Amyloid-β acts as a regulator of neurotransmitter release disrupting the interaction between Synaptophysin and Vamp2.

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Abstract

It is becoming increasingly evident that deficits in the cortex and hippocampus at early stages of dementia in Alzheimer's disease (AD) are associated to synaptic damage caused by oligomers of the toxic amyloid-β peptide (Aβ42). However, the underlying molecular and cellular mechanisms behind these deficits remain unknown. Here we provide evidence of a mechanism by which Aβ42 regulates neurotransmitter release. We provide evidence that application of Aβ42 in cultured neurones is followed by its internalisation and translocation to synaptic contacts. Interestingly, our results demonstrate that Aβ42 is translocated at the presynaptic terminal of glutamatergic synapses where it interacts with Synaptophysin. This interaction disrupts the complex formed between Synaptophysin and Vamp2, increasing the amount of primed vesicles, exocytosis and baseline transmission. Our observations provide a necessary and timely insight into the cellular mechanisms that underlie the initial pathological events that lead to synaptic dysfunction in Alzheimer's disease.

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 (Aβ) and intracellular neurofibrillary tangles (NFT) consisting of aggregates of hyperphosphorylated tau protein (Selkoe, 2001). Aβ, is derived from the proteolytic cleavage of the amyloid precursor protein (Thinakaran and Koo, 2008). The identification of Aβ 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 (Hardy and Allsop, 1991). Despite a clear association between Aβ build up and cognitive decline, a correlation between plaque deposition and the severity of dementia, could not be established. On the contrary, the cognitive decline is underlined by defects in synaptic plasticity (Gomez-Isla et al., 1997; Lambert et al., 1998; Selkoe, 2002; Cleary et al., 2005; Lesne et al., 2006) and by loss or dysfunction of synapses (Shrestha et al., 2006; Lacor et al., 2007; Shankar et al., 2007) that precede Aβ deposition and NFT formation (Hsia et al., 1999; Oddo et al., 2003; Oddo et al., 2006). It is now believed that small soluble Aβ oligomers, are responsible for early synaptic changes (Selkoe, 2008).

Although it is well established that Aβ affects long term potentiation (LTP) and long term depression (LTD), the causal mechanisms are still elusive(Hardy, 2009). LTP in the hippocampus, is blocked upon application of Aβ in an NMDAR-dependent manner (Cullen et al., 1997; Kim et al., 2001). Intriguingly though, at low concentrations Aβ induces LTP via an unknown mechanism (Puzzo et al., 2008). Furthermore, Aβ induces LTD and excitotoxicity mediated by NMDARs receptors (Harkany et al., 2000). 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 (Scholtzova et al., 2008; Puangthong and Hsiung, 2009; Klyubin

et al., 2011). This raises the possibility that Aβ's effects, could be due to an agonist action on NMDARs (Molnar et al., 2004). Since there is no conclusive evidence of a direct interaction between Aβ and NMDARs, proposals such as a reduction in glutamate uptake or an increase of glutamate release have been put forward to explain these findings (Arias et al., 1995; Harris et al., 1995; Kabogo et al., 2008; Puzzo et al., 2008; Abramov et al., 2009; Li et al., 2009; Li et al., 2011), but the cellular mechanisms underlying these defects are not clearly understood.

Here, we investigated the cellular and molecular mechanisms by which Aβ induces synaptic toxicity. We show that administration of Aβ42 peptides to mature hippocampal neurons is followed by its rapid internalisation. Subsequently, Aβ42 is detected at presynaptic terminals, where it can interact with Synaptophysin. We show that this interaction disrupts the Syp/Vamp2 complex, inducing an expansion of the primed synaptic vesicle pool and of baseline neurotransmission.

Results

Aβ interacts with Synaptophysin.

To examine the underlining mechanisms behind Aβ42 induced excitotoxicity, we designed a proteomic screen, based on an affinity column of Aβ42, 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, Synaptophysin (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 synaptopbrevin 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 Aβ42.

