Dysregulation of Ca\(^{2+}\) homeostasis is considered to contribute to the toxic action of the Alzheimer’s disease (AD)-associated amyloid-\(\beta\)-peptide (A\(\beta\)). 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, A\(\beta\) oligomers, calcium/Ca\(^{2+}\), InsP\(_3\)/IP\(_3\), InsP\(_3\) receptors/InsP\(_3\)Rs, endoplasmic reticulum/ER
2003; Blanchard et al., 2004; De Felice et al., 2007), to alter neuronal excitability which, in turn, influences the extent of Ca2+ influx (Good et al., 1996) and to induce dysregulation of endoplasmic reticulum (ER) Ca2+ 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., 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 Ca2+ mobilization from the ER to the increase in intracellular Ca2+ induced by oligomeric Aβ42, (2) the mechanism(s) by which Aβ42 elicited this effect, (3) the capacity for Aβ42 to mobilize Ca2+ directly from the ER. To avoid isolation of effects on the ER from other plasma membrane targets of Aβ42, model cells systems were used that allowed fundamental aspects of ER Ca2+ regulation to be studied. We determined that Ca2+ release from the ER contributed to the increase in intracellular Ca2+ concentration induced by oligomeric Aβ42. The Aβ42-induced Ca2+ elevation comprised InsP3 dependent and independent components. Using DT40 cells deficient in the three InsP3 isoforms that have 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 Ca2+ imaging experiments, cells were plated onto poly-L-lysine-coated coverslips at a density of 3.2 × 10⁵ cells/cm². For the MITT reduction assay, cells were plated at a density of 9 × 10⁵ cells/cm². To overexpress GFP-tagged type 1 InsP3 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 InsP3R isoforms (DT40 TKO) was performed as previously described (Tovey et al., 2006).

**PREPARATION OF Aβ42 OLIGOMERS**

Wild type and scrambled Aβ42 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 Aβ42 oligomers were prepared as previously described (Demuro et al., 2005) and remained stable for at least 3 weeks. Samples of Aβ1–42 scrambled peptide (KVKGLIDGAHIGDLVYEFMDSN SAIIFREGVAGHVVQVF) were prepared in the same way as Aβ42 oligomers. All Aβ samples were stored at 4°C and were used within 10–15 days of preparation. Toxicity of Aβ42 preparations was confirmed by MTT assay before use in Ca2+ imaging experiments (Figure S1A). The oligomeric nature of the Aβ42 preparation was established by surface plasmon resonance (SPR) spectroscopy using an antibody specific to oligomeric Aβ42 (Figure S1B). All Aβ42 concentrations stated are based on the molar mass of the peptide.

**LIVE CELL CA2+ IMAGING**

Methods for single cell analysis of intracellular Ca2+ 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 Ca2+ containing (121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 6 mM NaHCO3, 25 mM HEPES, 5.5 mM glucose, pH 7.3) or Ca2+ free (as for Ca2+ containing with 1.8 mM CaCl2 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 (Gryniewicz et al., 1985), using the maximum and minimum ratio values obtained through exposing cells sequentially to Ca2+free and Ca2+ containing imaging buffer to which 2 μM ionomycin had been added. Parameters analyzed from the Ca2+ 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.

InsP3-induced Ca2+ release (ICR) from permeabilized wild type and InsP3R null DT40 cells (three InsP3R 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 Ca2+ indicator mag-fluo-4 and Aβ-induced Ca2+ 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 Aβ_{42} on SH-SY5Y cells and was performed according to manufacturer’s instructions. Briefly, cells were incubated with Aβ_{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% CO2. 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. Aβ_{42} samples were considered to be toxic if 25–40% of cells remained metabolically healthy at an Aβ_{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 Aβ_{42}**

