

Amyloid- β Acts as a Regulator of Neurotransmitter Release Disrupting the Interaction between Synaptophysin and VAMP2

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Abstract

Background: It is becoming increasingly evident that deficits in the cortex and hippocampus at early stages of dementia in Alzheimer's disease (AD) are associated with synaptic damage caused by oligomers of the toxic amyloid- β peptide (A β 42). However, the underlying molecular and cellular mechanisms behind these deficits are not fully understood. Here we provide evidence of a mechanism by which A β 42 affects synaptic transmission regulating neurotransmitter release.

Methodology/Findings: We first showed that application of 50 nM A β 42 in cultured neurones is followed by its internalisation and translocation to synaptic contacts. Interestingly, our results demonstrate that with time, A β 42 can be detected at the presynaptic terminals where it interacts with Synaptophysin. Furthermore, data from dissociated hippocampal neurons as well as biochemical data provide evidence that A β 42 disrupts the complex formed between Synaptophysin and VAMP2 increasing the amount of primed vesicles and exocytosis. Finally, electrophysiology recordings in brain slices confirmed that A β 42 affects baseline transmission.

Conclusions/Significance: Our observations provide a necessary and timely insight into cellular mechanisms that underlie the initial pathological events that lead to synaptic dysfunction in Alzheimer's disease. Our results demonstrate a new mechanism by which A β 42 affects synaptic activity.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. The brain of AD patients is characterised by neuronal loss, the presence of extracellular senile plaques comprised of β -amyloid peptide (A β) and intracellular neurofibrillary tangles (NFT) consisting of aggregates of hyperphosphorylated tau protein [1]. A β is derived from the proteolytic cleavage of the amyloid precursor protein (APP) [2] and the identification of A β as the major component of senile plaques led to the hypothesis that its extracellular deposition could be a key factor in the progression of AD [3]. Despite a clear association between A β build up and cognitive decline, [4,5,6,7,8] a correlation between plaque deposition and the severity of dementia, could not be established. On the contrary, the cognitive decline appears to be underlined by defects in synaptic plasticity and by loss or dysfunction of synapses [9,10,11] that precede A β deposition and NFT formation [12,13,14]. It is now believed that small soluble A β oligomers, are responsible for early synaptic changes [15].

Although it is well established that A β affects long term potentiation (LTP) and long term depression (LTD), the causal

mechanisms are still elusive [16]. NMDAR dependent LTP in the hippocampus is blocked upon application of A β [7,17,18]. Intriguingly though, at low concentrations A β induces LTP possibly through α -7 nicotinic acetylcholine receptors [19]. Furthermore, A β induces LTD and excitotoxicity mediated by NMDARs receptors [20]. The importance of glutamate signalling via NMDARs as a causative event of dementia in AD is further demonstrated, by findings that memantine -a low affinity antagonist for NMDARs- results in behavioural improvement in AD model transgenic mice and is used as treatment of moderate AD [21,22,23]. This raises the possibility that the effects of A β could be due to an agonist action on NMDARs [24]. However, it is not fully established whether these effects are mediated directly through NMDARs. On the contrary evidence suggests that high concentration or prolonged exposure to A β 42 is required to establish a direct effect on AMPA or NMDA receptors [10,25,26,27]. Thus, it is unlikely that AMPA- and NMDA-receptors are directly affected at the earliest stages of AD pathology. Since there is no conclusive evidence of a direct interaction between A β and NMDARs, proposals such as a reduction in glutamate uptake or an increase of glutamate

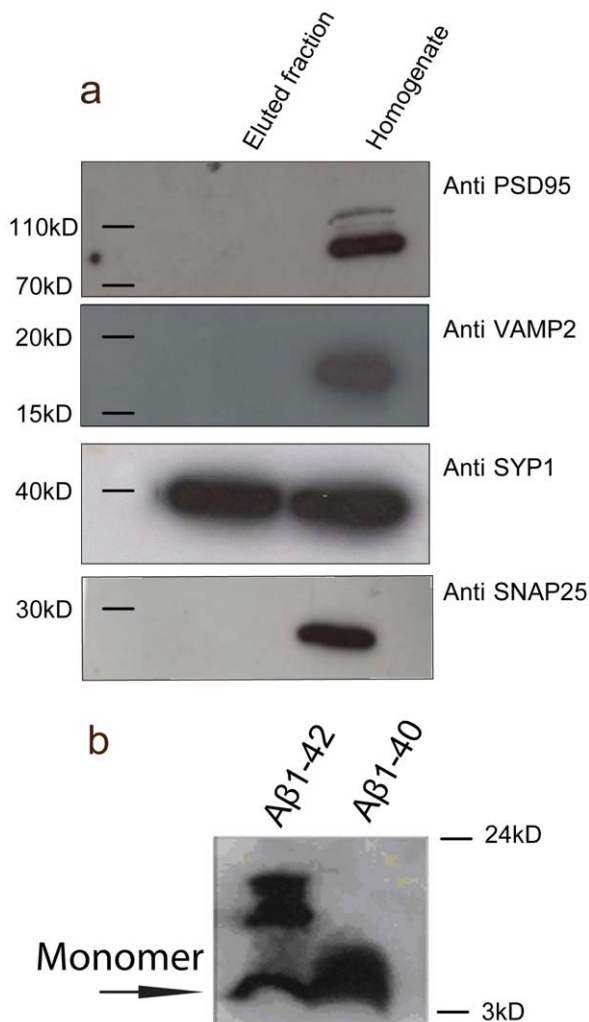


Figure 1. Identification of proteins interacting with A β by candidate approach. (a) Western blotting with primary antibodies against synaptic markers detected specific proteins in the starting material at their expected molecular weights. Only the primary antibody against Syp detected a specific protein in the fraction eluted from the A β affinity column. (b) Oligomerisation state of A β 42 compared to A β 40 revealed by western blotting. Monomeric A β peptides can be seen as a band at 4 kDa. Only A β 42 displayed visible low molecular weight aggregates.
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release have been put forward to explain these findings [19,28,29,30,31,32,33], but the cellular mechanisms underlying these defects are not clearly understood.

Here, we investigated cellular and molecular mechanisms by which A β induces synaptic toxicity. We show that administration of A β 42 peptides to mature hippocampal neurons is followed by its internalisation. Subsequently, A β 42 is detected at presynaptic terminals, where it can interact with Synaptophysin (Syp). We show that this interaction disrupts the Syp/VAMP2 complex and that this disruption could contribute to an expansion of the primed synaptic vesicle pool and of baseline neurotransmission.

Materials and Methods

Hippocampal Cell Culture

All animal experiments were performed according to Home Office regulations in compliance with the Animals Scientific Act

1986. Primary cultures of CA3-CA1 hippocampal neurons were prepared from E18 Sprague Dawley rat embryos. The experiments were performed in mature (21–28 days in vitro (DIV)) cultures. Neurons were seeded on poly-D-lysine (100 μ g/ml in 0.1 M borate buffer) and laminin (5 μ g/ml in PBS) coated coverslips at a density of 75,000 cells per coverslip and were maintained at 37°C, 5% CO₂ in Neurobasal media, supplemented with B27, L-glutamine (0.5 mM) and 100 units/ml penicillin/streptomycin.

Immunocytochemistry

Hippocampal cultures were rinsed once with PBS and fixed with 4% paraformaldehyde (PFA) in PBS. Fixed neurons were washed, permeabilised with 0.1% Tween-20 and 5% horse serum in PBS for 45 min at room temperature, and were incubated with primary antibodies overnight at 4°C. After washing, cells were incubated for two hours at room temperature with Alexa Fluor 488 or Alexa Fluor 555 (Molecular Probes, UK). Primary antibodies used were: Anti-A β 6E10 (ID Labs Ontario, Canada and Abcam Cambridge, UK), Anti-NR2A/B (Millipore Watford, UK). All other primary antibodies were supplied by Abcam. For internalisation assays, fixed cells were incubated with 6E10 followed by Alexa Fluor 488, then postfixed in 4% PFA for two minutes. Cells were re-incubated with 6E10 in the presence of 0.1% Tween-20, followed by the secondary antibody Alexa Fluor 555. In all experiments, the cells were rinsed, mounted with ProLong reagent, and visualised on a spin disc confocal system (CARV from Digital Imaging Solutions) with an EM-CCD camera (Rolera/QI Cam 3500) mounted in an Olympus X71 microscope with a 100 \times objective, using Image Pro 6.0 software. High magnification insets were produced using Adobe Photoshop.