Internalisation of Aβ42 in neurons

We then explored the significance of this interaction in dissociated neurons. For this interaction to occur *in vivo*, Aβ42 should be present inside the neurons, at the presynaptic terminal. Aβ42 accumulates intracellularly via two possible avenues; either it builds up because part of the intracellular pool is not secreted, or extracellular Aβ42 is internalised by neurons(LaFerla et al., 2007). Thus, we developed an internalisation assay to investigate the dynamics and the subcellular distribution of externally administered synthetic Aβ42. Synthetic Aβ42 is commercially available and readily aggregates in aqueous solution(El-Agnaf et al., 2000). Since soluble Aβ42 oligomers are believed to be the pathogenic peptides responsible for synaptic changes(Halene et al., 2009), we first ensured that oligomers were indeed present in our preparations, examining the aggregation state of Aβ42 by western blotting. Oligomeric Aβ42 species corresponding to trimers (molecular weight of approximately 12kDa), dimers (molecular weight of approximately 8kDa), as well as monomeric Aβ42 (molecular weight 4kDa) 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 Aβ42 to mature (21 DIV) dissociated hippocampal neurons.

Our results showed that incubation of dissociated hippocampal neurons with a range of concentrations of Aβ42 (50-500nM) for 20 min, followed by recovery periods of 15min, 2hrs and 4hrs allowed both, detection of Aβ42 and optimum survival of neurons. To look into the dynamics of the internalisation of Aβ42, sequential immuno-labelling of fixed neurons was employed to distinguish between surface and internalised Aβ42. Surface labelling was carried out using the 6E10 primary antibody (for Aβ detection) and the Alexa-Fluor 488 secondary antibody in the absence of detergent. The neurons were then post fixed and

intracellular Aβ42 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 2hrs of recovery Aβ42 was internalised. To quantify the extent of the internalisation, co-localisation levels were quantified by calculating the split Mander's co-localisation coefficients using the JACoP plugin for Image-J(Bolte and Cordelieres, 2006). 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 is highest (0.92±0.7 *n=6*) after the 15 min recovery time point, suggesting a high degree of co-localisation. Within a 2 hr recovery period, the Mander's coefficient dropped significantly (0.64±0.07 *n=10, student t-test p=0.0006*). Similarly, after 4hrs 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 that Aβ42 can be internalised over a 4h period (Saavedra et al., 2007).

Presynaptic localisation of Aβ42.

Since we identified Syp as a possible interacting partner of Aβ42, we investigated if such an interaction could mediate an internalisation/endocytosis of Aβ42 at synaptic contacts. Dissociated neurons were incubated with Aβ42 for 20mins and allowed to recover in fresh media for 15mins or 2hrs prior to fixation. If Aβ42 was internalised via its interaction with Syp, we would expect Aβ42 to be localised at presynaptic terminals soon after incubation with the peptide. Double immuno-labelling for Aβ42 and Syp after 15 min of recovery, did not reveal any significant overlap between Aβ42 and synaptic contacts marked by Syp labelling (fig 3a, a'). Thus, it is unlikely that Aβ42 is internalised at synaptic contacts via its interaction with Syp. After 2hrs of recovery though, a significant increase of

co-localisation between Aβ42 and Syp labelling was observed (fig 3b, b') suggesting that Aβ42 was localised at the presynaptic terminal. To further confirm our findings, we repeated the experiment using Vamp2 as an alternative presynaptic marker. Similar to our previous observations, after 15 min of recovery no overlapping pattern between Aβ42 and Vamp2 labelling was evident (fig 3c, c'), whereas after 2hrs of recovery, co-localisation between Vamp2 and Aβ42 was detected, further confirming its presence at pre-synaptic terminals. However, compared to Syp, co-localisation with Vamp2 was less evident (fig 3 d, d'). Furthermore, the pattern of Vamp2 staining after 2hrs of recovery, was unusual with visible stretches along the membrane (see brackets in fig 3d'') instead of the characteristic punctate pattern (fig 3c).

Aβ42 is specifically detected at glutamatergic synapses.

The biochemical interaction of Aβ42 with Syp and its co-localisation with presynaptic markers implies that the peptide has a role in the pre-synaptic terminal. To further confirm our results and to investigate if the peptide was present at specific synapses, we performed double immuno-labelling for Aβ42 with NMDARs in dissociated neurons incubated with Aβ42 for 20mins and allowed to recover in fresh media for 15mins or 2hrs prior to fixation.

