12, 24 and 48 h) under six conditions (control, A $\beta$ , A $\beta$ +thapsigargin, thapsigargin, A $\beta$ +BAPTA/AM and BAPTA/AM) in three experiments. Differences in fluorescent intensities between experimental conditions were similar at different time points, so we evaluated the data for statistical significance at the earliest time point in which we observed changes (Figure 3). We found significant changes in S100B and GFAP expression by 12 h after A $\beta$  exposure. The average fluorescence intensity of GFAP at this time point was  $492 \pm 12$  A.U. (arbitrary units) for controls and  $637 \pm 16$  for A $\beta$ -incubated cells ( $P < 0.001$ ,  $n = 10$  images per sample; see the legend to Figure 3), whereas for S100B the intensity level for A $\beta$  treated cells was  $602 \pm 21$  compared with  $435 \pm 6$  for controls. Thapsigargin and BAPTA by themselves had no effect on either GFAP or S100B expression levels relative to controls. For GFAP, the application of A $\beta$  in combination with thapsigargin or BAPTA significantly decreased the effects measured with A $\beta$  by itself and brought fluorescence levels back to those observed for untreated controls ( $458 \pm 9$  for A $\beta$  and thapsigargin and  $468 \pm 13$  for A $\beta$  and BAPTA,  $P < 0.001$  for



**Figure 2** Representative examples of the propagation of A $\beta$ -induced spontaneous intercellular calcium waves in astrocyte networks from two experiments

In most cases, waves propagated radially from an initiating focal locus (A, B). In some cases, however, waves travelled linearly in a specific direction across the field of view (C, D). (A, C) These figures show sequential frames from recorded movies as a function of time (with a time stamp in the lower right corner of each frame). Pseudo-colouring encodes the normalized relative concentration of the fluorescence signal displayed by the calcium indicator dye. (B, D) These figures are temporal projections for the propagating waves for the entire movies collapsed on to one frame overlaid on fields of view for each one. Pseudo-colouring in these figures encodes time in seconds. (E) A plot of the distance travelled by waves in microns against time in seconds for several representative experiments. Scale bars, 50  $\mu\text{m}$ .

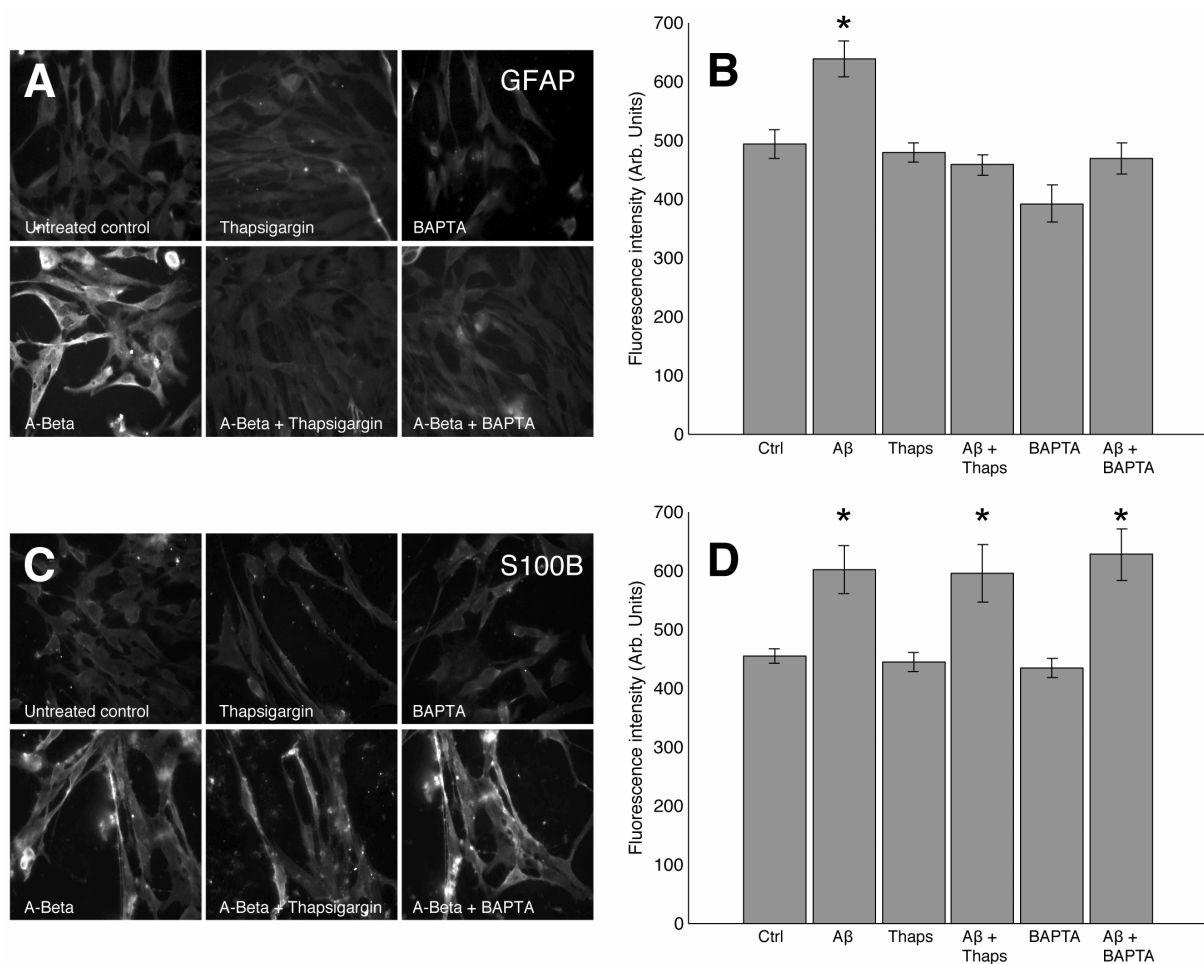
both). On the other hand, S100B expression levels did not change relative to A $\beta$  levels when challenged with A $\beta$  in combination with thapsigargin or BAPTA, which measured  $595 \pm 25$  and  $627 \pm 22$  A.U. respectively.