FM1-43FX Labelling

Following exposure to 50 nM of A β for 20 min and 2 hrs of recovery, 5 μ M of FM1-43FX dye (prepared in HBSS warmed to 37°C) was applied to the cells for 5 min, followed by application of a hypertonic 500 mM sucrose solution in HBSS with 5 μ M FM1-43FX. After 5 min the sucrose solution was replaced with the original FM1-43FX solution for 15 min. The cells were then washed twice for 15 min in HBSS to remove un-incorporated dye and were subsequently fixed in 4% paraformaldehyde. Cells were kept in the dark until utilised.

Protein Extracts and Immunoprecipitation

Hippocampi were isolated from the brains of two juvenile Sprague Dawley rats of either sex (weighing between 90 g and 140 g) and homogenized in 1 mL lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 2% Glycerol), containing a protease cocktail inhibitor (Roche, UK). Protein G Dynabeads[®] (25 μ L) (Invitrogen, UK) were incubated with 5 μ g of the precipitating monoclonal antibody for one hour at 4°C. Scrambled peptide and A β 42 were diluted in 100 μ l hippocampal protein extracts (~7 mg/ml) at a final concentration of 100 μ M and the mix was incubated with the antibody bound Dynabeads[®] at 4°C for 4 hrs. Samples were boiled for 5 min to elute bound proteins, which were then analysed by SDS-PAGE.

Immunoblot

Proteins were separated on NuPage 4–12% Bis-Tris gels at 200 V and transferred to nitrocellulose membrane at 100 V for 1 hr at 4°C in transfer buffer (200 mM Glycine, 25 mM Tris Base

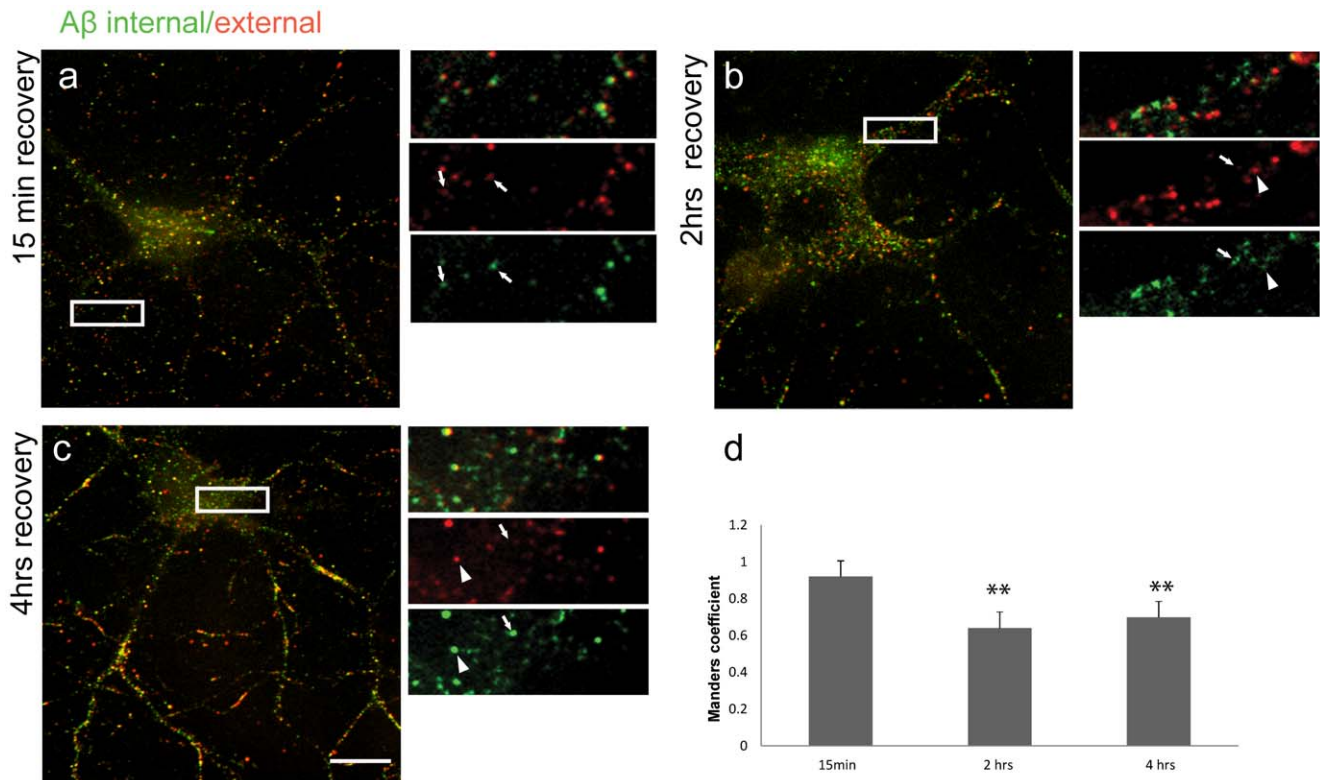


Figure 2. Representative images showing the dynamics of A β 42 internalisation by sequential immuno-labelling. Mature hippocampal neurons incubated with A β 42 for 20 min were left to recover for 15 min, 2 hrs and 4 hrs. (a) Labelling for external (red) and internalised (green) A β 42 after 15 min recovery period. Arrows at high magnification images of boxed area (right) point at examples of overlapping staining. A reduction in co-labelling was observed after 2 hrs (b) or 4 hrs (c) recovery. Arrowheads at high magnification images of boxed area (right) show examples of external and arrows of internalised A β 42. (d) Quantification of the extent of co-localisation shows a high degree of co-localisation at 15 min recovery ($M=0.92\pm 0.07$, $n=6$) compared to 2 hrs ($M=0.64\pm 0.07$, student t-test $**=0.0006$, $n=10$) and 4 hrs ($M=0.70\pm 0.08$, student t-test $*=0.0021$, $n=8$) of recovery. Scale bar = 10 μ m.

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20% Methanol). Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (50 mM Tris Base, 150 mM NaCl) containing 0.1% Tween 20, pH 7.5, for 1 hr and incubated overnight at 4°C with primary antibody (1:250) and 2 hrs with HRP-conjugated secondary antibody (1:1000). Membranes were developed with SuperSignal chemiluminescence kit according to instructions.

Hippocampal Slice Preparation

Field potential recordings were made from ventral sections of postnatal day 32–42 Sprague Dawley rats of either sex. Rats were deeply anaesthetised by halothane prior to decapitation, and the brain rapidly removed and submerged in oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) containing 135 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 1 mM MgCl₂, 10 mM Glucose and 26 mM NaHCO₃. The brain was hemisected, and ventral sections (400 μ m thick) prepared in aCSF on a Vibroslice (Campden Instruments Ltd). Slices were transferred and submerged in a holding chamber of continually oxygenated aCSF and left to recover for at least one hour. A β 42, A β 40 and scrambled A β peptide were added to the chamber to treat slices for 45 minutes prior to field EPSP recordings. Lyophilised fraction from 7PA2 cell media containing nanomolar amounts of A β oligomers was re-suspended in 100 ml aCSF in the tissue chamber to treat hippocampal slices for 45 min.

Electrophysiology

Recordings of field excitatory post synaptic potentials (fEPSPs) were made from the stratum radiatum in the CA1 region of the hippocampus using low resistance glass electrodes, in response to stimulation of the Schaffer collateral commissural pathway using a monopolar borosilicate glass electrode driven by a constant voltage stimulator (Digitimer) DC recordings were amplified using an AxoClamp2A (Molecular Devices) and a Neurolog NL104 (Digitimer Ltd) and signals filtered at 3 kHz prior to digitisation with a CED1401. Experiments were controlled using Signal 2 (CED Ltd) running on a connected PC. Electrodes were positioned just below the tissue surface (liquid interface setup) and slices were left to stabilise for 20 min. Input/output curves (or stimulation responses) were recorded, and the 10–90% slope measured, from a stimulus ranging from 10 V to 70 V with 3 successive events at each input level, 30 sec apart. To detect concentration dependent changes in neurotransmitter release, fEPSPs were initially evoked at 50 V with low frequency at 0.033 Hz in the presence of different concentrations of A β 42. For LTP recordings, a stable baseline was maintained for 25 min at 50% of the maximum fEPSP response, and LTP was induced by high frequency stimulation (HFS) consisting of three trains of 100 Hz, 10 sec apart. Potentiated synaptic response was subsequently recorded with low frequency stimulation every 30 sec for at least 90 min.

