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Alzheimer's disease-associated peptide $A\beta_{42}$ mobilizes ER Ca^{2+} via $InsP_3R$-dependent and -independent mechanisms

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Dysregulation of Ca^{2+} homeostasis is considered to contribute to the toxic action of the Alzheimer's disease (AD)-associated amyloid-β-peptide (Aβ). Ca^{2+} fluxes across the plasma membrane and release from intracellular stores have both been reported to underlie the Ca^{2+} fluxes induced by $A\beta_{42}$. Here, we investigated the contribution of Ca^{2+} release from the endoplasmic reticulum (ER) to the effects of $A\beta_{42}$ upon Ca^{2+} homeostasis and the mechanism by which $A\beta_{42}$ elicited these effects. Consistent with previous reports, application of soluble oligomeric forms of $A\beta_{42}$ induced an elevation in intracellular Ca^{2+}. The $A\beta_{42}$-stimulated Ca^{2+} signals persisted in the absence of extracellular Ca^{2+} indicating a significant contribution of Ca^{2+} release from the ER Ca^{2+} store to the generation of these signals. Moreover, inositol 1,4,5-trisphosphate ($InsP_3$) signaling contributed to $A\beta_{42}$-stimulated Ca^{2+} release. The Ca^{2+} mobilizing effect of $A\beta_{42}$ was also observed when applied to permeabilized cells deficient in $InsP_3$ receptors, revealing an additional direct effect of $A\beta_{42}$ upon the ER, and a mechanism for induction of toxicity by intracellular $A\beta_{42}$.

Keywords: Alzheimer's disease, Aβ oligomers, calcium/Ca^{2+}, $InsP_3/IP_3$, $InsP_3$ receptors/$InsP_3Rs$, endoplasmic reticulum/ER

INTRODUCTION

Alzheimer's disease (AD) is a progressive and irreversible brain disorder, which results in severe memory loss, behavioral as well as personality changes and a decline in cognitive abilities. While the most common type of AD remains idiopathic in origin, with age the most significant risk factor for disease onset (sporadic AD, sAD), ∼5% of cases show a Mendelian pattern of inheritance (familial AD, fAD). The amyloid β-peptide (Aβ) is hypothesized to be central to the pathogenesis of both sporadic and familial AD (Hardy and Selkoe, 2002). Aβ is a small, hydrophobic polypeptide, consisting of 39–42 amino acid residues, which occurs principally as a 40 or 42 amino acid peptide, $A\beta_{40}$ and $A\beta_{42}$, respectively (Zhang et al., 2011). An imbalance between the production and clearance of Aβ, as occurs in fAD and sAD, respectively, leads to the accumulation of Aβ and, in turn, to its aggregation. This aggregation process represents a critical step in the pathogenesis of AD because the neurotoxic properties of Aβ are associated only with aggregated forms of the peptide (Kuperstein et al., 2010). Protein aggregation is highly dynamic and involves a wide range of intermediate structures such as oligomers, comprising dimers, trimers, dodecamers, and higher-molecular weight complexes, before aggregating into protofibrils and finally into mature amyloid fibrils (Dobson, 2003).

A mounting body of evidence now suggests that soluble oligomeric forms of Aβ constitute the primary neurotoxic species rather than monomers or fibrils (Lambert et al., 1998; Chromy et al., 2003; Gong et al., 2003; Demuro et al., 2005; Klyubin et al., 2005). Soluble oligomers have proved toxic when applied to cultured cells and primary neuronal cultures *in vitro* (Lambert et al., 1998; Bucciantini et al., 2002; Dahlgren et al., 2002; Kayed et al., 2003; Whalen et al., 2005). In addition, they are capable of inducing cognitive deficits when administered *in vivo* (Cleary et al., 2005; Rowan et al., 2007) and adversely affect hippocampal LTP *in vivo* (Walsh et al., 2002; Cleary et al., 2005; Klyubin et al., 2009, 2011).

Dysregulation of intracellular Ca^{2+} homeostasis is associated with cell exposure to Aβ and likely underlies its neurotoxic effects (Bezprozvanny and Mattson, 2008; Green and Laferla, 2008; Berridge, 2010; Demuro et al., 2010). A number of mechanisms by which Aβ elicits its effects on intracellular Ca^{2+} homeostasis have been put forward. These include direct effects on the plasma membrane, where it has been proposed to destabilize its structure (Mueller et al., 1995; Mason et al., 1996), to induce a generalized increase in membrane permeability (Bucciantini et al., 2002; Kayed et al., 2003) or to insert into the membrane forming cation-conducting pores (Arispe et al., 1993; Mueller et al., 1995; Mason et al., 1996; Bucciantini et al., 2002; Kayed et al., 2003; Kawahara, 2004; Simakova and Arispe, 2006; Arispe et al., 2007; Demuro et al., 2011). Aβ has also been reported to activate plasma membrane receptors, including *N*-methyl-d-aspartate (NMDA) receptors coupled to Ca^{2+} influx (Guo et al., 1996; Dobson,

2003; Blanchard et al., 2004; De Felice et al., 2007), to alter neuronal excitability which, in turn, influences the extent of Ca^{2+} influx (Good et al., 1996) and to induce dysregulation of endoplasmic reticulum (ER) Ca^{2+} homeostasis (Ferreiro et al., 2004, 2006; Resende et al., 2008). In addition to acting from the extracellular space, where it accumulates in the diseased brain, Aβ also has an intracellular site of action (Wirths et al., 2004). Indeed, as a result of uptake from the extracellular space or via its intracellular synthesis and processing, Aβ has been reported to accumulate within the cell (Pierrot et al., 2004; Bayer and Wirths, 2011; Kaminski Schierle et al., 2011). This intracellular Aβ is also neurotoxic and has been shown to target the ER and the mitochondria, inducing a stress response and causing permeability transition, respectively (Yao et al., 2009; Umeda et al., 2011).

In this study, we investigated (1) the contribution of Ca^{2+} mobilization from the ER to the increase in intracellular Ca^{2+} induced by oligomeric $A\beta_{42}$, (2) the mechanism (s) by which $A\beta_{42}$ elicited this effect, (3) the capacity for $A\beta_{42}$ to mobilize Ca^{2+} directly from the ER. To allow isolation of effects on the ER from other plasma membrane targets of $A\beta_{42}$, model cells systems were used that allowed fundamental aspects of ER Ca^{2+} regulation to be studied. We determined that Ca^{2+} release from the intracellular ER substantially contributed to the increase in intracellular Ca^{2+} concentration induced by oligomeric $A\beta_{42}$. The $A\beta_{42}$-induced Ca^{2+} elevation comprised $InsP_3$ dependent and independent components. Using DT40 cells deficient in the three $InsP_3R$ isoforms that were permeabilized to allow direct access of $A\beta_{42}$ to the ER, we also demonstrated that it had the capacity to release Ca^{2+} from the ER independent of $InsP_3Rs$. Together, these data place the ER and Ca^{2+} released from it as central to the actions of both extracellular Aβ and Aβ that has reached an intracellular location.

MATERIALS AND METHODS

MATERIALS

Peptides were purchased from The American Peptide Company and rPeptide. Cell culture reagents and chemicals were from Invitrogen or Sigma, unless otherwise stated.