## DISCUSSION

We examined the effects of A $\beta$  on the dynamics of spontaneous intracellular calcium transients and intercellular calcium waves in networks of primary cortical astrocytes *in vitro*, and explored A $\beta$ -induced changes in the expression of

GFAP and S100B. A $\beta$  increased the frequency of intracellular calcium transients in individual cells and the total number of activated astrocytes. It also induced delayed spontaneous intercellular calcium waves. The recruitment of participating cells and the dynamics of observed waves were spatially heterogeneous, in the sense that not all cells within the area affected by the wave were necessarily activated, and waves displayed distinct radial or longitudinal directional propagation patterns. We also showed that changes in GFAP and S100B expression levels are affected by A $\beta$  calcium levels (Inoue, 2008; Small et al., 2009; Smith et al., 2009).

Our present results are the first to demonstrate that A $\beta$  is able to produce temporally delayed spontaneous intercellular



**Figure 3** Changes in immunocytochemical labelling for GFAP (A, B) and S100B (C, D) intermediate filament proteins 12 h after dosing for different experimental conditions (B, D) Shown are mean fluorescence values, with the error bars representing 95% confidence bounds in A.U. based on image analysis of the micrographs (see the Materials and methods section). Western blot gels were done to confirm protein expression for the different conditions but we did not optimize for or quantify protein levels using this method. Fluorescence data were acquired using the same exposure and gain setting in every case, and the mean fluorescence intensities of the cells were extracted as recorded on the camera for each image. The camera sensor recorded intensity values on a 12-bit scale in A.U., from 0 to 4095. The results shown are means  $\pm$  S.E.M. \* $P < 0.001$ .

calcium waves in isolated astrocytes independent of the contribution of other putative signalling cues. Astrocytic intercellular calcium waves have previously been associated with neuropathological phenomena such as spreading depression (Haughey and Mattson, 2003; Peters et al., 2003). A $\beta$ -induced waves in our experiments exhibited propagation distances and speeds similar to previously reported values in the range of 20–100  $\mu$ m and 5–20  $\mu$ m/s (Dani et al., 1992; Zanotti and Charles, 1997; Hamilton et al., 2008). The spatial heterogeneity of advancing wave fronts observed in both radial and longitudinal waves, in which not all astrocytes within the range of a passing wave were necessarily activated or displayed intracellular calcium transients, suggests that functional signalling through astrocyte networks is not a passive phenomenon but spatially and functionally complex (Sul et al., 2004; Macdonald et al., 2008). This is consistent with

previous experimental and theoretical work from our laboratory (Macdonald et al., 2008; Yu et al., 2009). Mechanistically, the generation and modulation of these waves is not clear and will require further investigation. For example, ATP release from astrocytes is enhanced in the presence of calcium and is stored and released from dense core granules independent of gap junctional release (Coco et al., 2003), which could putatively play a role in modulating A $\beta$ -induced calcium waves. Indeed, ATP vesicular release has been implicated as the principal mechanism underlying intercellular calcium waves in astrocyte networks, with a minimal contribution through gap junctions (Coco et al., 2003; Bowser and Khakh, 2007; Pangrsic et al., 2007; Zhang et al., 2007).