Experiments were first performed to validate the Ca^{2+} mobilizing properties of oligomeric Aβ_{42} over the concentration range of its toxicity. Application of Aβ_{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 Aβ_{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 Aβ_{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 Aβ_{42} (Figures 1Bi,iii,v). To test cell viability as well as to determine whether metabolotropic 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 Aβ_{42} or to a vehicle control (10%) (Figures 1Bi,iv,vi). At 10 μM Aβ, however, the number of cells responding to CCH was significantly reduced (Figure 1Bi). 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 Aβ_{42} (Figures 1Biv,vi). This observation suggested that exposure to Aβ_{42} oligomers was depleting the intracellular CCH-sensitive ER Ca^{2+} store. These Ca^{2+} mobilizing effects of oligomeric Aβ_{42} were significantly greater than observed in cells exposed to Aβ_{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 Aβ_{42} was selected for use in subsequent experiments.

**Aβ_{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 Aβ_{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 Aβ_{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 Aβ_{42} (Figures 2Bii,Cii,Di).

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

**Aβ_{42} OLIGOMERS MOBILIZE Ca^{2+} FROM INTRACELLULAR STORES**

The reduced magnitude of CCH-induced Ca^{2+} signals observed in cells previously exposed to oligomeric Aβ_{42} suggested that this form of Aβ_{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 Aβ_{42}-induced Ca^{2+} transients. To determine the contribution of extracellular Ca^{2+} and Ca^{2+} influx to Aβ_{42} oligomer-induced Ca^{2+} transients, we performed experiments using Ca^{2+}-free imaging buffer. Under these conditions, Aβ_{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 3Aii) of Aβ_{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 Aβ_{42} oligomer-induced Ca^{2+} response, the peak amplitude and the integral of the Ca^{2+} responses to CCH applied following Aβ_{42} oligomer exposure were significantly decreased by removal of extracellular Ca^{2+} from the imaging buffer (CCH, after Aβ_{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 Aβ_{42}. Indeed, the peak amplitude and the integral of CCH-induced Ca^{2+} responses elicited in Ca^{2+} free buffer were significantly greater in naive cells (CCH, no Aβ_{42}) than when Aβ_{42} oligomers were previously applied (Figures 3Aiv,vi). Since Aβ_{42} oligomer-induced Ca^{2+} transients were not significantly affected by removal of extracellular Ca^{2+}, these results suggest...
Jensen et al. Mobilization of ER Ca$^{2+}$ by Aβ$_{42}$

**FIGURE 1 | Aβ$_{42}$ 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 Aβ$_{42}$ oligomers followed by 100 μM CCH. A trace taken from cells in which Aβ$_{42}$ oligomers were substituted with double-distilled water (dd H$_2$O; 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 Aβ$_{42}$ oligomers, dd H$_2$O and CCH is presented as (Bi,ii) percentage of responding cells, (Biii,iv) peak amplitude and (Bv,vii) integral of the response. Aβ$_{42}$ oligomer-induced Ca$^{2+}$ transients were normalized to the responses induced with the highest concentration (10 μM) of the respective Aβ$_{42}$ 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 | Aβ$_{42}$ 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 Aβ$_{42}$ 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.
that oligomeric Aβ42 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 to the SERCA pump inhibitor thapsigargin (Tg; 2 μM, 15 min) prior to the application of Aβ42. In the absence of replete ER Ca\(^{2+}\) stores, Aβ42-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 Aβ42 oligomers mobilize Ca\(^{2+}\) from the ER.

**Aβ42-INDUCED Ca\(^{2+}\) RELEASE OCCURS IN PART THROUGH INSP\(_3\)Rs**

Having determined that Aβ42 oligomers mobilize Ca\(^{2+}\) from the ER, we aimed to identify the mechanism by which Ca\(^{2+}\) release occurs. We therefore tested whether Aβ42 was causing Ca\(^{2+}\) release from the ER through activation of InsP\(_3\)Rs 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 Aβ42 oligomer-mediated Ca\(^{2+}\) release was ruled out.