CELL CULTURE

Human neuroblastoma SH-SY5Y cells were cultured in F-12 Nutrient Mixture (Ham) containing FBS (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), non-essential amino acids (0.1 mM), and L-glutamine (2 mM). Prior to all experiments, SH-SY5Y cells were cultured overnight in Opti-MEM Reduced Serum Medium, containing FBS (1.5%), penicillin (100 units/ml), streptomycin (1.0 μg/ml), non-essential amino acids (0.1 mM), and L-glutamine (2 mM). For live-cell Ca^{2+} imaging experiments, cells were plated onto poly-L-lysine-coated coverslips at a density of 3.2×10^4 cell/cm^2. For the MTT reduction assay, cells were plated at a density of 9×10^3 cells/cm^2. To overexpress GFP-tagged type 1 $InsP_3$ 5′-Phosphatase (GFP-5′P) or GFP (Peppiatt et al., 2004; Higazi et al., 2009), cells were infected with adenovirus for 8 h prior to overnight culture. Culture of DT40 cells and DT40 cells deficient in the three $InsP_3R$ isoforms (DT40 TKO) was performed as previously described (Tovey et al., 2006).

PREPARATION OF $A\beta_{42}$ OLIGOMERS

Wild type and scrambled $A\beta_{42}$ were obtained at a purity of >95%. Peptide mass was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and peptides from the same batch were used throughout. Samples of synthetic $A\beta_{42}$ oligomers were prepared as previously described (Demuro et al., 2005) and remained stable for at least 3 weeks. Samples of $A\beta_{1-42}$ scrambled peptide (KVKGLIDGAHIGDLVYEFMDSN SAIFREGVGAGHVHVAQVEF) were prepared in the same way as $A\beta_{42}$ oligomers. All Aβ samples were stored at 4°C and were used within 10–15 days of preparation. Toxicity of $A\beta_{42}$ preparations was confirmed by MTT assay before use in Ca^{2+} imaging experiments (**Figure S1A**). The oligomeric nature of the $A\beta_{42}$ preparation was established by surface plasmon resonance (SPR) spectroscopy using an antibody specific to oligomeric $A\beta_{42}$ (**Figure S1B**). All $A\beta_{42}$ concentrations stated are based on the molar mass of the peptide.

LIVE CELL Ca^{2+} IMAGING

Methods for single cell analysis of intracellular Ca^{2+} concentration were as previously described (Peppiatt et al., 2003). Cells were loaded at 37°C with 2 μM of the acetoxymethyl (AM) ester form of fura-2 for 30 min followed by an equivalent period in dye free media to allow de-esterification of the indicator. Imaging experiments were performed using either Ca^{2+}-containing (121 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 6 mM $NaHCO_3$, 25 mM HEPES, 5.5 mM glucose, pH 7.3) or Ca^{2+} free (as for Ca^{2+} containing with 1.8 mM $CaCl_2$ replaced with 1 mM EGTA) buffer as indicated. Fura-2 imaging was carried out using an imaging system configured around a Nikon TE300 inverted epi-fluorescence microscope equipped with a 20 × 0.75 NA multi-immersion objective. Samples were illuminated by alternate excitation at 340 and 380 nm using a Sutter filter changer (340HT15 and 380HT15; Sutter Industries) and emitted light was filtered at >460 nm (1 ratio pair per 2 s). Images were captured using a Hamamatsu ORCA ER CCD camera. The imaging system was controlled with Ultraview software (PerkinElmer Life Sciences Ltd., UK). Acquired images were processed with Ultraview software and analyzed in MATLAB. Background subtracted fura-2 ratios were calibrated according to standard procedures (Grynkiewicz et al., 1985), using the maximum and minimum ratio values obtained through exposing cells sequentially to Ca^{2+} free and Ca^{2+} containing imaging buffer to which 2 μM ionomycin had been added. Parameters analyzed from the Ca^{2+} responses included the peak amplitude, the time to peak and the integral of the response (the area under the curve) and the percentage of responding cells.

$InsP_3$-induced Ca^{2+} release (IICR) from permeabilized wild type and $InsP_3R$ null DT40 cells (three $InsP_3R$ isoforms deleted by homologous recombination; DT40 TKO) (Sugawara et al., 1997) was performed as previously described (Tovey et al., 2006). Briefly, the ER of cells was loaded with the low-affinity Ca^{2+} indicator mag-fluo-4 and Aβ-induced Ca^{2+} release was measured

from the saponin-permeabilized cells using a fluorescence plate reader (FlexStation 3, Molecular Devices).

MTT REDUCTION ASSAY

The Cell Titer 96 Non-Radioactive Cell Proliferation Assay (Promega) was used to validate the cytotoxic effect of $A\beta_{42}$ on SH-SY5Y cells and was performed according to manufacturer's instructions. Briefly, cells were incubated with $A\beta_{42}$ ($n = 4$) for 24 h prior to the addition of the MTT dye solution and a further 4 h incubation at 37°C, 5% CO_2. Thereafter, the solubilization/stop solution was added and incubated overnight at room temperature. Absorbances were read at 570 nm with a reference wavelength of 650 nm using a fluorescence plate reader (Synergy HT, BIO-TEK). The data is expressed as the percentage of MTT reduction relative to both live- and dead-cell controls and thus represents the percentage of viable cells. $A\beta_{42}$ samples were considered to be toxic if 25–40% of cells remained metabolically healthy at an $A\beta_{42}$ concentration of 1 μM and if more than 50% remained metabolically healthy at a concentration of 100 nM.

STATISTICAL ANALYSIS

Data is presented as the mean value of the combined datasets ± SEM. Statistical significance was determined by Student's *t*-test (two-tailed). Data was accepted as significant when $p < 0.05$ and is denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

RESULTS

INTRACELLULAR Ca^{2+} IS ELEVATED IN CELLS EXPOSED TO OLIGOMERIC $A\beta_{42}$

Experiments were first performed to validate the Ca^{2+} mobilizing properties of oligomeric $A\beta_{42}$ over the concentration range of its toxicity. Application of $A\beta_{42}$ spanning its cytotoxic range (1, 5 and 10 μM) caused an elevation in intracellular Ca^{2+} (**Figure 1A**). The increase in cytosolic Ca^{2+} concentration immediately followed the addition of $A\beta_{42}$, developed to a peak within minutes of application and subsequently returned to baseline, despite the continued presence of the peptide. No Ca^{2+} responses were detected when $A\beta_{42}$ below 1 μM was applied (data not shown). Between 1 μM and 10 μM Aβ, the number of responding cells, the peak amplitude and the integral of the Ca^{2+} responses increased in a concentration-dependent manner. The number of responding cells reached 100% at 5 μM $A\beta_{42}$ (**Figures 1Bi,iii,v**). To test cell viability as well as to determine whether metabotropic Ca^{2+} signaling was affected by Aβ, carbachol (CCH) was applied subsequent to Aβ. CCH elicited Ca^{2+} responses in 100% of cells pre-exposed to 1 or 5 μM oligomeric $A\beta_{42}$ or to a vehicle control (10%) (**Figures 1Bii,iv,vi**). At 10 μM Aβ, however, the number of cells responding to CCH was significantly reduced (**Figure 1Bii**). The peak amplitude and integral of the Ca^{2+} responses to CCH subsequently applied were inversely related to the magnitude of the Ca^{2+} responses elicited by oligomeric $A\beta_{42}$ (**Figures 1Biv,vi**). This observation suggested that exposure to $A\beta_{42}$ oligomers was depleting the intracellular CCH-sensitive ER Ca^{2+} store. These Ca^{2+} mobilizing effects of oligomeric $A\beta_{42}$ were significantly greater than observed in cells exposed to $A\beta_{42}$ that had been prepared in a manner to yield a monomeric form of the peptide (**Figures S2, S1B**). From these results, due to its potency in mobilizing Ca^{2+} whilst preserving agonist responses, a concentration of 5 μM oligomeric $A\beta_{42}$ was selected for use in subsequent experiments.