Double immuno-labelling for Aβ42 and NMDARs after 2hrs recovery revealed that Aβ42 was often juxtaposed to NMDARs (fig 4b), further confirming its presynaptic localisation. In addition, after 15 min of recovery, labelling for Aβ42 and NMDARs was largely unrelated (fig 4a) suggesting that Aβ42 can be detected at synapses after prolonged recovery period. However, these result were unexpected since previous reports have demonstrated that incubation of dissociated neurons with Aβ peptides results entirely in its post-synaptic localisation(Lacor et al., 2004; Lacor et al., 2007; Pellistri et al., 2008). Since neurotransmitter release has been associated with rapid increase in the extracellular levels of Aβ42(Cirrito et al., 2005), we considered the possibility that the observed presynaptic localisation of Aβ42 could be transient, followed by its

release into the synaptic cleft in an activity dependent manner, where it subsequently can interact with postsynaptic targets. To test this hypothesis dissociated hippocampal neurons incubated with Aβ42 and allowed to recover for 4h were depolarised with 50mM KCl to induce neurotransmitter release. Double immuno-labelling for Aβ42 and NMDARs showed that a substantial amount of Aβ42 was still juxtaposed to NMDARs (fig 4c), suggesting either that Aβ42 remained presynaptic after neurotransmitter release or that it is released slowly at quantities below our detection threshold.

Aβ42 disrupts the Syp/Vamp2 complex

We next investigated the unusual distribution of Vamp2 in neurons exposed to Aβ42 (fig 3 d''). It has been shown that Syp interacts with Vamp2 regulating its synaptic distribution(Pennuto et al., 2003). Thus, we hypothesised that the diffuse distribution of Vamp2 could be a consequence of Aβ42 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 100μM aggregated Aβ42, or in the presence of a similar concentration of control, scrambled Aβ40(Kowall et al., 1992) (fig 5a). A substantial reduction of co-immunoprecipitated Syp was observed in the presence of Aβ42, compared to the amount of co-immunoprecipitated Syp in the presence of the scrambled peptide (compare fig 5a lane 3 and 4) suggesting that Aβ42 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(Pennuto et al., 2003), we compared the extent of co-localisation between these two proteins in neurons exposed to Aβ42 and control neurons to investigate if Aβ42 disrupts their interaction in living neurons as well. Double immuno-labelling of Vamp2 and Syp in control untreated neurons (fig 5b) and in neurons recovering for 15min after exposure to Aβ42 (fig 5c) showed the expected extensive co-localisation between the two proteins suggesting that Aβ42 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 Aβ42 after 2h of recovery (fig 5d) showed stretches of Vamp2 immunoreactivity devoid of Syp labelling (i.e. areas between arrows in figure 5d). High magnification of boxed area in fig 5d demonstrates in detail Vamp2 labelling independent of Syp in what appears to be membrane fragments (brackets in fig 5d''). This diffuse Vamp2 labelling is indicative of a disruption in the formation of the Vamp2/Syp complex *in vivo* (Pennuto et al., 2003) demonstrating that Aβ42 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 5e) suggesting the diffuse Vamp2 labelling is specifically induced by Aβ42 and not by the addition of any peptide in the medium. Quantification of the extent of co-localisation between Vamp and Syp, by calculating the Mander's coefficient, confirmed that their co-localisation was specifically reduced in neurons exposed to Aβ42(fig 5f).

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)(Calakos and Scheller, 1994; Pennuto et al., 2002; Arthur and Stowell, 2007). Therefore, disruption of the Vamp2-Syp complex at synaptic contacts by Aβ42 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 either with Aβ42 or with the scrambled peptide were incubated with FM1-43FX, a fluorescent lipophilic dye that labels membranes. Using a hypertonic sucrose solution (500mM 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(Gaffield and Betz, 2006). Incorporation of the dye at synaptic contacts was confirmed

with immuno-labelling for Syp. Images of mature (21DIV) untreated hippocampal neurons (fig 6a) and neurons treated either with Aβ42 (fig 6b) or scrambled peptide as a negative control (fig 6c) were taken consecutively leaving the microscope/camera settings unaltered.