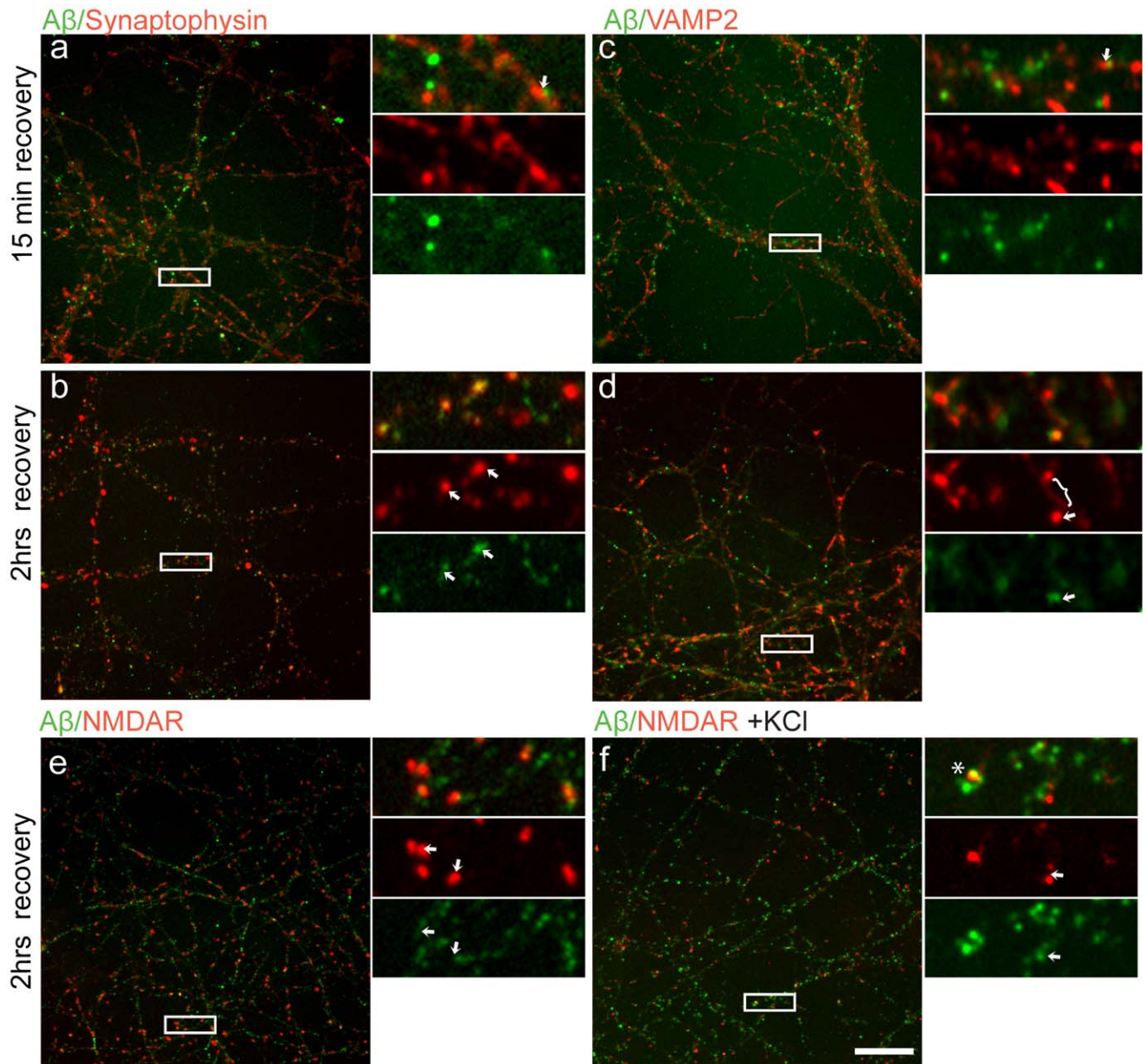


Figure 3. Representative images demonstrating presynaptic localisation of A β 42 (green) in mature hippocampal neurons, incubated with A β 42 for 20 min and left to recover for various time points. After 15 min recovery, A β 42 and Syp (a) or A β 42 and VAMP2 (c) labelling is distinct and often juxtaposed (as detailed in high magnifications of boxed areas (right), $n=15$). After 2 hrs of recovery co-localisation of A β 42 with Syp (b) and VAMP2 (d) can be observed ($n=39$ for each condition). Arrows at high magnification images of boxed area (right) point at examples of Syp (b) or VAMP2 (d) positive synaptic contacts that are also positive for A β 42. Bracket in high magnification images of boxed area (d) shows diffuse VAMP2 staining evident after the 2 hrs recovery period. After 2 hrs of recovery A β 42 is often juxtaposed to NMDAR (e) (even after depolarisation (f) ($n=10$). Arrows in high magnification images of boxed area point at examples of A β 42 juxtaposed to NMDAR. Scale bar = 10 μ m.

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Synthetic A β Peptides

A β 42 (American Peptide USA) was prepared as described previously [34] and stored in 0.1 M Tris pH7.4 at a stock concentration of 100 μ M at -20° C. Neurons were incubated with 50 nM of peptide for 20 min. Following treatment, the media was replaced with fresh supplemented Neurobasal and the cells were left to recover for the specified times.

Cell-Derived A β

7PA2 cells (a kind gift from D.M. Walsh, University College Dublin) [35], are a CHO cell line stably expressing human APP₇₅₁ carrying the V717F AD causing mutation. Cell cultures of 90% confluency were washed with serum free DMEM followed by incubation for 18 hrs in serum free DMEM. The conditioned medium was collected, cleared of cells by centrifugation ($134 \times g_{av}$ for 10 mins), and protease inhibitors were added to the supernatant. A β oligomers were separated by size exclusion