$A\beta_{42}$ OLIGOMER-INDUCED Ca^{2+} TRANSIENTS ARE PEPTIDE SEQUENCE SPECIFIC

As a control for the application of peptide, experiments were also performed using a scrambled Aβ sequence, which had been prepared in the same manner as the wild type $A\beta_{42}$. Although significantly less toxic than the wild type sequence (**Figure S1A**), scrambled Aβ peptide also evoked Ca^{2+} responses in all cells (**Figure 2Ai**). However, consistent with its lower toxicity, both the amplitude and the integral of the Ca^{2+} transients elicited by scrambled Aβ were significantly lower than those induced by oligomeric $A\beta_{42}$ and, in addition, they required a significantly longer time to reach peak (**Figures 2Bi,Ci,Di**). Furthermore, concordant with the less potent effect of scrambled Aβ in mobilizing intracellular Ca^{2+}, the amplitude and integral of CCH-induced Ca^{2+} transients elicited following prior exposure to scrambled Aβ were significantly greater than those stimulated following prior exposure to oligomeric $A\beta_{42}$ (**Figures 2Bii,Cii,Dii**).

Taken together, the comparison of the effects of Aβ scramble and oligomeric $A\beta_{42}$ demonstrates that the amino acid sequence of $A\beta_{42}$ has potent Ca^{2+} mobilizing properties, which are distinct from the action of Aβ scramble.

$A\beta_{42}$ OLIGOMERS MOBILIZE Ca^{2+} FROM INTRACELLULAR STORES

The reduced magnitude of CCH-induced Ca^{2+} signals observed in cells previously exposed to oligomeric $A\beta_{42}$ suggested that this form of $A\beta_{42}$ was exerting an effect on intracellular Ca^{2+} stores. Therefore, we tested the relative contributions of Ca^{2+} influx from the extracellular space and its release from intracellular stores to $A\beta_{42}$-induced Ca^{2+} transients.

To determine the contribution of extracellular Ca^{2+} and Ca^{2+} influx to $A\beta_{42}$ oligomer-induced Ca^{2+} transients, we performed experiments using Ca^{2+}-free imaging buffer. Under these conditions, $A\beta_{42}$ oligomers retained their ability to induce Ca^{2+} responses, with 100% of cells responding (**Figure 3Ai**). While no significant difference was observed in the peak amplitude (**Figure 3Aiii**) of $A\beta_{42}$ oligomer-induced Ca^{2+} transients, the integral of the response was significantly decreased in the absence of extracellular Ca^{2+} (**Figure 3Av**).

In contrast to the $A\beta_{42}$ oligomer-induced Ca^{2+} response, the peak amplitude and the integral of the Ca^{2+} responses to CCH applied following $A\beta_{42}$ oligomer exposure were significantly decreased by removal of extracellular Ca^{2+} from the imaging buffer (CCH, after $A\beta_{42}$; **Figures 3Aiv,vi**). This effect on the CCH-induced Ca^{2+} responses is likely due to lack of store-operated Ca^{2+} entry, which would replenish the Ca^{2+} released from stores by $A\beta_{42}$. Indeed, the peak amplitude and the integral of CCH-induced Ca^{2+} responses elicited in Ca^{2+} free buffer were significantly greater in naïve cells (CCH, no $A\beta_{42}$) than when $A\beta_{42}$ oligomers were previously applied (**Figures 3Aiv,vi**). Since $A\beta_{42}$ oligomer-induced Ca^{2+} transients were not significantly affected by removal of extracellular Ca^{2+}, these results suggest

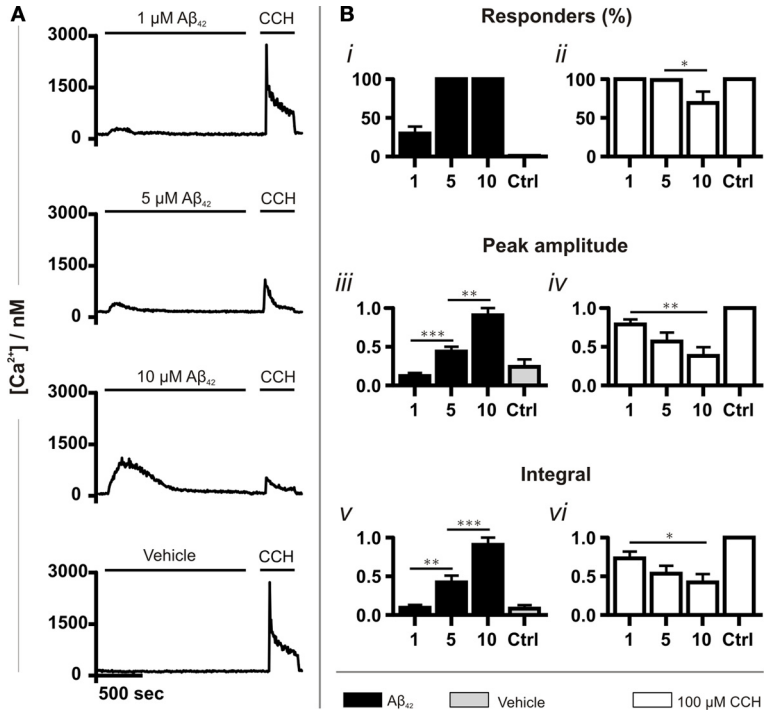

FIGURE 1 | $A\beta_{42}$ oligomers induce Ca^{2+} transients in a concentration-dependent manner. (A) Example fura-2 Ca^{2+} traces of SH-SY5Y cells exposed to a concentration range of $A\beta_{42}$ oligomers followed by 100 μM CCH. A trace taken from cells in which $A\beta_{42}$ oligomers were substituted with double-distilled water (dd H_2O; vehicle) is also shown (for each group, $n > 744$ cells). **(B)** Quantitative analysis of the Ca^{2+} responses illustrated in A. The magnitude of Ca^{2+} responses elicited by $A\beta_{42}$ oligomers, dd H_2O and CCH is presented as **(Bi,ii)** percentage of responding cells, **(Biii,iv)** peak amplitude and **(Bv,vi)** integral of the response. $A\beta_{42}$ oligomer-induced Ca^{2+} transients were normalized to the responses induced with the highest concentration (10 μM) of the respective $A\beta_{42}$ preparation. CCH-induced Ca^{2+} responses were normalized to control experiments conducted on the same experimental day. Bar graphs are mean ± SEM from at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