In untreated or control treated neurons, although internalisation of the dye was evident in the cell soma, the amount of internalised dye at synaptic contacts was not significant (n=20, fig 6a, b). On the contrary, neurons exposed to Aβ42 consistently displayed a marked increase of the internalised dye at synaptic contacts (n=20), indicative of an increase in the number of vesicles primed to the presynaptic membrane (fig 6c). These results strongly support our hypothesis that Aβ42 increases the amount of primed vesicles at the presynaptic terminal. The question now was how this would affect synaptic transmission. To test this, we recorded excitatory post-synaptic responses (fEPSPs) from the CA1 region of hippocampal slices in response to electrical stimulation of the Schaffer collateral pathway, in the presence or absence of Aβ42. First, we noticed a concentration dependent enhancement in basic transmission at concentrations ranging from 300pM to 1nM Aβ42 (fig 7a). We subsequently investigated in detail this effect by increasing electrical stimulation in slices exposed to 1nM Aβ42. Our results showed a progressive enhancement ($p=0.044$ at 50V) of recorded fEPSPs, in treated as compared with untreated, control slices (fig 7b) indicative of an increased efficacy of the Schaffer collateral synapse. We then confirmed the specificity of this enhanced efficacy either by chelation of Aβ42 using an excess of a specific antibody (6E10), or by using the scrambled peptide. Co-incubation with 6E10 and Aβ42 produced similar recordings to untreated slices (fig 7c). Furthermore, incubation of slices with the scrambled peptide had no effect (fig 7d) further confirming that the enhancement of fEPSPs was specific to Aβ42.

To ensure that our results were not an artefact, we investigated in separate experiments if the same Aβ42 peptide used above could block LTP in hippocampal CA1 region. Our results showed that indeed it could reproduce the

established block of LTP (fig 7e, f) confirming that the effect we recorded was not an artefact. To unambiguously confirm that the enhancement of synaptic transmission was induced by Aβ, we repeated our experiments with cell-derived Aβ peptides, from 7PA2 cells, [Chinese-hamster ovary (CHO) cells that express the V717F mutant human APP] that produce and secrete significant amounts of low-n oligomers Aβ peptides(Podlisny et al., 1995). Our results with the cell derived peptides were similar to the ones obtained with the synthetic peptide suggesting that the effect we recorded was not an artefact of the synthetic peptide (Fig 8) but was rather a physiological function of the peptide.

Discussion

Synaptic localisation of Aβ42.

Whilst extensive information in the literature shows that synaptic failure precedes cognitive decline in AD(Selkoe, 2002) the cellular and molecular events underlying this synaptic dysfunction remain obscure. The data presented here provide important insight into the mechanisms by which Aβ42 affects synaptic activity. First, we show that the presynaptic protein Synaptophysin interacts with Aβ42 *in vitro*. We then establish the significance of this interaction by showing that Syp and externally applied Aβ42 co-localise in living neurons. Studies with transgenic animal models for AD, have supported the presence of intraneuronal Aβ (iAβ) prior to the appearance of extracellular deposits, suggesting that iAβ is a significant contributor to the onset of learning and memory deficits(LaFerla et al., 2007; Li et al., 2007). Despite an apparent relationship between the intracellular pool of Aβ and the extracellular deposits(Oddo et al., 2004; Oddo et al., 2006) the precise mechanism by which the peptide enters the neuron is still unknown. Physical interaction between Aβ42 and the α7 acetylcholine receptor(Nagele et al., 2002) or apoE(Zerbinatti et al., 2006) has made these proteins candidates for a receptor mediated transfer of Aβ into the cell. On the other hand, there is evidence that Aβ 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(Kandimalla et al., 2009). Thus, we considered if an interaction between Syp and Aβ42 at the presynaptic terminal could be an alternative mechanism to internalise the peptide. We examined the dynamics of the co-localisation of these proteins and found no supporting evidence since there was no indication of synaptic localisation of Aβ42 in neurons that were left to recover for only 15min. Interestingly though, the absence of co-localisation at early time points compared to the co-localisation we observed after 2hrs of recovery, shows that Aβ42 requires time to accumulate at the presynaptic terminal. It is thus likely that Aβ42 is translocated to the presynaptic terminal by an unknown transport mechanism. Co-labelling for Aβ42 and NMDARs further confirmed that Aβ42 is present at presynaptic terminals, and similar to other reports, it is detected at glutamatergic synapses(Lacor et al., 2007), suggesting the existence of an internalisation mechanism specific to glutamatergic neurons.