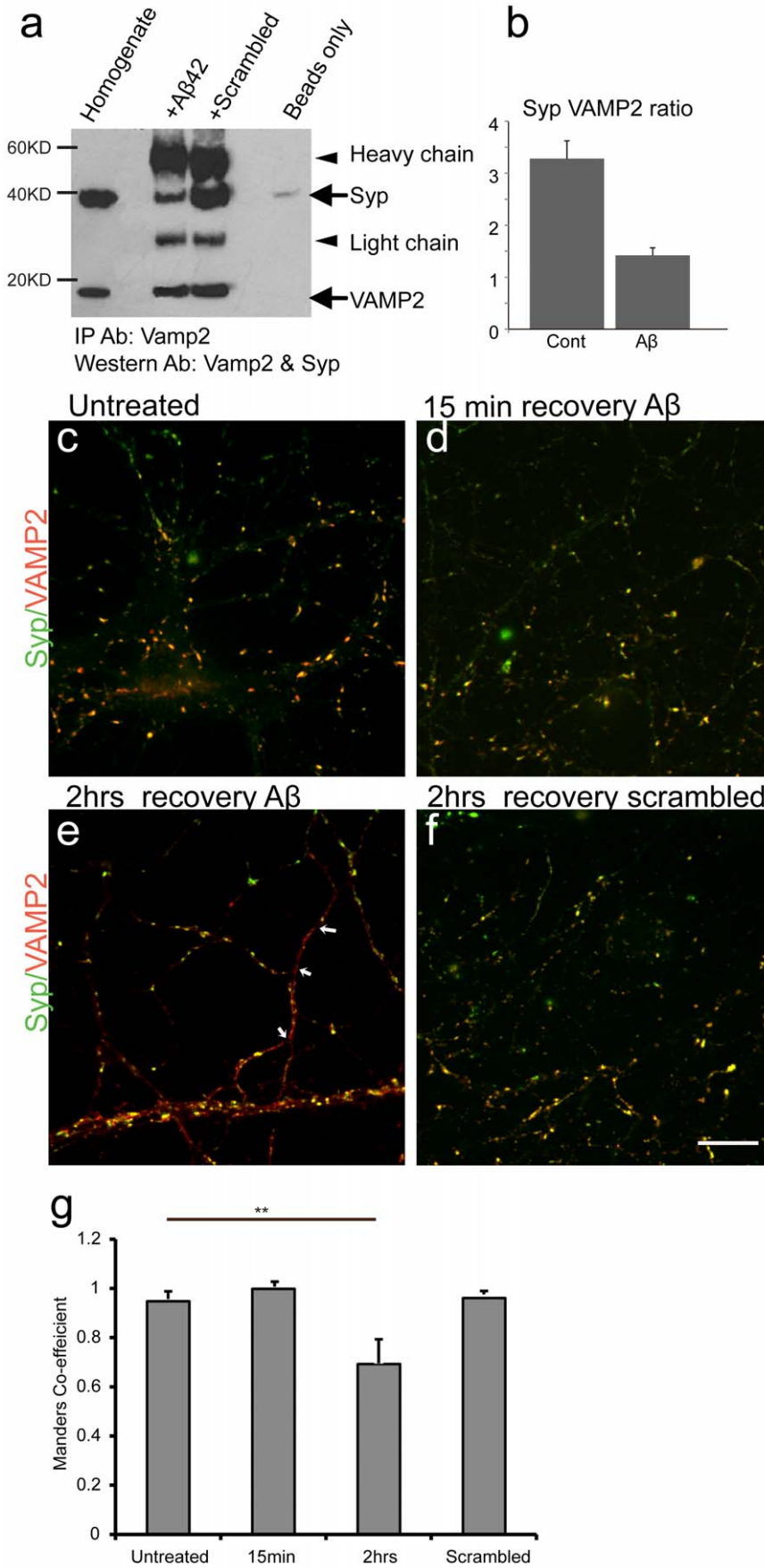


Figure 4. Disruption of the Syp/VAMP2 complex. (a) Immunoprecipitation of VAMP2 from hippocampal extracts assayed by western blotting for VAMP2 and Syp. A band of 19 kDa corresponding to VAMP2 and a band of 38 kDa, corresponding to Syp were detected in all conditions. A reduction in immunoprecipitated Syp was induced by A β 42 (lane 3) compared to control (scrambled A β , lane 4). (b) The intensity ratio between Syp and VAMP2 is reduced in the presence of A β 42 ($n=3$, student t -test $p=0.0026$). (c) co-labelling for Syp and VAMP2 in untreated hippocampal neurons, in neurons treated either with A β 42 and allowed to recover for 15 min (d) or with scrambled peptide (f) display a significant degree of co-localisation. (e) A β 42 treated neurons allowed to recover for 2 hrs display stretches of VAMP2 immunoreactivity (indicated by arrows). (g) Quantification of the extent of co-localisation between Syp and VAMP2. A β 42 treated neurons left to recover for 2 hrs showed decreased level of co-localisation ($M=0.85\pm 0.09$, $n=24$) compared to control ($M=0.961\pm 0.03$, $n=24$, student t -test $**=0.0003$), whereas neurons left to recover for 15 min ($M=0.99\pm 0.02$, $n=24$) or incubated with the scrambled peptide ($M=0.99\pm 0.01$, $n=18$) did not display differences compared to control. Scale bar = 10 μ m.

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chromatography as described [36]. Briefly, 1 ml of concentrated CM from each cell line was injected onto a Superdex 75 10/30 HR column attached to a AKTA HPLC system (Amersham) and eluted at a 0.4 mL/min. flow rate with 50 mM ammonium acetate (pH 8.5). The eluent was monitored at 280 nm and 230 nm and collected in fractions (0.5 ml). 50 μ l of each fraction was used in a double ELISA assay to determine A β oligomer content as previously described [37]. The remaining fractions containing A β peptides were pooled together and used to treat hippocampal slices.

Results

A β Interacts with Synaptophysin

To examine the underlying mechanisms behind A β 42 induced excitotoxicity, we designed a proteomic screen, based on an affinity column of A β 42, to identify synaptic proteins from hippocampal extracts that bind to the peptide. Bound proteins were first characterised by a candidate approach using antibodies against specific synaptic proteins to probe Western blots. Among various proteins examined, Syp, a protein present at synaptic vesicles (SV) was detected at high levels (fig. 1a). Antibodies against additional pre-synaptic markers, such as synaptosomal-associated protein 25 (SNAP25) and vesicle associated protein 2 (VAMP2 or synaptobrevin 2) failed to detect any protein in the eluted fractions. In addition, antibodies against postsynaptic proteins, such as the post synaptic density protein PSD95 were also used and again no bands were observed (fig. 1a). These results suggest that Syp is a strong candidate to be a specific interacting partner of A β 42.

Internalisation of A β 42 in Neurons

We then explored the significance of this interaction in dissociated neurons. For this interaction to occur *in vivo*, A β 42 should be present inside the neurons, at the presynaptic terminal. A β 42 accumulates intracellularly via two possible avenues; either it builds up because part of the intracellular pool is not secreted, or extracellular A β 42 is internalised by neurons [38]. Thus, we developed an internalisation assay to investigate the dynamics and the subcellular distribution of externally administered synthetic A β 42. Synthetic A β 42 is commercially available and readily aggregates in aqueous solution [34]. Since soluble A β 42 oligomers are believed to be the pathogenic peptides responsible for synaptic changes [36], we first ensured that oligomers were indeed present in our preparations, examining the aggregation state of A β 42 by Western blotting. Oligomeric as well as monomeric A β 42 species were consistently detected (fig. 1b). We then established the optimum conditions that would allow detection of the peptide via immuno-labelling and also ensure optimum survival of the neurons, applying different concentrations of A β 42 to mature (21 DIV) dissociated hippocampal neurons.

Our results showed that incubation of dissociated hippocampal neurons with 50 nM of A β 42 for 20 min, followed by recovery

periods of 15 min, 2 hrs and 4 hrs allowed both, detection of A β 42 and optimum survival of neurons. To look into the dynamics of the internalisation of A β 42, sequential immuno-labelling of fixed neurons (treated with 50 nM of A β 42) was employed to distinguish between surface and internalised A β 42. Surface labelling was carried out using the 6E10 primary antibody (for A β detection) and the Alexa-Fluor 488 secondary antibody in the absence of detergent. The neurons were then post fixed and intracellular A β 42 was detected by repeating the procedure using the same primary antibody and the Alexa-Fluor 555 secondary antibody in the presence of 0.1% Tween-20 to permeabilise the cells. After 15 min of recovery, a high degree of co-labelling was observed, suggesting the peptide remained extracellular (fig. 2a). However, with increased recovery time, although co-labelling was still evident, additional signal was increasingly observed after permeabilisation (fig. 2b, c) showing that after 2 hrs of recovery A β 42 had been internalised. To quantify the extent of the internalisation, the co-localisation was quantified by calculating the split Mander's co-localisation coefficients using the JACoP plugin for Image-J [39]. Mander's co-efficient measures the coincidence of two signals, even when the intensities in both channels are significantly different. Our results (fig. 2d) show that the Mander's coefficient was highest (0.92 ± 0.7 $n=6$) after the 15 min recovery time point, suggesting a high degree of co-localisation. Within a 2 hrs recovery period, the Mander's coefficient dropped significantly (0.64 ± 0.07 $n=10$, student t -test $p=0.0006$). Similarly, after 4 hrs of recovery, the Mander's coefficient was lower than the 15 min recovery period (0.70 ± 0.08 $n=8$, student t -test $p=0.0021$) confirming our observation that A β 42 can be detected intracellularly over a 4 h period [40].

Presynaptic Localisation of A β 42

Since we identified Syp as a possible interacting partner of A β 42, we examined if such an interaction could mediate the internalisation/endocytosis of A β 42 at synaptic contacts. Dissociated neurons were incubated with A β 42 (50 nM) for 20 min and allowed to recover in fresh media for 15 min or 2 hrs prior to fixation. If A β 42 was internalised via its interaction with Syp, we would expect A β 42 to be localised at presynaptic terminals soon after incubation with the peptide. Double immuno-labelling for A β 42 and Syp after 15 min of recovery, did not reveal any significant overlap between A β 42 and presynaptic contacts marked by Syp labelling (fig. 3a, $n=15$). On the contrary, A β labelling was often detected juxtaposed to Syp (arrow in high magnification of fig. 3a) suggesting a postsynaptic localisation of A β [11,41]. After 2 hrs of recovery however, co-localisation between A β 42 and Syp labelling in a fraction of synapses was evident (fig. 3b, $n=39$). These results showed that although internalisation of A β 42 via an interaction with Syp is unlikely, the peptide can be detected pre-synaptically after an extended period of time. To further confirm our findings, we repeated the experiment using VAMP2 as an alternative presynaptic marker.