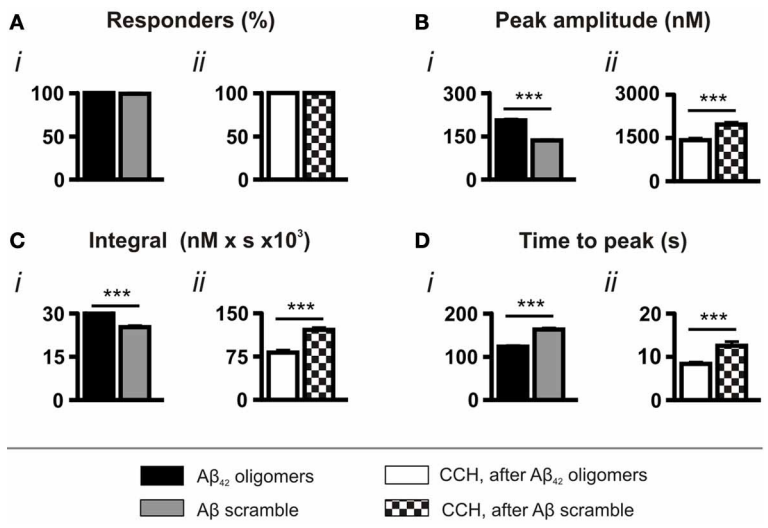

FIGURE 2 | $A\beta_{42}$ oligomer-induced Ca^{2+} transients are sequence specific. Bar charts illustrating the magnitude of Ca^{2+} responses elicited by SH-SY5Y cells following the application of 5 μM $A\beta_{42}$ oligomers or Aβ scramble and 100 μM CCH ($n > 370$ cells). Data is presented as **(A)** percentage of responding cells, **(B)** peak amplitude, **(C)** integral of the response, **(D)** time to peak. Bar graphs are mean ± SEM from at least three independent experiments. $^{***}p < 0.001$.

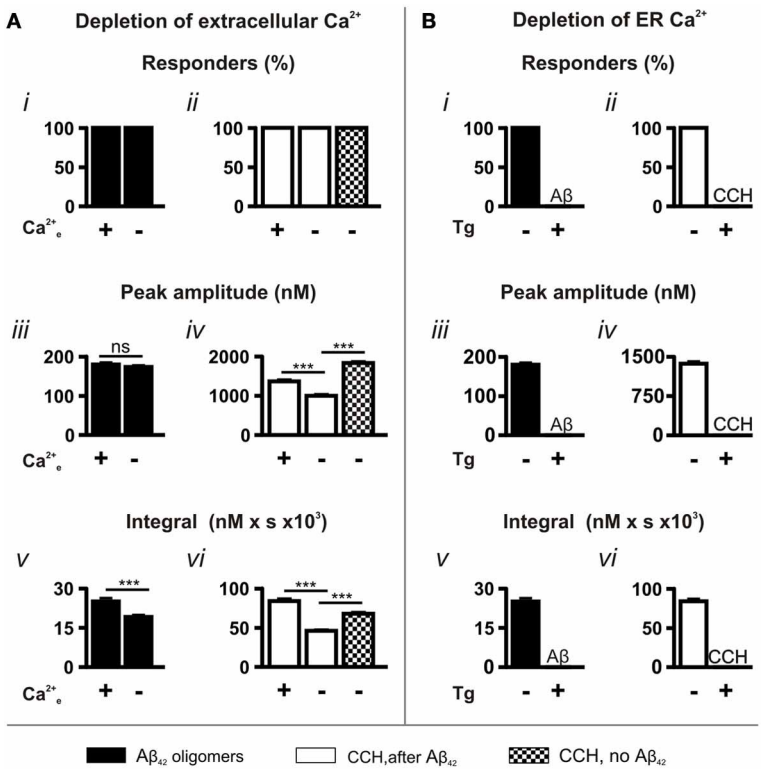

FIGURE 3 | $A\beta_{42}$ oligomer-induced Ca^{2+} transients arise through release from the ER Ca^{2+} store. Bar charts illustrating the magnitude of Ca^{2+} responses elicited following the manipulation of **(A)** extracellular ($n > 218$ cells) and **(B)** ER Ca^{2+} concentrations ($n > 512$ cells). The magnitude of Ca^{2+} responses elicited by 5 μM $A\beta_{42}$ oligomers and 100 μM CCH is presented as **(i,ii)** percentage of responding cells, **(iii,iv)** peak amplitude and **(v,vi)** integral of the response. Bar graphs are mean ± SEM from at least three independent experiments. $^{***}P < 0.001$.

that oligomeric $A\beta_{42}$ and CCH mobilize Ca^{2+} from a common intracellular Ca^{2+} pool.

The requirement of Ca^{2+} release from the ER Ca^{2+} store for the Ca^{2+} transients elicited by Aβ-induced was next investigated. To this end, ER Ca^{2+} stores were depleted by exposure of cells the SERCA pump inhibitor thapsigargin (Tg; 2 μM, 15 min) prior to the application of $A\beta_{42}$. In the absence of replete ER Ca^{2+} stores, $A\beta_{42}$-induced Ca^{2+} transients were completely abrogated (**Figures 3Bi,iii,v**). Similarly, CCH-induced Ca^{2+} responses were eliminated in Tg-treated cells (**Figures 3Bii,Biv,Bvi**), confirming the effect of Tg. Taken together, these experiments establish that $A\beta_{42}$ oligomers mobilize Ca^{2+} from the ER.

$A\beta_{42}$-INDUCED Ca^{2+} RELEASE OCCURS IN PART THROUGH $INSP_3Rs$

Having determined that $A\beta_{42}$ oligomers mobilize Ca^{2+} from the intracellular ER Ca^{2+} store, we aimed to identify the mechanism by which Ca^{2+} release occurs. We therefore tested whether $A\beta_{42}$ was causing Ca^{2+} release from the ER through activation of $InsP_3R$ or ryanodine receptor (RyR) Ca^{2+} release channels localized to this organelle.

Although SH-SY5Y cells have been reported to express functional RyRs, application of caffeine (10 mM), an agonist of the three RyR isoforms (10 mM) did not elicit a Ca^{2+} response in the SH-SY5Y cells used in this study (**Figure S2A**). Furthermore, the neuronally-expressed type 2 RyR could not be detected by immunoblot analysis (**Figure S2B**). Based on these observations, a role for RyR2 in $A\beta_{42}$ oligomer-mediated Ca^{2+} release was ruled out.

SH-SY5Y cells express $InsP_3Rs$ and elicit robust Ca^{2+} responses to $InsP_3$-generating agonists including CCH (Tovey et al., 2001) (**Figures 1–3**). Therefore, we focused our investigation on the contribution of $InsP_3Rs$ to $A\beta_{42}$-induced Ca^{2+} transients. To abrogate $InsP_3$-mediated Ca^{2+} responses, $InsP_3$ signaling was inhibited pharmacologically with 10 mM caffeine (Parker and Ivorra, 1991; Bezprozvanny et al., 1994) or was prevented by adenoviral-mediated overexpression of GFP-5′P, which metabolizes the second messenger $InsP_3$ to inactive $InsP_2$ (Higazi et al., 2009). To exclude the contribution of Ca^{2+} influx to the $A\beta_{42}$ oligomer-induced Ca^{2+} transients, these experiments were performed in the absence of extracellular Ca^{2+}.

Caffeine application did not affect the number of cells exhibiting Ca^{2+} responses following $A\beta_{42}$ oligomer application, with 100% of cells responding (**Figure 4B**). However, caffeine significantly decreased the peak amplitude and the integral of the $A\beta_{42}$ oligomer-induced Ca^{2+} transients (**Figure 4B**). In contrast, Aβ scramble-induced Ca^{2+} transients were unaffected by caffeine application (**Figure 4C**). Ca^{2+} responses to 0.5 μM CCH were abolished by caffeine, demonstrating its inhibitory effect upon IICR (**Figures 4A–C**).