Unlike the data presented here, several reports demonstrate exclusive co-localisation between Aβ42 and postsynaptic markers(Lacor et al., 2004; Lacor et al., 2007). In such a study, Lacor and colleagues found 92% of exogenously applied Aβ co-localised with the post-synaptic protein PSD-95 and the majority of the peptide was found juxtaposed to Synaptophysin(Lacor et al., 2004). We believe that the difference in our results reflects differences in our experimental procedures. For instance, a plausible explanation for our differences could be that we allowed cells to recover for two to four hours after exposure to Aβ42. This recovery period proved to be essential since localisation at presynaptic terminals could only be detected after prolonged recovery periods. In addition, Lacor *et.al.* showed that only high molecular weight species of Aβ peptides could be detected post-synaptically whereas our Aβ preparation was enriched in low oligomeric forms. Taken together, we believe that these results rather complement each other demonstrating different roles of Aβ.

Mechanism of synaptic toxicity

Confusing data regarding the effect of Aβ42 in synaptic dysfunction such as increase or suppression of spontaneous activity are often found in the literature (Hartley et al., 1999; Nimmrich et al., 2008). 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 ambiguousness of the causal mechanisms behind these effects has further challenged consistent interpretation of these data. We believe that our findings regarding the interaction between Aβ42 and Syp may help resolve some of these inconsistencies. Syp is a synaptic vesicle (SV) protein implicated in regulating the formation of the fusion pore complex (FPC) during SV fusion and exocytosis. It does so by forming hetero-dimeric complexes with Vamp2 (Synaptobrevin 2)(Calakos and Scheller, 1994), a SNARE protein(Sollner et al., 1993). 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 diffused localisation of Vamp2 along the axonal membrane(Pennuto et al., 2003). Here we demonstrate that Aβ42 disrupts the interaction between Vamp2 and Syp both in vitro and in dissociated neurons (fig 5). Two possible mechanisms could account for this disruption: either Aβ42 disrupts a signalling pathway that regulates the dynamics of this interaction at the TGN, or it competes with Vamp2 for binding to Syp. Our IP data demonstrated that Aβ42 directly competes with Vamp2 for binding to Syp supporting the latter hypothesis. We also noticed that this disruption was more pronounced in less mature neurones (14 DIV) when synapses are not yet fully mature and there is increased traffic of structural components towards the developing synapses. Furthermore, we have no evidence supporting the presence of Aβ42 at the TGN at an early time point.

In addition to correctly sorting Vamp2 at the synapse, Syp also prevents it from participating in the formation of the FPC(Edelmann et al., 1995; Pennuto et al., 2002). Thus, if Aβ42 competes with Vamp2 for binding to Syp at the synapse,

an increase in the number of primed vesicles at the presynaptic membrane would be expected due to an increased availability of Vamp2. We verified this by showing that inducing the fusion of primed SV in the presence of the lipophilic FM1-43FX dye results in an increase of endocytosed dye at the synapses of neurons incubated with Aβ42. Aβ42 induced changes to SV recycling has previously been reported. For instance, Kelly et.al. proposed that Aβ disrupts SV endocytosis, resulting in depleted SV pools(Kelly and Ferreira, 2007). From a different perspective though, this depletion could be due to deregulation of SNARE complex formation ensuing from an increased availability of primed SVs. Indeed, the enhancement of single-shock fEPSPs by Aβ42 in living synapses within hippocampal slices suggested an increased availability of releasable synaptic vesicles. 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 under our experimental conditions the well-established disruption of LTP by Aβ42.