SH-SY5Y cells express InsP\(_3\)Rs 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\(_3\)Rs to Aβ42-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 prevented by adenoviral-mediated overexpression of GFP-

Caffeine application did not affect the number of cells exhibiting Ca\(^{2+}\) responses following Aβ42 oligomer application, with 100% of cells responding (Figure 4B). However, caffeine significantly decreased the peak amplitude and the integral of the Aβ42 oligomer-induced Ca\(^{2+}\) transients, 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).
When compared to the magnitude of Ca\textsuperscript{2+} (1994), it also acts on targets other than the InsP\textsubscript{3}R such as Ca\textsuperscript{2+} significantly decreased by overexpression of GFP-5 (Toescu et al., 1992; Taylor and Broad, 1998). Therefore, to investigate further the role of InsP\textsubscript{3} signaling in the generation of A\textsubscript{β} transients elicited by A\textsubscript{β}, the expression upon the 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\textsubscript{3}Rs (Bezprozvanny et al., 1994), it also acts on targets other than the InsP\textsubscript{3}R 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\textsubscript{3} signaling in the generation of A\textsubscript{β} oligomer-induced Ca\textsuperscript{2+} transients, InsP\textsubscript{3} signaling was inhibited by GFP-5P overexpression. Using this strategy, InsP\textsubscript{3}-mediated Ca\textsuperscript{2+} signals induced by CCH were prevented, validating this approach for suppression of InsP\textsubscript{3} signaling (Figure 5A). As observed for caffeine, however, GFP-5P overexpression did not prevent A\textsubscript{β} oligomer-induced Ca\textsuperscript{2+} transients, with 100% of cells responding (Figure 5B). However, the peak amplitude and the integral of A\textsubscript{β} oligomer-induced Ca\textsuperscript{2+} transients were significantly decreased by overexpression of GFP-5P (Figure 5B) when compared to the magnitude of Ca\textsuperscript{2+} transients in control cells, expressing GFP alone. Significantly, A\textsubscript{β} scramble-induced Ca\textsuperscript{2+} transients were not affected by GFP-5P overexpression with no significant impact of its expression upon the peak amplitude or the integral of A\textsubscript{β} scramble-induced Ca\textsuperscript{2+} transients (Figure 5C). Taken together, these results demonstrate that Ca\textsuperscript{2+} transients elicited by A\textsubscript{β} oligomers arise as a result of release from the ER intracellular Ca\textsuperscript{2+} store and that activation of InsP\textsubscript{3}Rs contributes to this effect.

**A\textsubscript{β} oligomer-induced Ca\textsuperscript{2+} leak from the ER**

The data presented above indicates that externally applied A\textsubscript{β} rapidly induces an increase on cytosolic Ca\textsuperscript{2+} that involves InsP\textsubscript{3}-dependent and -independent Ca\textsuperscript{2+} release from the ER. Since A\textsubscript{β} 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\textsuperscript{2+} from the ER when directly applied. We also tested whether InsP\textsubscript{3}Rs were required for its intracellular action.

To this end, an established permeabilized cell high-throughput functional assay of ER Ca\textsuperscript{2+} release was used (Tovey et al., 2006). This model uses as substrate for specific analysis of ER Ca\textsuperscript{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\textsubscript{3}R isoforms have been deleted by homologous recombination (DT40 TKO), allows the requirement for InsP\textsubscript{3}Rs for A\textsubscript{β} oligomers to trigger Ca\textsuperscript{2+} release. Since A\textsubscript{β} 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\textsuperscript{2+} from the ER when directly applied. We also tested whether InsP\textsubscript{3}Rs were required for its intracellular action.

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.