Synaptophysin/FM 1-43FX

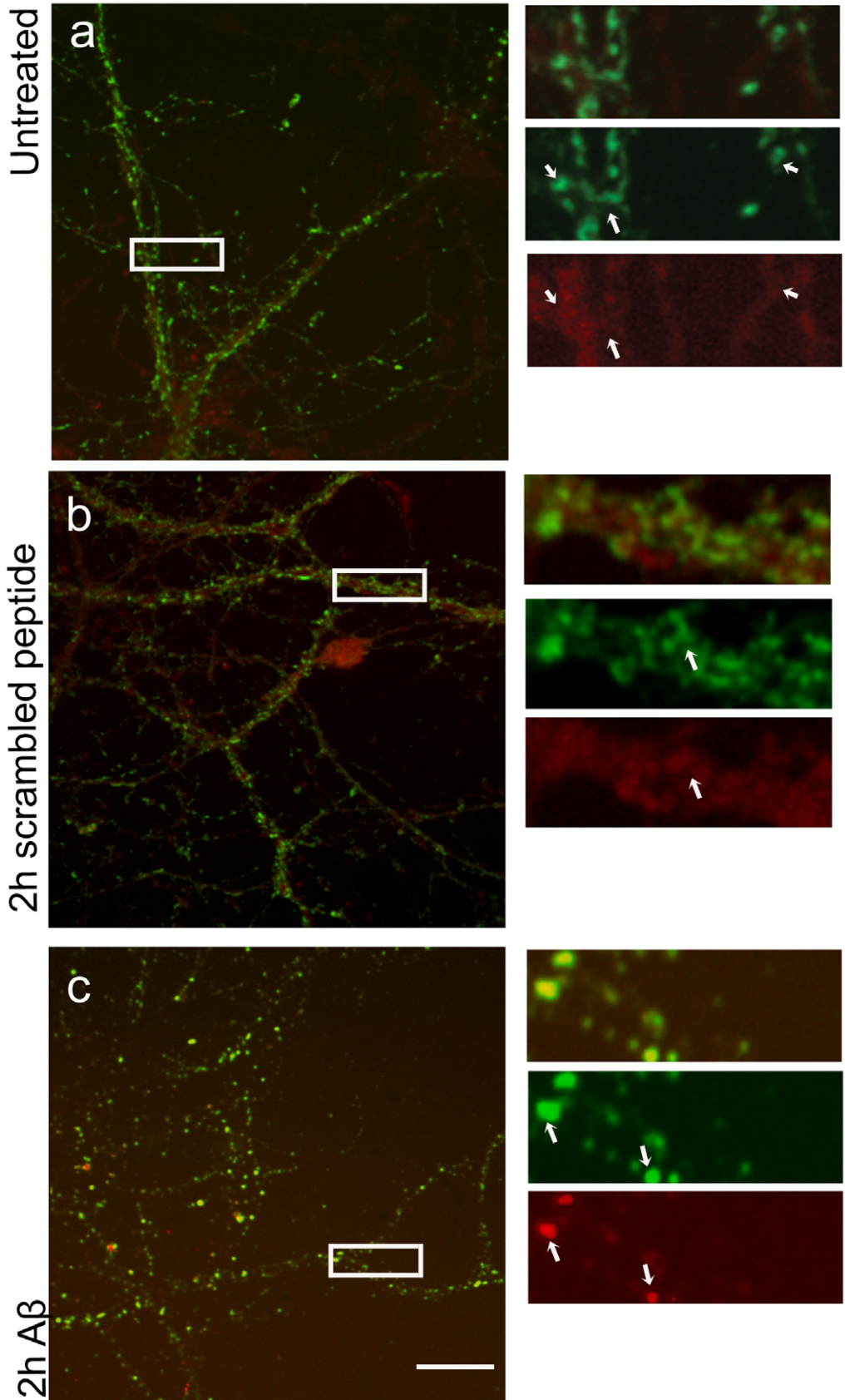


Figure 5. Fusion of primed vesicles at synaptic contacts marked by labelling for Syp (green) is visualised by the extent of internalisation of FM1-43FX dye (red). High magnification images of boxed areas are shown on the right. Internalised dye at synapses is near background levels in untreated ($n = 20$) (a) as well as in neurons treated with the scrambled peptide ($n = 20$) (b). Arrows in a and b point at synaptic contacts labelled with Syp. (c) Exposure to A β 42 followed by 2 hrs of recovery results in a significant increase in internalised dye at synaptic contacts ($n = 20$). Arrows in c point at synaptic contacts labelled with Syp. Scale bar = 10 μ m.
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Similar to our previous observations, after 15 min of recovery no overlapping pattern between A β 42 and VAMP2 labelling was evident (fig. 3c, $n = 15$), whereas after 2 hrs of recovery, co-localisation between VAMP2 and A β 42 could be detected (fig. 3d, $n = 39$), further confirming its presence at pre-synaptic terminals. However, compared to Syp, the pattern of VAMP2 staining after 2 hrs of recovery was unusual, with visible stretches along the membrane (see brackets in high magnification of fig. 3d) instead of the characteristic punctate pattern (fig. 3c). Finally, double immuno-labelling for A β 42 and NMDARs in dissociated neurons incubated with 50 nM of A β 42 for 20 min and allowed to recover in fresh media for 2 hrs prior to fixation showed that A β 42 could be detected alongside as well as juxtaposed to NMDARs confirming its presynaptic localisation (arrows in fig. 3e). However, these data were unexpected since previous reports have demonstrated that incubation of dissociated neurons with A β peptides results in its postsynaptic localisation [11,41,42]. Since neurotransmitter release has been associated with a rapid increase in the extracellular levels of A β 42 [43], we considered the possibility that the observed presynaptic localisation of A β 42 could be transient, followed by its release into the synaptic cleft in an activity dependent manner. Subsequently the released A β 42 would interact with postsynaptic targets. To test this hypothesis, dissociated hippocampal neurons exposed to 50 nM of A β were depolarised after 2 hrs of recovery. The neurons were treated with 50 mM KCl for 10 min to induce neurotransmitter release. Double immuno-labelling for A β 42 and NMDARs showed that a substantial amount of A β 42 was still juxtaposed to, NMDARs (fig. 3f). Quantification of the extent of co-localization between A β and NMDARs by calculating the Mander's co-efficient, did not reveal any statistically significant differences between neurons exposed to A β 42 (0.296 ± 0.05) and neurons exposed to A β 42 and depolarised after recovery (0.348 ± 0.03 , student t-test $p = 0.098$, $n = 10$). These results suggest that the presynaptic localisation of A β 42 was not transient since a substantial amount of the peptide remained presynaptic even after induction of neuronal activity.

A β 42 Disrupts the Syp/VAMP2 Complex

The biochemical interaction of A β 42 with Syp and its presence at the presynaptic terminal suggests that the peptide may have a pre-synaptic effect. To explore this further, we looked closer at the unusual distribution of VAMP2 in neurons exposed to A β 42 (high magnification of fig. 3 d). It has been shown that Syp interacts with VAMP2 regulating its synaptic distribution [44]. Thus we hypothesised that the diffuse distribution of VAMP2 could be a consequence of A β 42 interfering with the formation of the VAMP2/Syp complex. To test this, we immunoprecipitated VAMP2 complexes from hippocampal extracts either in the presence of an excess of A β 42, or in the presence of a similar concentration of control, scrambled A β (fig. 4a). A substantial reduction of co-immunoprecipitated Syp was observed in the presence of A β 42 (fig. 4b, $n = 3$), compared to the amount of co-immunoprecipitated Syp in the presence of the scrambled peptide suggesting that A β 42 disrupts the interaction between VAMP2 and Syp *in vitro*.

Since it has been shown that disruption of the interaction between VAMP2 and Syp induces the axonal distribution of

VAMP2 [44], we compared the extent of co-localisation between these two proteins in neurons exposed to A β 42 and control neurons to investigate if A β 42 disrupts their interaction in living neurons as well. Double immuno-labelling of VAMP2 and Syp in control neurons (fig. 4c) and in neurons recovering for 15 min after exposure to 50 nM of A β 42 (fig. 4d) showed the expected extensive co-localisation between the two proteins suggesting that A β 42 had little or no effect on the VAMP2-Syp complex during the first 15 min of recovery. On the contrary, VAMP2 labelling in neurons exposed to A β 42 after 2 hrs of recovery (fig. 4e) showed stretches of VAMP2 immunoreactivity devoid of Syp labelling (i.e. areas between arrows in figure 4e). This diffuse VAMP2 labelling is indicative of a disruption in the formation of the VAMP2/Syp complex *in vivo* [44] demonstrating that A β 42 interferes with the stability of the interaction between VAMP2 and Syp. The co-localisation of VAMP2 and Syp was not affected in neurons exposed to similar concentration of the scrambled peptide (fig. 4f) suggesting the diffuse VAMP2 labelling is specifically induced by A β 42 and not by the addition of any non specific peptide in the medium. Quantification of the extent of co-localisation between Vamp and Syp, by calculating the Mander's co-efficient, confirmed that their co-localisation was specifically reduced in neurons exposed to A β 42 and allowed to recover for 2 hrs (fig. 4g).