These results complement the work by Kuchibhotla and colleagues (Kuchibhotla et al., 2009). These authors eliminated putative effects of A $\beta$ -induced neuronal hyperactivity on

astrocytic calcium signalling by blocking neuronal activity with TTX (tetrodotoxin), concluding that the resultant observed astrocytic calcium waves were due to the direct effect of A $\beta$  on the astrocyte network. However, microglia could also modulate astrocytic calcium signalling independent of any neuronal contribution, and have been shown to migrate and cluster near A $\beta$  plaque-forming areas in the hippocampus and neocortex (Frautschy et al., 1998; Stalder et al., 1999; Hasegawa et al., 2001). Microglia internalize microaggregates of A $\beta$  and secrete pro-inflammatory factors including IL-1 $\beta$  (interleukin-1 $\beta$ ), TNF (tumour necrosis factor) and nitric oxide, which in turn affect calcium homeostasis and transients in surrounding neurons and astrocytes (Meda et al., 1995; Weldon et al., 1998; Colton et al., 2000; Benveniste et al., 2001; Lee et al., 2002; Morita et al., 2003). Furthermore, microglia derived from AD patients show significantly higher basal [Ca<sup>2+</sup>]<sub>i</sub> (McLarnon et al., 2005), and can also cause calcium elevations in neighbouring cells by regulating the amount of extracellular calcium (Barger and Basile, 2001; McLarnon et al., 2006). Our results here show that astrocytes by themselves are capable of spontaneously forming signalling networks by initiating and propagating intercellular calcium waves under stress conditions similar to those present in AD in the absence of signalling factors and cues from other cell types. The shorter distance and lower speed of the waves in our experiments compared with those reported by Kuchibhotla et al. (2009) may be due to an augmented effect caused by other cell types or signalling factors *in vivo* missing from the reduced *in vitro* preparations we studied. The molecular and cellular mechanisms that produce these waves and any effects on the pathophysiology of AD are yet to be confirmed *in vivo* and will require much further investigation.

Lastly, in our experiments, A $\beta$  induced changes in the protein expression of both S100B and GFAP by 12 h after exposure to the peptide. Measured relative changes of normalized fluorescence intensity levels of anti-GFAP and anti-S100B signals in immunocytochemistry have been used as reliable markers associated with reactive gliosis (Dyer and Cepko, 2000; Wu et al., 2003; Pathak et al., 2009). We also observed a dependency between [Ca<sup>2+</sup>]<sub>i</sub> and the expression levels of GFAP after exposure to A $\beta$ . Reducing [Ca<sup>2+</sup>]<sub>i</sub> by chelating with BAPTA or applying thapsigargin reduced GFAP levels to those measured under control conditions. Thus it appears that changes in GFAP expression are dependent on changes in calcium homeostasis induced by A $\beta$ , rather than a direct effect of A $\beta$  itself. S100B levels, on the other hand, were not affected by application of BAPTA or thapsigargin, suggesting a potentially more direct effect of A $\beta$  on S100B up-regulation. Interestingly, GFAP plays a role in trafficking of dense core vesicles containing ANF (atrial natriuretic factor) (Pangrsic et al., 2007); and since intercellular calcium waves in astrocytes are mediated primarily by vesicular ATP, this could play a role in the pathological propagation of signals throughout the astrocytic network.

The pathophysiology of AD is multidimensional and complex. While previous efforts have focused on the effects of A $\beta$  on neurons, the results we present here add to other work implicating a potential, but as yet poorly understood,

role for astrocytes in the pathogenesis of AD. The picture that is emerging, however, is one where astrocytes directly and robustly respond to the presence of A $\beta$  in a number of ways known to affect the health and function of neurons. It is likely that a complete understanding of the pathology of AD will require an understanding of the metabolic and functional responses and effects that astrocytes have. Such an understanding may suggest novel pharmacological targets to either slow down or reverse the resultant neuronal death.

## FUNDING

This work was supported by funds from the NINDS (National Institute of Neurological Disorders and Stroke) at the NIH [National Institutes of Health; NS054736].

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Received 29 June 2009/23 November 2009; accepted 8 December 2009

Published as Immediate Publication 14 December 2009, doi 10.1042/AN20090035