![Figure 4](https://example.com/figure4.png)

**Figure 4** | A\textsubscript{β} oligomer-induced Ca\textsuperscript{2+} release is sensitive to caffeine.

(A) Imaging protocol employed to investigate the involvement of InsP\textsubscript{3}Rs in A\textsubscript{β} oligomer-mediated Ca\textsuperscript{2+} release from the ER. InsP\textsubscript{3}Rs were inhibited by co-administration of caffeine. (B,C) Bar charts illustrating the magnitude of Ca\textsuperscript{2+} signals induced by CCH were prevented, validating this approach for suppression of InsP\textsubscript{3} signaling (Figure 5A). As observed for caffeine, however, GFP-5P overexpression did not prevent A\textsubscript{β} oligomer-induced Ca\textsuperscript{2+} transients, with 100% of cells responding (Figure 5B). However, the peak amplitude and the integral of A\textsubscript{β} oligomer-induced Ca\textsuperscript{2+} transients were significantly decreased by overexpression of GFP-5P (Figure 5B) when compared to the magnitude of Ca\textsuperscript{2+} transients in control cells, expressing GFP alone. Significantly, A\textsubscript{β} scramble-induced Ca\textsuperscript{2+} transients were not affected by GFP-5P overexpression with no significant impact of its expression upon the peak amplitude or the integral of A\textsubscript{β} scramble-induced Ca\textsuperscript{2+} transients (Figure 5C). Taken together, these results demonstrate that Ca\textsuperscript{2+} transients elicited by A\textsubscript{β} oligomers arise as a result of release from the ER intracellular Ca\textsuperscript{2+} store and that activation of InsP\textsubscript{3}Rs contributes to this effect.

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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 A\(\beta\) 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 A\(\beta\) scramble in wild-type DT40 cells (\(p = 0.0056\)) and DT40 TKO cells (\(p = 0.0045\)) was observed, indicating that A\(\beta\)-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 A\(\beta\) 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 A\(\beta\)-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 A\(\beta\) affects Ca\(^{2+}\) homeostasis, the mechanisms underlying this action of A\(\beta\) 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 A\(\beta\) 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 A\(\beta\) 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 A\(\beta\) 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

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**FIGURE 5** | A\(\beta_{42}\) oligomer-induced Ca\(^{2+}\) release occurs is reduced by InsP\(_3\) 5P 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\(_3\) 5P. (B,C) Bar charts illustrating the magnitude of Ca\(^{2+}\) responses elicited by SH-SY5Y cells infected with InsP\(_3\) 5P or GFP alone following the application of 5 \(\mu\)M A\(\beta_{42}\) oligomers (n > 207 cells) (B) or A\(\beta\) scramble (n > 115 cells) (C) and 0.5 \(\mu\)M 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. *\(P < 0.05\).
Major contributor to the toxicity and 

to affect ion conductance of membranes (Capone et al., 2002; Yoshiike et al., 2008). For example, oligomeric forms of the peptide, however, may reflect the previously described intrinsic mobilizing properties of an oligomeric/amyloid peptide (Bucciantini et al., 2013). Although Ca²⁺ entry from the extracellular space was a component of the Ca²⁺ elevation induced by Aβ₄₂ in this study, the greatest contribution was due to release from the ER. Moreover, the lack of an effect of removal of extracellular Ca²⁺ upon the initial peak of the Ca²⁺ response or the number of responding cells suggested that Ca²⁺ entry across the plasma membrane was secondary to Ca²⁺ release from the ER. Since Aβ₄₂ was acting to deplete the ER stores, the Ca²⁺ influx could arise via a store-operated Ca²⁺ entry pathway. These observations are not, however, incompatible with an additional mechanism for Ca²⁺ entry via plasma membrane pores formed by Aβ₄₂, which have been shown to require a longer period to develop (Demuro et al., 2011). Whether the Ca²⁺ fluxes associated with the formation of membrane pores, which were generally local to the pore and of a relatively small magnitude, contribute to the global Ca²⁺ transient is not clear (Demuro et al., 2011).