Physiological Consequences in Synaptic Transmission

Further to its role in sorting VAMP2, Syp also interacts with VAMP2 at the presynaptic terminal and it has been proposed that it regulates the participation of VAMP2 in the SNARE complex during the formation of the fusion pore complex (FPC) [45,46,47]. Therefore, disruption of the association between VAMP2 and Syp at synaptic contacts by A β 42 could induce the formation of the FPC resulting in an increase of primed SVs. To investigate this hypothesis, untreated mature hippocampal neurons or neurons treated for 15 min either with 50 nM of A β 42 or with the inverted control peptide, were incubated after 2 hrs of recovery with FM1-43 FX, a fluorescent lipophilic dye that labels membranes. Using a hypertonic sucrose solution (500 mM sucrose in HBSS) primed vesicles were induced to fuse to the presynaptic membrane and neurons were left to recover in the presence of the dye to allow for its uptake at sites of SV recycling [48]. Incorporation of the dye at synaptic contacts was confirmed with immuno-labelling for Syp. Images of mature (21 DIV) untreated hippocampal neurons (fig. 5a) and neurons treated either with scrambled peptide as a negative control (fig. 5b) or with A β 42 (fig. 5c) were taken consecutively leaving the microscope/camera settings unaltered. In untreated neurons or in neurons exposed to the control inverted peptide, uptake of the dye at synaptic contacts was near background levels, although internalisation of the dye was evident in the cell soma ($n = 20$, fig. 5 a, b). On the contrary, in neurons exposed to A β 42, the dye uptake was more pronounced at a fraction of synaptic contacts (fig. 5c, $n = 20$), indicative of an increase in the number of vesicles primed to the presynaptic membrane. These results strongly suggest that A β 42 increases the amount of primed vesicles at the presynaptic terminal. The question now was if this increase would contribute to synaptic transmission. Although our results show that A β 42 could be detected at the presynapse via immuno-labelling after 2 hrs of

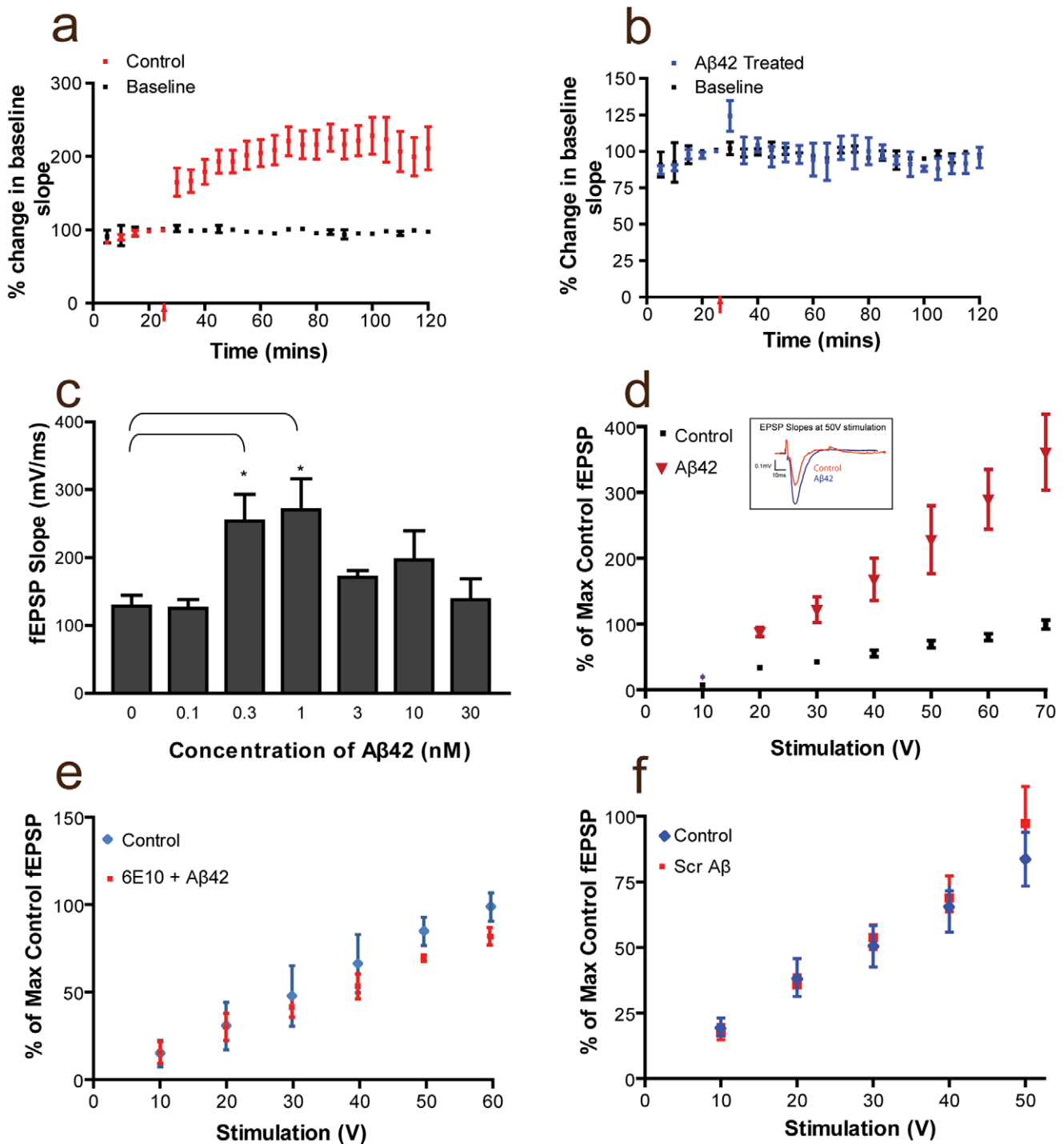


Figure 6. Neurotransmitter release is affected by Aβ. (a) Induction of CA1-LTP after HFS in control hippocampal slices (Two-way ANOVA $p=0.0006$ $n=5$). (b) Induction of CA1-LTP after HFS is blocked in hippocampal slices after a 45 min bath application of Aβ42. (c) fEPSPs recorded in the CA1 in response to a 50 V stimulus of the Schaffer collateral commissural pathway showed a concentration dependent effect of Aβ in fEPSPs. Only mid-range concentrations of 0.3 nM and 1 nM were able to increase the fEPSP slope ($p=0.028$ and $p=0.048$ respectively with student t-test, $n=5$ for each concentration tested). (d) Treatment with Aβ42 (45 min) increases the stimulation response compared to control, untreated slices over a range of stimuli. Results are presented as the percentage of the maximum fEPSP from control recordings ($n\geq 5$ each). Inlet in shows a trace of EPSP slopes between a control slice and a slice treated with Aβ42 stimulated at 50 V. (e) Chelation of Aβ42 using 6E10, a specific antibody for the peptide, abrogates the enhancement at all input values ($n=5$). (f) Scrambled Aβ did not induce increased responses compared to controls. doi:10.1371/journal.pone.0043201.g006

recovery, it has been demonstrated that incubation of slices with Aβ42 for a shorter period of time, is necessary and sufficient to induce detectable synaptic defects [19,49]. To account for possible

differences in the physiology of neurons between hippocampal slices and dissociated neurons, we adopted a similar approach to investigate the effect of Aβ42 on synaptic transmission. Consistent