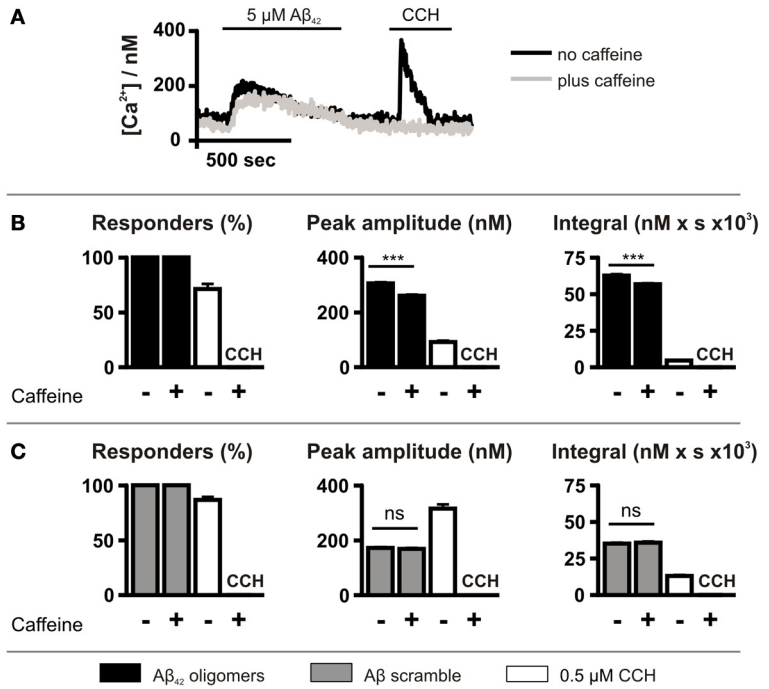

FIGURE 4 | $A\beta_{42}$ oligomer-induced Ca^{2+} release is sensitive to caffeine. **(A)** Imaging protocol employed to investigate the involvement of $InsP_3Rs$ in $A\beta_{42}$ oligomer-mediated Ca^{2+} release from the ER. $InsP_3Rs$ were inhibited by co-administration of caffeine. **(B,C)** Bar charts illustrating the magnitude of Ca^{2+} responses elicited by SH-SY5Y cells following the application of 5 μM $A\beta_{42}$ oligomers ($n > 780$ cells) **(B)** or Aβ scramble ($n > 144$ cells) **(C)** and 0.5 μM CCH ($n = 512$ cells) in the presence or absence of 10 mM caffeine. Data is presented as percentage of responding cells, peak amplitude and integral of the response. Bar graphs are mean ± SEM from at least three independent experiments. $^{***}P < 0.001$.

Although caffeine inhibits $InsP_3Rs$ (Bezprozvanny et al., 1994), it also acts on targets other than the $InsP_3R$ such as cyclic nucleotide phosphodiesterases and phospholipase C (PLC) (Toescu et al., 1992; Taylor and Broad, 1998). Therefore, to investigate further the role of $InsP_3$ signaling in the generation of $A\beta_{42}$ oligomer-induced Ca^{2+} transients, $InsP_3$ signaling was inhibited by GFP-5′P overexpression. Using this strategy, $InsP_3$-mediated Ca^{2+} signals induced by CCH were prevented, validating this approach for suppression of $InsP_3$ signaling (**Figure 5A**). As observed for caffeine, however, GFP-5′P overexpression did not prevent $A\beta_{42}$ oligomer-induced Ca^{2+} transients, with 100% of cells responding (**Figure 5B**). However, the peak amplitude and the integral of $A\beta_{42}$ oligomer-induced Ca^{2+} transients were significantly decreased by overexpression of GFP-5′P (**Figure 5B**) when compared to the magnitude of Ca^{2+} transients in control cells, expressing GFP alone. Significantly, Aβ scramble-induced Ca^{2+} transients were not affected by GFP-5′P overexpression with no significant impact of its expression upon the peak amplitude or the integral of Aβ scramble-induced Ca^{2+} transients (**Figure 5C**). Taken together, these results demonstrate that Ca^{2+} transients elicited by $A\beta_{42}$ oligomers arise as a result of release from the ER intracellular Ca^{2+} store and that activation of $InsP_3Rs$ contributes to this effect.

$A\beta_{42}$ OLIGOMER-INDUCED Ca^{2+} LEAK FROM THE ER

The data presented above indicates that externally applied $A\beta_{42}$ rapidly induces an increase on cytosolic Ca^{2+} that involves $InsP_3$-dependent and -independent Ca^{2+} release from the ER. Since $A\beta_{42}$ has also been shown to elicit some of its cytotoxic effects as a result of intracellular accumulation (Wirths et al., 2004), we investigated whether it mobilized Ca^{2+} from the ER when directly applied. We also tested whether $InsP_3Rs$ were required for its intracellular action.

To this end, an established permeabilized cell high-throughput functional assay of ER Ca^{2+} release was used (Tovey et al., 2006). This model uses as substrate for specific analysis of ER Ca^{2+} release, a plasma membrane-permeabilized preparation of the DT40 chicken B-lymphocyte cell line. A derivative of this cell line in which the 3 $InsP_3R$ isoforms have been deleted by homologous recombination (DT40 TKO), allows the requirement for $InsP_3Rs$ for Ca^{2+} release to be tested (Sugawara et al., 1997). Cell permeabilization and substantial dilution in intracellular buffer rules out the contribution of endogenously generated $InsP_3$ to signaling in this assay. Using this assay, a significantly greater $InsP_3$ independent ER Ca^{2+} leak was observed in both wild-type ($p = 0.002$) and DT40 TKO cells ($p = 0.0195$) exposed to $A\beta_{42}$ oligomers compared to the passive Ca^{2+} leak detected in each cell type (**Figures 6A,B**). The maximal Ca^{2+} leak rate induced by $A\beta_{42}$ oligomers was not significantly different between wild-type and DT40 TKO cells ($p = 0.2606$, **Figure 6C**), suggesting that $InsP_3Rs$ were not required for $A\beta_{42}$ oligomers to trigger Ca^{2+} release.