Analysis of the mechanisms underlying Ca²⁺ release from the ER revealed that while InsP₃Rs contributed to Aβ₄₂-induced Ca²⁺ release from the ER in intact cells, the greater part of the Ca²⁺ elevation induced by Aβ₄₂ was due to an alternative mechanism. However, IICR did not contribute to the Ca²⁺ responses induced by scrambled peptide. From these results, we concluded that Aβ₄₂-induced Ca²⁺ release from the ER comprises an Aβ₄₂ sequence-specific component, which is InsP₃-dependent, and a second component, which is peptide sequence- and InsP₃-independent. Comparison of these Aβ₄₂ and Aβ₄₂ scrambled datasets reveals that although the InsP₃-dependent component of the total Aβ₄₂ signal is relatively minor, when considered as a fraction of the Aβ₄₂-specific Ca²⁺ signal (i.e., Aβ₄₂—Aβ₄₂ scrambled Ca²⁺ transient), its importance is increased.

Our demonstration of the participation of InsP₃ signaling in Aβ₄₂-induced Ca²⁺ responses provides robust evidence in support of this pathway in Aβ₄₂-mediated Ca²⁺ signals thus far. In particular, the use of InsP₃ 5′-phosphatase overexpression to suppress InsP₃ signaling is a highly selective strategy, overcoming issues regarding incomplete knockdown of InsP₃Rs and contribution of the isoforms not targeted when using siRNA approaches. The inhibition of Ca²⁺ signals by caffeine is also consistent with a role for the InsP₃ signaling pathway in the Ca²⁺ mobilizing effects of Aβ (Parker and Ivorra, 1991; Bezprozvanny et al., 1994). Not only does caffeine inhibit InsP₃Rs directly (Bezprozvanny et al., 1994), by also inhibiting PLC, caffeine is a potent inhibitor of InsP₃ generation (Taylor and Broad, 1998). These findings are consistent with the reduction in the Aβ₄₂-induced Ca²⁺ transient observed following application of the PLC inhibitor U73122 (Resende et al., 2008) although U73122 has numerous non-specific effects. The mechanism by which InsP₃ signaling is engaged by Aβ₄₂ in this study remains to be established. Since the effects of inhibition of InsP₃ signaling persist in the absence of extracellular Ca²⁺, activation of PLC and InsP₃ generation by Aβ₄₂-stimulated Ca²⁺ influx can be excluded. Thus, a more likely scenario would involve Aβ₄₂ engagement of a PLC-coupled G protein-coupled-receptor (GPCR). Indeed, a number of different GPCRs, including metabotropic glutamate receptors, are activated by Aβ₄₂, contributing to modulation of LTP, Aβ₄₂ 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 InsP3Rs or to alter InsP3 generation/metabolism. Since significant intracellular Aβ42 accumulation would require up to 1 h (Bucciantini et al., 2004; Kaminski Schierle et al., 2011), it is unlikely that this endocytosed Aβ42 contributes to the acute modulation of Ca2+ fluxes observed in this study and elsewhere in intact cells. Endocytosis of Aβ42 may, however, contribute to the more chronic effects on Ca2+ homeostasis as well as cytotoxicity previously reported (Ferreiro et al., 2004, 2006; Resende et al., 2008). The possibility that Aβ42 could directly affect ER Ca2+ homeostasis from an intracellular location was therefore also considered. Using a permeabilized cell assay to allow control of cytosolic conditions and access of Aβ to the ER, an Aβ42-stimulated Ca2+ efflux from the ER was observed. Unlike that observed for intact cells, the difference between Aβ42 and Aβ40 scrambled was dramatic, revealing a highly specific effect of Aβ42 upon ER Ca2+ mobilization. These effects were observed in the absence of exogenous InsP3 suggesting that the effects were InsP3R-independent. The extensive dilution of cytosol following permeabilization of the DT40 cells would also likely preclude a contribution of Aβ42-stimulated InsP3 generation. More significantly, InsP3Rs were not required for the Ca2+ mobilizing properties of Aβ42, since deficiency in all three InsP3R isoforms did not affect the Ca2+ mobilizing properties of Aβ42. The absence of a requirement for InsP3Rs for Aβ42-stimulated Ca2+ flux in the permeabilized cell system does not rule out the possibility that IICR contributes to Ca2+ fluxes and toxicity mediated by intracellular Aβ42. Indeed, by activating Ca2+-sensitive PLC and generation of InsP3, Ca2+ mobilized by Aβ42 could promote IICR. Consistent with this notion, microinjected Aβ42 was recently shown to promote Ca2+ signals in Xenopus oocytes in a manner that involved InsP3 generation (Demuro and Parker, 2013).