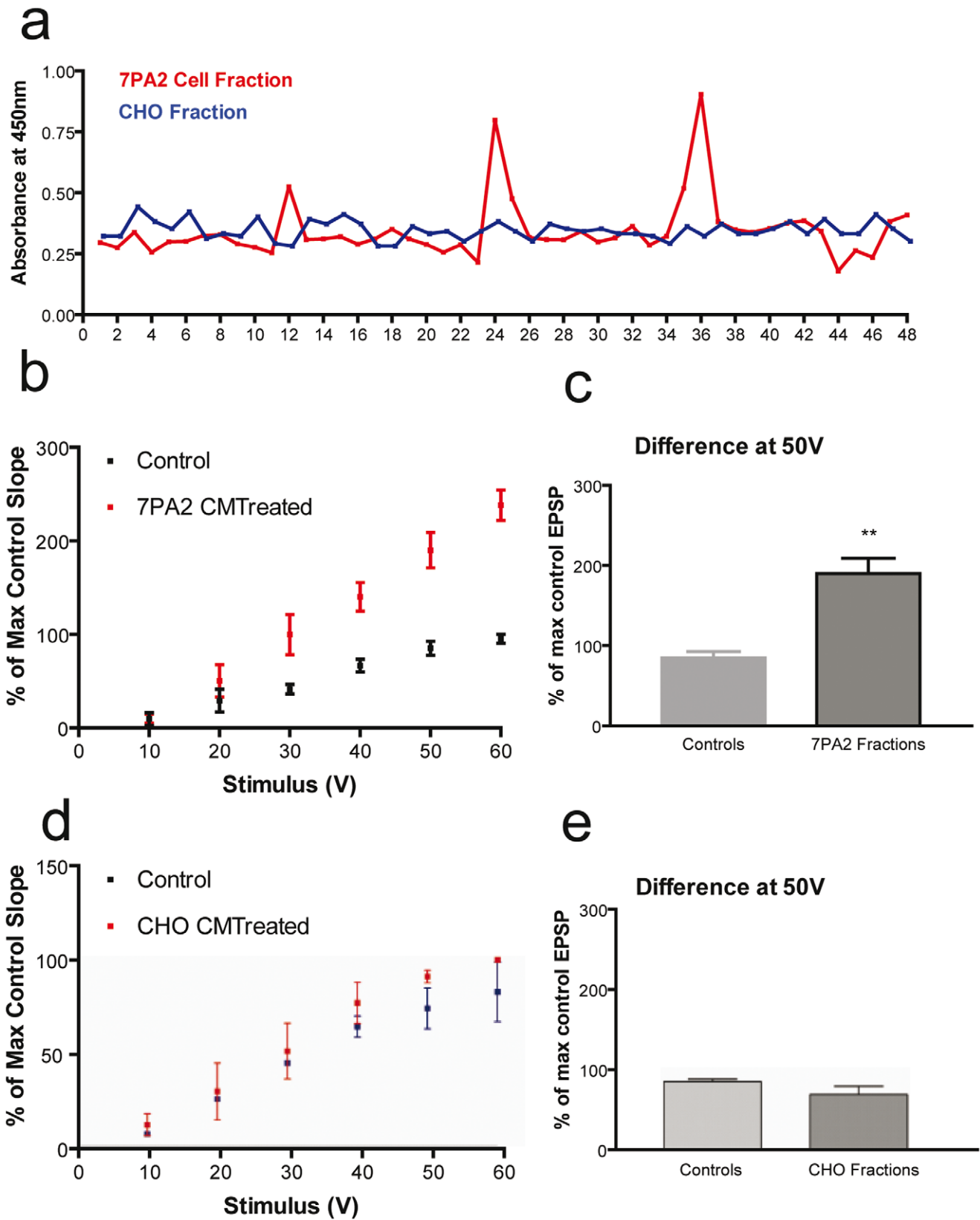


Figure 7. ELISA assay from fractionated conditioned media (CM) from 7PA2 cells and control CHO cells detected A β oligomers in the conditioned medium of 7PA2 cells but not of CHO control cells (a). Hippocampal slices were incubated with CM from fractions 12, 24 and 36 of control as well as 7PA2 cells. fEPSPs were recorded in response to Schaffer collateral commissural pathway stimulation at a range of stimulus from 10 V through to 60 V (b and d). Incubation with cell-derived A β , increased the response to stimulation at the CA1 synapse (b), compared to slices treated with conditioned media from the control CHO cells (d). The results are expressed as a percentage of the maximum fEPSP

slope from control slices. Graphs showing representative results at 50 V in slices exposed to 7 PA conditioned medium (c) and to control CHO conditioned medium (e) show that cell derived A β increased fEPSP (student's t-test $p=0.003$ $n=5$), whereas the control medium had no effect at 50 V ($n=5$) (e).

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with previous studies, hippocampal slices incubated with A β 42 for 45 min prior to recording showed that A β 42 inhibited the induction of NMDAR-dependent LTP in the hippocampal CA1 region (fig. 6a, b). Next, we investigated if under the same conditions A β 42 affected basal transmission. We compared fEPSPs recorded from the CA1 region of hippocampal slices in response to electrical stimulation of the Schaffer collateral pathway, in the presence or absence of A β 42. First, we noticed a concentration dependent enhancement in basic transmission at concentrations ranging from 300 pM to 1 nM A β 42 (fig. 6c). We subsequently investigated this effect in detail by increasing electrical stimulation in slices exposed to 1 nM A β 42. Our results showed a progressive enhancement of fEPSPs recorded in treated slices compared to untreated control slices (fig. 6d, $p=0.044$ at 50 V with student's t-test, $n=5$), indicative of an increased efficacy of the Schaffer collateral synapse. We then confirmed the specificity of this enhanced efficacy either by chelation of A β 42 using an excess of an A β 42 specific antibody (200 ng/ μ l of 6E10 mAb), or by using equal concentrations of the scrambled peptide. Co-incubation with 6E10 and A β 42 produced similar recordings to untreated slices (fig. 6e, $n=5$). Furthermore, incubation of slices with the scrambled peptide had no effect (fig. 6f, $n=5$) further confirming that the enhancement of fEPSPs was specific to A β 42.

To unambiguously confirm that the enhancement of synaptic transmission was induced by A β , we repeated our experiments with cell-derived A β peptides, from 7PA2 cells. These Chinese-hamster ovary cells that express the V717F mutant human APP secrete low (nanomolar) levels of oligomeric A β peptides [35,50]. Incubation of slices with cell derived oligomers provided similar results to those obtained with the synthetic peptide, suggesting that the enhancement of fEPSPs is not an artefact of the synthetic peptide (Fig. 7) but rather a physiological function of A β .

Discussion

Synaptic Localisation of A β 42

Whilst extensive information in the literature shows that synaptic failure precedes cognitive decline in AD [4] the cellular and molecular events underlying this synaptic dysfunction are still not fully resolved. The data presented here provide an insight into one mechanism by which A β 42 affects synaptic activity. First, we show that the presynaptic protein Syp interacts with A β 42 *in vitro*. We then establish the significance of this interaction by showing that Syp and externally applied A β 42 can co-localise in living neurons. Studies with transgenic animal models of AD, have supported the presence of intraneuronal A β (iA β) prior to the appearance of extracellular deposits, suggesting that iA β is a significant contributor to the onset of learning and memory deficits [38,51]. Despite an apparent relationship between the intracellular pool of A β and the extracellular deposits [13,52] the precise mechanism by which the peptide enters the neuron is still under investigation. Physical interaction between A β 42 and the α 7 acetylcholine receptor [53] or apoE [54] suggests that these proteins are candidates for a receptor mediated transfer of A β into the cell. On the other hand, there is evidence that A β peptides cross the neuronal membrane passively, via non-endocytic and energy independent pathways, most likely due to its ability to biophysically interact with lipids at the neuronal membrane [55]. Thus, we considered the possibility that an interaction between

Syp and A β 42 at the presynaptic terminal could be an alternative mechanism for its internalisation. We examined the dynamics of the co-localisation of these proteins and found no evidence to support the hypothesis that interaction of A β 42 with Syp provides an alternative mechanism for the internalisation of the peptide, since there was no indication of presynaptic localisation of A β 42 in neurons that were left to recover for only 15 min. Interestingly though, the absence of co-localisation at early time points compared to the co-localisation we observed 2 hrs after the removal of A β from our cultures, shows that A β 42 can accumulate at the presynaptic terminal with time. It is therefore likely that, at least in part, the presynaptic localisation of A β 42 could be due to its translocation at the presynaptic terminal by an unknown transport mechanism. Co-labelling for A β 42 and NMDARs further confirmed that A β 42 could be detected at presynaptic as well as at postsynaptic terminals.