Aβ scramble did not increase the rate of the Ca^{2+} leak in DT40 cells ($p = 0.2528$) or in DT40 TKO cells ($p = 0.0993$) compared

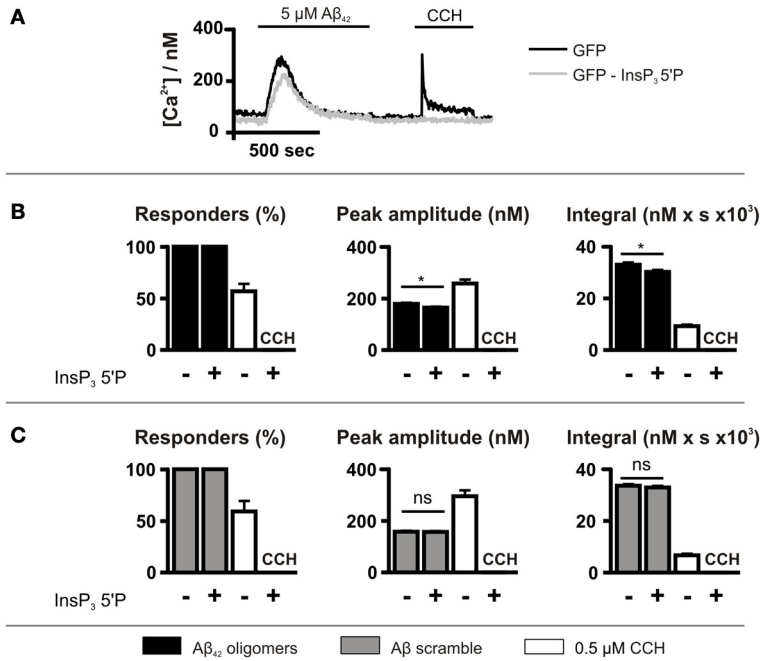

FIGURE 5 | $A\beta_{42}$ oligomer-induced Ca^{2+} release occurs is reduced by $InsP_3$ 5′P expression. (A) Imaging protocol employed to investigate the involvement of $InsP_3$Rs in $A\beta_{42}$ oligomer-mediated Ca^{2+} release from the ER. $InsP_3$ was metabolized by overexpression of $InsP_3$ 5′P. **(B,C)** Bar charts illustrating the magnitude of Ca^{2+} responses elicited by SH-SY5Y cells infected with $InsP_3$ 5′P or GFP alone following the application of 5 μM $A\beta_{42}$ oligomers ($n > 207$ cells) **(B)** or Aβ scramble ($n > 115$ cells) **(C)** and 0.5 μM CCH ($n > 55$ cells). Data is presented as percentage of responding cells, peak amplitude and integral of the response. Bar graphs are mean ± SEM from at least three independent experiments. $^*P < 0.05$.

to the passive Ca^{2+} leak observed in each cell type (**Figure 6B**), and thus there was no significant difference in the maximal Ca^{2+} leak rate following Aβ scramble application between these two cell types ($p = 0.2522$, **Figure 6C**). Importantly, a significant difference between the Ca^{2+} leak rates triggered by exposure to $A\beta_{42}$ oligomers and Aβ scramble in wild-type DT40 cells ($p = 0.0056$) and DT40 TKO cells ($p = 0.0045$) was observed, indicating that Aβ-induced Ca^{2+} leak from the ER is dependent and specific to the amino acid sequence of $A\beta_{42}$. Taken together, these results suggest that $A\beta_{42}$ oligomers trigger a Ca^{2+} leak from the ER, which does not depend upon a direct interaction with $InsP_3$Rs.

DISCUSSION

Here we show that the oligomeric form of the AD-associated peptide $A\beta_{42}$ has potent Ca^{2+} mobilizing properties and we identify mechanisms responsible for its action. Using both intact and permeabilized cell assays to investigate the effects of extracellular and internalized $A\beta_{42}$, respectively, we establish that Ca^{2+} release from the ER makes the greatest contribution to the Ca^{2+} mobilizing effects of $A\beta_{42}$. The $InsP_3$ signaling pathway also contributes to the Ca^{2+} mobilizing properties of oligomeric $A\beta_{42}$ in intact cells. $InsP_3$Rs were not required for $A\beta_{42}$-stimulated Ca^{2+} flux in permeabilized cells ruling out a direct regulation of $InsP_3$Rs by $A\beta_{42}$.

Central to the Ca^{2+} hypothesis of amyloid toxicity is the property of Aβ to induce Ca^{2+} elevations in its target cells. This sets in motion a cascade of events, which culminates in neuronal death. Ever since this hypothesis was put forward more than 20 years ago (Khachaturian, 1989, 1994), numerous reports have described Aβ-induced changes in intracellular Ca^{2+} in a number of cell types including primary neurons and astrocytes as well as neuroblastoma cell lines (Abramov et al., 2004b; Demuro et al., 2005). While there is general consensus that Aβ affects Ca^{2+} homeostasis, the mechanisms underlying this action of Aβ are many. Contributing to this diversity are the different experimental models used, the peptide sequence applied, the conformational state of the peptide and the method used for peptide preparation. Indeed, a number of shorter Aβ sequences have been employed in *in vitro* studies and depletion of ER Ca^{2+} store content reported (Ferreiro et al., 2004, 2008). Since $A\beta_{42}$ is considered to be more relevant to the pathology of AD, we focused on its effects on intracellular Ca^{2+} homeostasis. Not only is an accumulation of $A\beta_{42}$ observed in AD, this longer and more hydrophobic peptide is also more prone to self-assemble than $A\beta_{40}$, the other principle length at which Aβ occurs. As a result, $A\beta_{42}$ exerts a greater degree of neurotoxicity (Jarrett and Lansbury, 1993). Consistent with the growing body of evidence that soluble oligomeric forms of Aβ constitute the primary neurotoxic species (Walsh et al., 2002; Gong et al., 2003; Cleary et al., 2005; Klyubin et al., 2005), this species of $A\beta_{42}$ potently induced Ca^{2+} fluxes and cytotoxicity in this study (**Figures 1**, **2** and **Figure S2**). Highlighting the requirement for appropriate peptide controls when studying $A\beta_{42}$, Ca^{2+} release and cytotoxicity was also induced by a scrambled peptide sequence of $A\beta_{42}$, although the magnitude of these responses was significantly lower than that induced by the wild type sequence. From these

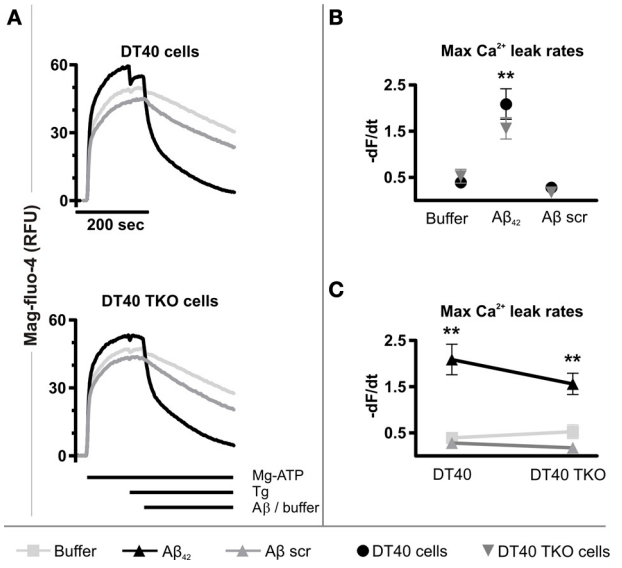

FIGURE 6 | $A\beta_{42}$ oligomers stimulate Ca^{2+} release from the ER of permeabilized cells. (A) Representative traces of mag-fluo-4 fluorescence (relative fluorescence units, RFU) in permeabilized DT40 cells and DT40 TKO cells, illustrating changes in ER luminal Ca^{2+} levels as a function of time in response to $A\beta_{42}$ oligomers, Aβ scramble and buffer alone. Data represents the mean of three measurements. **(B,C)** Initial quantitative analysis of the maximum Ca^{2+} leak rates from the ER of permeabilized DT40 cells and DT40 TKO cells triggered by $A\beta_{42}$ oligomers, Aβ scramble and buffer, respectively. The maximal Ca^{2+} leak rate was calculated by taking the maximal value of the first derivative of the fluorescence values to the time. Data were calculated as −dF/dt and represents the mean Ca^{2+} leak rate obtained from three measurements in RFU over time (RFU/s). $^{**}p < 0.01$.