In support to our results, several reports have shown an Aβ-dependent increase in the number of vesicles available at the presynaptic active zone (Puzzo et al., 2008; Abramov et al., 2009; Parodi et al., 2010) or that glutamate release is enhanced by Aβ40 (Cuevas et al., 2011) , suggesting that regulation of NT release at the pre-synaptic level could be an important aspect of the physiological role of Aβ. Furthermore, increasing evidence suggest that Aβ could exert its effects in synaptic plasticity by excessive activation of NMDARs. For instance it has been shown that inhibition of LTP by Aβ can be through a mechanism involving both, excessive activation of extrasynaptic NMDARs or defects in glutamate uptake (Harris et al., 1995; Li et al., 2009; Li et al., 2011). Our results allow us to propose a model to describe the mechanism behind these observations (fig 9). We propose that low molecular weight Aβ oligomers at low concentrations increase the association of SV with the presynaptic membrane. It does so by disrupting the Vamp2-Syp complex through its interaction with Syp.

Consequently, Vamp2 becomes available to participate in the formation of SNARE complexes increasing the amount of primed SVs. Long term exposure and/or high levels of Aβ42 would consistently deregulate glutamate release, disrupting LTP (Kelly and Ferreira, 2007; Parodi et al., 2010; Li et al., 2011) and inducing excitotoxicity(Hynd et al., 2004). This model can also explain findings that show a "bell shaped" relationship between Aβ and synaptic plasticity(Abramov et al., 2009). Finally, at later stages, increased release of Aβ42 from presynaptic sites would induce additional effects at the post-synaptic membrane (Cirrito et al., 2005).

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 severe side effects. Thus, understanding the cellular mechanism behind the deregulation of glutamatergic neurotransmission might provide alternative therapeutic targets. Our data suggest a cellular mechanism that can account for the deregulation of glutamatergic synaptic transmission. Although it is quite possible that we have demonstrated only one of several cellular events disrupted by Aβ, understanding the causative events behind its toxicity is essential, in order to design appropriate therapeutic strategies to target the symptoms of AD more efficiently.

Materials and Methods

Hippocampal cell culture: All animal experiments were performed according to home office regulations in appliance with the Animals Scientific Act 1986. Primary cultures of CA3-CA1 hippocampal neurons were prepared from E18 Wistar rat embryos. The experiments were performed in mature (21–28 days in vitro (DIV)) cultures. Neurons were seeded on poly-D-lysine (100µg/ml in 0.1M borate buffer) and laminin (5µg/ml in PBS) coated coverslips at a density of 75,000 cells per coverslip and were maintained at 37°C, 5% CO_2 in Neurobasal media, supplemented with B27, L-glutamine (0.5mM) and 100units/ml penicillin/streptomycin.

Immunocytochemistry: Hippocampal cultures were rinsed once with PBS and fixed with 4% paraformaldehyde (PFA) in PBS. The coverslips were washed, permeabilised with 0.1% Tween-20 and 5% horse serum in PBS for 45 min at room temperature, followed by incubation 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). Primary antibodies used were: Anti-Aβ 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 Flour 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 100x objective, using Image Pro 6.0 software. High magnification inlets were produced using Adobe photoshop.

FM1-43FX Labelling: Following exposure to Aβ, a 5uM of FM1-43FX dye (prepared in HBSS warmed to 37°C) was applied to the cells for 5mins, followed

by application of a hypertonic 500mM sucrose solution in HBSS with 5uM FM1-43FX. After 5mins the sucrose solution was replaced with the original FM1-43FX solution for 15mins. The cells were then washed twice for 15mins 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 (weighing between 90g and 140g) and homogenized in 1mL lysis buffer (25mM HEPES pH7.5, 150mM NaCl, 1% NP-40, 10mM $MgCl_2$, 1mM EDTA, 2% Glycerol), containing Sigma protease cocktail inhibitor. Protein G Dynabeads® (25uL) (Invitrogen) were incubated with 5µgr of the precipitating monoclonal antibody for one hour at 4°C. Scrambled peptide and Aβ42 were diluted in 100 µl hippocampal protein extracts (~7mg/ml) at a final concentration of 100µM and the mix was incubated with the antibody bound dynabeads at 4°C for 4 hours. Samples were boiled for 5mins to elute bound proteins, which were then analysed by SDS-PAGE.