In contrast to the data presented here, several reports demonstrate exclusive co-localisation between A β 42 and postsynaptic markers [11,41]. In one such study, Lacor and colleagues found 92% of exogenously applied A β co-localised with the postsynaptic protein PSD-95 and the majority of the peptide was found juxtaposed to Syp [41]. We believe that the difference in our results reflects differences in our experimental procedures. For instance, a plausible explanation is that we allowed cells to recover for two to four hours after exposure to A β 42. This recovery period (in the absence of the peptide) proved to be essential for the detection of the peptide at presynaptic terminals. In addition, Lacor *et al.* showed that only high molecular weight species of A β peptides could be detected post-synaptically whereas our A β preparation was enriched in low oligomeric forms. We suggest that taken together, these results complement each other, demonstrating two different roles of A β at the synapse, one presynaptic and the other postsynaptic.

Mechanism of Synaptic Toxicity

Confusing data regarding the effect of A β 42 in synaptic dysfunction such as increase or suppression of spontaneous activity are often found in the literature [56,57]. The underlying problem in resolving these inconsistencies has been to understand the mechanisms by which differences in the amount/preparation of the peptides can affect the outcome of an experiment. Furthermore, the ambiguity of the causal mechanisms behind these effects has further challenged consistent interpretation of these data. We believe that our findings regarding the presynaptic localisation, the interaction between A β 42 and Syp and its effect in neurotransmitter release may help resolve some of these inconsistencies. Syp was the first SV protein to be characterised [58,59]. Since then, various studies have implicated Syp in exocytosis, in regulating the formation of the fusion pore complex (FPC), in synapse formation and in endocytosis of SVs [45,46,47,60,61,62,63]. However, studies using Syp KO mice failed to show an apparent SV recycling phenotype [64,65]. The lack of an obvious phenotype was attributed to the redundant function of its isoforms Synaptogyrin (syg) and Synaptoporin. Indeed, mice lacking both Syp and Syg expression showed reduced LTP [66] although the mechanism responsible for this reduction is still not clear.

One of the best characterised roles of Syp is its interaction with VAMP2 [45], a SNARE protein [67]. The complex between

VAMP2 and Syp is formed at the trans-Golgi network (TGN), and the formation of this complex is necessary and sufficient to recruit VAMP2 to synaptic contacts. Disruption of this interaction results in diffuse localisation of VAMP2 along the axonal membrane [44]. Here we demonstrate that A β 42 disrupts the interaction between VAMP2 and Syp both *in vitro* and in dissociated neurons (fig. 4). Two possible mechanisms could account for this disruption: either A β 42 disrupts a signalling pathway that regulates the dynamics of this interaction at the TGN, or it competes with VAMP2 for binding to Syp. Our immunoprecipitation data demonstrate that A β 42 directly competes with VAMP2 for binding to Syp supporting the latter hypothesis. We also observed that this disruption was more pronounced in less mature neurons (14 DIV) when synapses are not yet fully mature and there is increased traffic of structural components towards the developing synapses. In addition to correctly sorting VAMP2 at the synapse, Syp also regulates the retrieval kinetics of VAMP2 during endocytosis [63]. Thus, if A β 42 competes with VAMP2 for binding to Syp at the synapse, it is likely that it will also affect SV recycling. A β 42 induced changes to SV recycling has previously been reported. Kelly *et.al.* proposed that A β disrupts SV endocytosis, a phenotype consistent with reduced binding of Syp to VAMP2 in A β treated neurons [68]. Finally, it has also been reported that VAMP2 dissociates from Syp prior to exocytosis suggesting that Syp prevents VAMP2 from participating in the formation of the FPC [46,69]. Furthermore, it has also been shown that A β induces an extensive depletion of the SV pool, indicative of an increased participation of SVs in neurotransmitter release [68]. Thus, we hypothesised that disruption of the interaction between Syp and VAMP2 would increase the availability of VAMP2, contributing towards an increase in the number of primed vesicles at the presynaptic membrane. We verified this by showing that fusion of primed SV in the presence of FM1-43FX results in an increase of endocytosed dye at the synapses of neurons incubated with A β 42. In addition, the enhancement of single-shock fEPSPs by A β 42 in synapses within hippocampal slices further suggested an increased availability of releasable SVs. What is more, the same effect was reproduced by naturally produced amyloid peptides demonstrating that this effect is independent of the peptide source. It is important to note that we were also able to reproduce the well-established disruption of LTP by A β 42 under the same experimental conditions. In support of our results, several reports have shown an A β -dependent increase in the number of vesicles available at the presynaptic active zone [19,28,70] or that glutamate release is enhanced by A β peptides [71]. These results suggest that regulation of NT release at the pre-synaptic level could be an important aspect of the physiological role of A β . Furthermore, increasing evidence suggests that A β could exert its effects in synaptic plasticity by excessive activation of NMDARs. For instance, it has been shown that inhibition of LTP by A β can be via a mechanism involving excessive activation of extrasynaptic NMDARs as well as due to defects in glutamate uptake [29,32,33]. A key question that remains to be answered though is to what extent the interaction between A β and Syp that we report here, is necessary and sufficient to induce the observed increase in synaptic transmission, or to what extent this interaction is a contributing factor affecting the homeostasis of the presynaptic terminal. Although our data clearly demonstrate that A β is detected at the presynapse where it can interact with Syp affecting neurotransmitter release, its presynaptic effects are likely to be more complex. For instance, it has been shown that A β inhibits presynaptic P/Q Ca channels suppressing spontaneous synaptic activity [57,72]. Interestingly, a series of studies demonstrated that A β can also

have a positive effect on synaptic transmission in a concentration dependent manner. It has been shown that picomolar concentrations of A β 42 enhance neurotransmitter release during HFS [19] and can also activate presynaptic α 7-nAChRs [73]. Finally, Puzzo *et.al.* showed that newly formed endogenous A β is essential for NT release from the presynaptic terminal [74], demonstrating further that at least in part, A β exerts its function at the pre-synapse. The data presented here could contribute towards our understanding of the mechanism behind these observations. It is possible that the disruption of the Syp/VAMP2 complex contributes towards an increase in NT release, allowing VAMP2 to participate more readily in the formation of SNARE complexes deregulating NT release by increasing the amount of primed SVs. Moreover, following exocytosis, the ability of Syp to initiate SV endocytosis via its interaction with VAMP2 [62,63] would be reduced. Such a mechanism can explain both the positive contribution of A β to NT release as well as the reported disruption of endocytosis and the depletion of SVs induced by A β [19,68,74]. Furthermore our data are also supported by findings that show a “bell shaped” relationship between A β and synaptic plasticity [28]. Consequently, long term exposure and/or high levels of A β 42 would consistently deregulate glutamate release, disrupting LTP [33,68,70] and inducing excitotoxicity [75]. Finally, at later stages, increased release of A β 42 from presynaptic sites [43] would induce additional effects at the postsynaptic membrane.

An increasing number of reports in the past years demonstrate that glutamatergic synaptic transmission via AMPA, NMDA and metabotropic glutamate receptors is crucial in the pathogenesis of AD. Targeting these receptors for AD therapy although beneficial comes with side effects. Thus, understanding the cellular mechanism behind the deregulation of glutamatergic neurotransmission might provide alternative therapeutic targets. Furthermore, in a recent publication, Puzzo *et.al.* pointed out that impairment of LTP at early stages of the disease is not necessarily linked to interference with the function of AMPAR or NMDAR and only increased concentrations of A β or prolonged exposure to the peptide showed a direct impairment of the receptors [25]. Our data further support this observation since we showed that brief exposure to A β 42 at concentrations that are not known to directly interfere with the receptors, results in LTP impairment that is likely induced by pre-synaptic defects. Taken together, these data suggest that A β 42 is involved in modulation of synaptic activity by interfering with multiple pre- and postsynaptic mechanisms in a concentration and time dependent manner.

Although our data showed the effect of A β 42 on a cellular mechanism that can contribute to the deregulation of glutamatergic synaptic transmission, it is quite possible that we have demonstrated only one of several cellular events disrupted by A β . Nevertheless, our data make an important contribution in elucidating the pleiotropic function of A β . Understanding the causative events behind its toxicity is essential in order to design appropriate therapeutic strategies to target the symptoms of AD more efficiently.

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Author Contributions

Conceived and designed the experiments: PA RME. Performed the experiments: CLR SS. Analyzed the data: CLR SS RME PA. Contributed reagents/materials/analysis tools: PA PWB BMA. Wrote the paper: PA.

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