results, we concluded that the peptide sequence of $A\beta_{42}$ was the major contributor to the toxicity and Ca^{2+} mobilizing properties. The temporal properties of the Ca^{2+} transients we observed were reminiscent of those reported elsewhere, being relatively slow in reaching peak and returning to baseline levels after a few minutes (Demuro et al., 2005; Simakova and Arispe, 2006). The return of these Ca^{2+} signals to baseline does, however, suggest that the Ca^{2+} elevations induced by $A\beta_{42}$ were not immediately toxic. The Ca^{2+} mobilizing properties of the scrambled peptide, however, may reflect the previously described intrinsic properties of an oligomeric/amyloid peptide (Bucciantini et al., 2002; Yoshiike et al., 2008). For example, oligomeric forms of polyQ and insulin have been shown to induce Ca^{2+} transients (Demuro et al., 2005). The solvent HFIP used for preparation of the peptide has also previously been shown to exhibit cytotoxicity and to affect ion conductance of membranes (Capone et al., 2009).

Both Ca^{2+} influx from the extracellular space and release from ER-localized intracellular stores have been reported to be induced by Aβ and involved in its toxic action (Blanchard et al., 2004; Ferreiro et al., 2004, 2006; Kayed et al., 2004; Demuro et al., 2005, 2011; Kelly and Ferreira, 2006; Simakova and Arispe, 2006; Arispe et al., 2007; De Felice et al., 2007; Resende et al., 2008; Demuro and Parker, 2013). Although Ca^{2+} entry from the extracellular space was a component of the Ca^{2+} elevation induced by $A\beta_{42}$ in this study, the greatest contribution was due to release from the ER. Moreover the lack of an effect of removal of extracellular Ca^{2+} upon the initial peak of the Ca^{2+} response or the number of responding cells suggested that Ca^{2+} entry across the plasma membrane was secondary to Ca^{2+} release from the ER. Since $A\beta_{42}$ was acting to deplete the ER stores, the Ca^{2+} influx could arise via a store-operated Ca^{2+} entry pathway. These observations are not, however, incompatible with an additional mechanism for Ca^{2+} entry via plasma membrane pores formed by $A\beta_{42}$, which have been shown to require a longer period to develop (Demuro et al., 2011). Whether the Ca^{2+} fluxes associated with the formation of membrane pores, which were generally local to the pore and were of a relatively small magnitude, contribute to the global Ca^{2+} transient is not clear (Demuro et al., 2011).

Analysis of the mechanisms underlying Ca^{2+} release from the ER revealed that while $InsP_3Rs$ contributed to $A\beta_{42}$-induced Ca^{2+} release from the ER in intact cells, the greater part of the Ca^{2+} elevation induced by $A\beta_{42}$ was due to an alternative mechanism. However, IICR did not contribute to the Ca^{2+} responses induced by scrambled peptide. From these results, we concluded that $A\beta_{42}$-induced Ca^{2+} release from the ER comprises an $A\beta_{42}$ sequence-specific component, which is $InsP_3$-dependent, and a second component, which is peptide sequence- and $InsP_3$-independent. Comparison of these $A\beta_{42}$ and $A\beta_{42}$ scrambled datasets reveals that although the $InsP_3$-dependent component of the total $A\beta_{42}$ signal is relatively minor, when considered as a fraction of the $A\beta_{42}$-specific Ca^{2+} signal (i.e., $A\beta_{42}$—$A\beta_{42}$ scrambled Ca^{2+} transient), its importance is increased.

Our demonstration of the participation of $InsP_3$ signaling in $A\beta_{42}$-induced Ca^{2+} responses provides robust evidence in support of this pathway in $A\beta_{42}$-mediated Ca^{2+} signals thus far. In particular, the use of $InsP_3$ 5′phosphatase overexpression to suppress $InsP_3$ signaling is a highly selective strategy, overcoming issues regarding incomplete knockdown of $InsP_3Rs$ and contribution of the isoforms not targeted when using siRNA approaches. The inhibition of Ca^{2+} signals by caffeine is also consistent with a role for the $InsP_3$ signaling pathway in the Ca^{2+} mobilizing effects of Aβ (Parker and Ivorra, 1991; Bezprozvanny et al., 1994). Not only does caffeine inhibit $InsP_3Rs$ directly (Bezprozvanny et al., 1994), by also inhibiting PLC, caffeine is a potent inhibitor of $InsP_3$ generation (Taylor and Broad, 1998). These findings are consistent with the reduction in the $A\beta_{42}$-induced Ca^{2+} transient observed following application of the PLC inhibitor U73122 (Resende et al., 2008) although U73122 has numerous non-specific effects. The mechanism by which $InsP_3$ signaling is engaged by $A\beta_{42}$ in this study remains to be established. Since the effects of inhibition of $InsP_3$ signaling persist in the absence of extracellular Ca^{2+}, activation of PLC and $InsP_3$ generation by $A\beta_{42}$-stimulated Ca^{2+} influx can be excluded. Thus, a more likely scenario would involve $A\beta_{42}$ engagement of a PLC-coupled G protein coupled-receptor (GPCR). Indeed, a number of different GPCRs, including metabotropic glutamate receptors, are activated by $A\beta_{42}$, contributing to modulation of LTP, $A\beta_{42}$ synthesis and processing and cytotoxicity (Wang et al., 2004; Thathiah and De Strooper, 2011).

The internalization of Aβ from the extracellular space (Bucciantini et al., 2004; Pierrot et al., 2004; Wirths et al., 2004; Kaminski Schierle et al., 2011) raises a further possibility that Aβ acts to either directly activate/sensitize $InsP_3Rs$ or to alter $InsP_3$ generation/metabolism. Since significant intracellular $A\beta_{42}$ accumulation would require up to 1 h (Bucciantini et al., 2004; Kaminski Schierle et al., 2011), it is unlikely that this endocytosed $A\beta_{42}$ contributes to the acute modulation of Ca^{2+} fluxes observed in this study and elsewhere in intact cells. Endocytosis of $A\beta_{42}$ may, however, contribute to the more chronic effects on Ca^{2+} homeostasis as well as cytotoxicity previously reported (Ferreiro et al., 2004, 2006; Resende et al., 2008). The possibility that $A\beta_{42}$ could directly affect ER Ca^{2+} homeostasis from an intracellular location was therefore also considered. Using a permeabilized cell assay to allow control of cytosolic conditions and access of $A\beta_{42}$ to the ER, an $A\beta_{42}$-stimulated Ca^{2+} efflux from the ER was observed. Unlike that observed for intact cells, the difference between $A\beta_{42}$ and $A\beta_{42}$ scrambled was dramatic, revealing a highly specific effect of $A\beta_{42}$ upon ER Ca^{2+} mobilization. These effects were observed in the absence of exogenous $InsP_3$ suggesting that the effects were $InsP_3R$-independent. The extensive dilution of cytosol following permeabilization of the DT40 cells would also likely preclude a contribution of $A\beta_{42}$-stimulated $InsP_3$ generation. More significantly, $InsP_3Rs$ were not required for the Ca^{2+} mobilizing properties of $A\beta_{42}$, since deficiency in all three $InsP_3R$ isoforms did not affect the Ca^{2+} mobilizing properties of $A\beta_{42}$. The absence of a requirement for $InsP_3Rs$ for $A\beta_{42}$-stimulated Ca^{2+} flux in the permeabilized cell system does not rule out the possibility that IICR contributes to Ca^{2+} fluxes and toxicity mediated by intracellular $A\beta_{42}$. Indeed, by activating Ca^{2+}-sensitive PLC and generation of $InsP_3$, Ca^{2+} mobilized by $A\beta_{42}$ could promote IICR. Consistent with this notion, microinjected $A\beta_{42}$ was recently shown to promote Ca^{2+} signals in Xenopus oocytes in a manner that involved $InsP_3$ generation (Demuro and Parker, 2013).