The depletion of the ER Ca2+ store by Aβ42 has important implications for the mechanisms of its toxicity. Depletion of ER Ca2+ 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 (Verkhatsky, 2005; Xu et al., 2005; Mekabi et al., 2011). The engagement of InsP3Rs during Aβ42-stimulated depletion of ER Ca2+ may be of greater consequence. Specifically, InsP3R-induced Ca2+ release from the ER and its subsequent sequestration by neighboring mitochondria could lead to mitochondrial Ca2+ 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-SYSY neuroblastoma cell line and permeabilized DT40 B-lymphocytes in this study, rather than primary neurons allowed careful dissection of the role of ER Ca2+ signaling to Aβ-induced Ca2+ signals independent from Ca2+ 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 Ca2+ mobilizing properties of Aβ42 are neuron-independent and do not require the expression of any other of its reported targets. Fundamental aspects of the Ca2+ mobilizing properties of Aβ42 were further revealed and exemplified by the Aβ42-stimulated Ca2+ flux from the InsP3R-deficient ER of permeabilized DT40 B-lymphocytes. These latter data demonstrate for the first time that Aβ42 has the capacity to directly induce Ca2+ flux from the ER. Given the importance of the ER and InsP3Rs in neuronal functions, future studies will be required to test whether InsP3Rs 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 Aβ42 oligomers. (A) Assessment of toxicity of homogeneous Aβ preparations. Bar chart illustrating the cytotoxic effects of Aβ42 preparations upon SH-SYSY 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 Aβ42 oligomers and scrambled Aβ at the respective concentrations. (B) Schematic diagram illustrating the epitopes of Aβ42 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). (Bi,iii) Senssorgams 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 Aβ42 was prepared by mixing a 1:10 molar ratio of biotinylated and unbiotinylated Aβ42. In preparation for the binding studies, Aβ42 was injected onto a streptavidin chip at a concentration of 10 μM to immobilize Aβ42 by streptavidin-biotin
coupling. The streptavidin chip of flow cell (Fc) 2 was partially (50%) and of Fc-4 fully saturated (100%) with Aβ42 oligomers. As a control, the surface of Fc-3 was partially saturated (50%) with Aβ24 monomers. Antibodies (Bii) A11 and (Bii) 12F4 were injected over the immobilized Aβ42 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 Aβ42 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 Aβ42 only.

**Figure S2** | Comparison of Ca2+ responses elicited by Aβ42 oligomers and monomers in SH-SY5Y cells. (A) Imaging protocol employed to assess the effects of homogeneous preparations of Aβ42 on the Ca2+ signaling capacity of fluo-4-loaded SH-SY5Y cells. Cellular Ca2+ responses were recorded by wide-field epifluorescence. The magnitude of Ca2+ responses elicited by 5 μM Aβ42 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 see Figure S1B). All Aβ42 concentrations stated were based on the molar mass of the peptide.

**Figure S3** | Human neuroblastoma SH-SY5Y cells lack RyR expression. (A) Representative Ca2+ responses following the application of 10 mM caffeine, indicating that cells lack RyRs (n = 239 cells). However, SH-SY5Y cells do exhibit InsP3-mediated Ca2+ 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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