Immunoblot: Proteins were separated on NuPage 4–12% Bis-Tris gels at 200V and transferred to nitrocellulose membrane at 100Vfor one hour at 4°C in transfer buffer (200mM Glycine, 25mM Tris Base 20% Methanol). Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (50mM Tris Base, 150mM NaCl) containing 0.1% Tween 20, pH 7.5, for one hour and incubated overnight at 4°C with primary antibody (1:250) and two hours 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 Wistar rats. Rats were deeply anesthetised by halothane prior to decapitation, and the brain rapidly removed and submerged in oxygenated (95% O_2, 5%CO_2) artificial cerebrospinal fluid (aCSF) containing 135mM NaCl, 3mM KCl, 1.25mM NaH_2PO_4, 1mM $MgCl_2$, 10mM Glucose and 26mM $NaHCO_3$. The brain was hemisected, and ventral sections

(400µm thick) prepared in aCSF on a Vibroslice (Campden Instruments Ltd). Slices were transferred to a holding chamber of continually oxygenated aCSF and left to recover for at least one hour. 1nM final concentration of Aβ42, Aβ40 and scrambled Aβ peptide was added to the chamber to treat slices for 45 minutes prior to field EPSP recordings. One lyophilised fraction from 7PA2 cell media known to contain Aβ oligomers was re-suspended in 100ml aCSF in the tissue chamber to treat hippocampal slices for 45 minutes.

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 3kHz prior to digitisation with a CED1401. Experiments were controlled using Signal 2 (CED Ltd) running on PC. Electrodes were positioned just below the tissue surface and slices were left to stabilise for 20 minutes. Input/output curves (or stimulation responses) were recorded, and the 10-90% slope measured, from a stimulus ranging from 10V to 70V with 3 successive events at each input level, 30 seconds apart. To detect concentration dependent changes in neurotransmitter release, fEPSPs were initially evoked at 50V with low frequency at 0.033 Hz in the presence of different concentrations of Aβ42. For LTP recordings, a stable baseline was maintained for 25 minutes at 50% of the maximum fEPSP response, and LTP was induced by high frequency stimulation consisting of three trains of 100Hz, 10 seconds apart. Low frequency stimulation every 30 seconds for at least 90 minutes recorded the potentiated synaptic response.

Synthetic Aβ peptides: Aβ42 (American Peptide USA) was prepared as described previously (El-Agnaf et al., 2000) and stored in 0.1M Tris pH7.4 at a stock concentration of 100µM at -20°C. Neurons were incubated with 50nM of

peptide for 20 minutes. Following treatment, the media was replaced with fresh supplemented Neurobasal and the cells were left to recover for the specified times.

Cell-Derived Aβ: 7PA2 cells are a CHO cell line stably expressing human APP_{751} carrying the V717F AD causing mutation. The Aβ peptides produced and secreted into the media of cultured 7PA2 cells were collected, concentrated and Aβ oligomers were separated by Size Exclusion Chromatography as previously described(Sian et al., 2000; Townsend et al., 2006)

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C.L.R. performed the majority of the experiments, SS contributed to the experimental procedure. P.A. was the main supervisor of the project designing the experiments and writing the paper. R.M.E. supervised physiology experiments. W.P.B. and B.M.A. assisted in the supervision of C.L.R.

References

Abramov E, Dolev I, Fogel H, Ciccotosto GD, Ruff E, Slutsky I (2009) Amyloid-beta as a positive endogenous regulator of release probability at hippocampal synapses. Nat Neurosci 12:1567-1576.

Arias C, Arrieta I, Tapia R (1995) beta-Amyloid peptide fragment 25-35 potentiates the calcium-dependent release of excitatory amino acids from depolarized hippocampal slices. J Neurosci Res 41:561-566.

Arthur CP, Stowell MH (2007) Structure of synaptophysin: a hexameric MARVEL-domain channel protein. Structure 15:707-714.

Bolte S, Cordelieres FP (2006) A guided tour into subcellular colocalization analysis in light microscopy. J Microsc 224:213-232.