The depletion of the ER Ca^{2+} store by $A\beta_{42}$ has important implications for the mechanisms of its toxicity. Depletion of ER Ca^{2+} stores results in the accumulation of unfolded proteins and activation of the ER stress response, which via caspase 12 activation and Bap31 cleavage can subsequently induce mitochondrial apoptotic cascades (Verkhratsky, 2005; Xu et al., 2005; Mekahli et al., 2011). The engagement of $InsP_3Rs$ during $A\beta_{42}$-stimulated depletion of ER Ca^{2+} may be of greater consequence. Specifically, $InsP_3R$-induced Ca^{2+} release from the ER and its subsequent sequestration by neighboring mitochondria could lead to mitochondrial Ca^{2+} overload, permeability transition and death (Csordas et al., 2006). These pathways also lead to increased reactive oxygen species generation, which is commonly observed in AD (Ferreiro et al., 2004, 2008; Arduino et al., 2009; Clark et al., 2010).

The use of SH-SY5Y neuroblastoma cell line and permeabilized DT40 B-lymphocytes in this study, rather than primary neurons allowed careful dissection of the role of ER Ca^{2+} signaling to Aβ-induced Ca^{2+} signals independent from Ca^{2+} fluxes that may arise in neurons as a result of electrical or synaptic activity. Moreover, using this cell line, contributions from other Aβ targets described in neurons such as NMDA receptors are excluded. Analogous to a number of other studies in electrically non-excitable primary and cultured cells including Xenopus oocytes (Demuro and Parker, 2013) astrocytes and PC12 cells (Abramov et al., 2003, 2004a; Simakova and Arispe, 2006), our data indicates that certain of the Ca^{2+} mobilizing properties of $A\beta_{42}$ are neuron-independent and do not require the expression of any other of its reported targets. Fundamental aspects of the Ca^{2+} mobilizing properties of $A\beta_{42}$ were further revealed and exemplified by the $A\beta_{42}$ -stimulated Ca^{2+} flux from the $InsP_3R$-deficient ER of permeabilized DT40 B-lymphocytes. These latter data demonstrate for the first time that $A\beta_{42}$ has the capacity to directly induce Ca^{2+} flux from the ER. Given the importance of the ER and $InsP_3Rs$ in neuronal functions, future studies will be required to test whether $InsP_3Rs$ contribute to Aβ-mediated neuronal pathology.

AUTHOR CONTRIBUTIONS

Laura E. Jensen: substantial contributions to conception and design, acquisition, analysis and interpretation of data as well as writing of manuscript. H. Llewelyn Roderick: substantial contributions to conception and design, interpretation of data as well as writing of manuscript. Geert Bultynck and Tomas Luyten: designed, acquired, analysed and interpreted data of **Figure 6**. Hozeefa Amijee: designed, acquired and interpreted data of **Figure S2.** Martin D. Bootman: proof-read manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnmol.2013.00036/abstract

Figure S1 | Validating the toxicity and conformation of $A\beta_{42}$ oligomers. (A) Assessment of toxicity of homogeneous Aβ preparations. Bar chart illustrating the cytotoxic effects of $A\beta_{42}$ preparations upon SH-SY5Y cells determined using the MTT assay. Data is expressed as a percentage of MTT reduced by test samples to the dead cell controls following 24-h treatment with $A\beta_{42}$ oligomers and scrambled Aβ at the respective concentrations. **(Bi)** Schematic diagram illustrating the epitopes of $A\beta_{42}$ recognized by the conformation dependent, anti-oligomer antibody, A11 (Kayed et al., 2003), and the sequence dependent, anti-amyloid antibody, 12F4 (Parvathy et al., 2001). **(Bii,iii)** Sensorgrams obtained from surface plasmon resonance spectroscopy, as described (Maezawa et al., 2008). A Biacore T-100, equipped with four flow cells on a sensor chip, was used for these real-time binding studies. Biotinylated $A\beta_{42}$was prepared by mixing a 1:10 molar ratio of biotinylated and unbiotinylated $A\beta_{42}$. In preparation for the binding studies, $A\beta_{42}$ was injected onto a streptavidin chip at a concentration of 10 μM to immobilize $A\beta_{42}$by streptavidin-biotin

coupling. The streptavidin chip of flow cell (Fc) 2 was partially (50%) and of Fc-4 fully saturated (100%) with $A\beta_{42}$ oligomers. As a control, the surface of Fc-3 was partially saturated (50%) with $A\beta_{42}$monomers. Antibodies **(Bii)** A11 and **(Biii)** 12F4 were injected over the immobilized $A\beta_{42}$ of each flow cell at a concentration of 50 μg/ml and 10 μg/ml, respectively. The injection of the anti-oligomer antibody, A11, was followed by a regeneration step prior to injection of 12F4. The binding of injected antibodies, present in the flow phase, to the immobilized $A\beta_{42}$was measured by response units (RU) elicited. All values were corrected for the RU obtained from the reference cell, flow cell 1, which was saturated with biotinylated $A\beta_{42}$ only.

Figure S2 | Comparison of Ca^{2+} responses elicited by $A\beta_{42}$ oligomers and monomers in SH-SY5Y cells. (A) Imaging protocol employed to assess the effects of homogeneous preparations of $A\beta_{42}$ on the Ca^{2+} signaling capacity of fluo-4-loaded SH-SY5Y cells. Cellular Ca^{2+} responses were recorded by wide-field epifluorescence. The magnitude of Ca^{2+} responses elicited by 5 μM $A\beta_{42}$ monomers and oligomers and the subsequent application of 100 μM CCH is presented as **(B)** percentage of responding cells, **(C)** peak amplitude and **(D)** integral of the response. Soluble Aβ monomers and Aβ oligomers were prepared as previously described (Demuro et al., 2005). This method of Aβ preparation reportedly results in homogeneous populations of Aβ monomers and oligomers (also **Figure S1B**). All $A\beta_{42}$ concentrations stated were based on the molar mass of the peptide.

Figure S3 | Human neuroblastoma SH-SY5Y cells lack RyR expression. (A) Representative Ca^{2+} trace illustrating that SH-SY5Y cells do not elicit Ca^{2+} responses following the application of 10 mM caffeine, indicating that cells lack RyRs ($n = 239$ cells). However, SH-SY5Y cells do exhibit $InsP_3$-mediated Ca^{2+} responses. **(B)** Immunoblot analysis corroborating the lack of RyR2 expression in SH-SY5Y cells. RyR2 expression is observed in control samples of adult hippocampal tissue and primary hippocampal cultures maintained for 4, 8, 11, and 15 days *in vitro* (DIVs).

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