Calakos N, Scheller RH (1994) Vesicle-associated membrane protein and synaptophysin are associated on the synaptic vesicle. J Biol Chem 269:24534-24537.

Cirrito JR, Yamada KA, Finn MB, Sloviter RS, Bales KR, May PC, Schoepp DD, Paul SM, Mennerick S, Holtzman DM (2005) Synaptic activity regulates interstitial fluid amyloid-beta levels in vivo. Neuron 48:913-922.

Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ, Ashe KH (2005) Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat Neurosci 8:79-84.

Cuevas ME, Haensgen H, Sepulveda FJ, Zegers G, Roa J, Opazo C, Aguayo LG (2011) Soluble Abeta1-40 Peptide Increases Excitatory Neurotransmission and Induces Epileptiform Activity in Hippocampal Neurons. J Alzheimers Dis.

Cullen WK, Suh YH, Anwyl R, Rowan MJ (1997) Block of LTP in rat hippocampus in vivo by beta-amyloid precursor protein fragments. Neuroreport 8:3213-3217.

Edelmann L, Hanson PI, Chapman ER, Jahn R (1995) Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. Embo J 14:224-231.

El-Agnaf OM, Mahil DS, Patel BP, Austen BM (2000) Oligomerization and toxicity of beta-amyloid-42 implicated in Alzheimer's disease. Biochem Biophys Res Commun 273:1003-1007.

Gaffield MA, Betz WJ (2006) Imaging synaptic vesicle exocytosis and endocytosis with FM dyes. Nat Protoc 1:2916-2921.

Gomez-Isla T, Hollister R, West H, Mui S, Growdon JH, Petersen RC, Parisi JE, Hyman BT (1997) Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. Ann Neurol 41:17-24.

Halene TB, Ehrlichman RS, Liang Y, Christian EP, Jonak GJ, Gur TL, Blendy JA, Dow HC, Brodkin ES, Schneider F, Gur RC, Siegel SJ (2009) Assessment of NMDA receptor NR1 subunit hypofunction in mice as a model for schizophrenia. Genes Brain Behav 8:661-675.

Hardy J (2009) The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. J Neurochem 110:1129-1134.

Hardy J, Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci 12:383-388.

Harkany T, Abraham I, Timmerman W, Laskay G, Toth B, Sasvari M, Konya C, Sebens JB, Korf J, Nyakas C, Zarandi M, Soos K, Penke B, Luiten PG (2000) beta-amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis. Eur J Neurosci 12:2735-2745.

Harris ME, Carney JM, Cole PS, Hensley K, Howard BJ, Martin L, Bummer P, Wang Y, Pedigo NW, Jr., Butterfield DA (1995) beta-Amyloid peptide-derived, oxygen-dependent free radicals inhibit glutamate uptake in cultured astrocytes: implications for Alzheimer's disease. Neuroreport 6:1875-1879.

Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, Selkoe DJ (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. J Neurosci 19:8876-8884.

Hsia AY, Masliah E, McConlogue L, Yu GQ, Tatsuno G, Hu K, Kholodenko D, Malenka RC, Nicoll RA, Mucke L (1999) Plaque-independent disruption of

neural circuits in Alzheimer's disease mouse models. Proc Natl Acad Sci U S A 96:3228-3233.

Hynd MR, Scott HL, Dodd PR (2004) Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. Neurochem Int 45:583-595.

Kabogo D, Rauw G, Amritraj A, Baker G, Kar S (2008) beta-Amyloid-related peptides potentiate K+-evoked glutamate release from adult rat hippocampal slices. Neurobiol Aging 31:1164-1172.

Kandimalla KK, Scott OG, Fulzele S, Davidson MW, Poduslo JF (2009) Mechanism of neuronal versus endothelial cell uptake of Alzheimer's disease amyloid beta protein. PLoS ONE 4:e4627.

Kelly BL, Ferreira A (2007) Beta-amyloid disrupted synaptic vesicle endocytosis in cultured hippocampal neurons. Neuroscience 147:60-70.

Kim JH, Anwyl R, Suh YH, Djamgoz MB, Rowan MJ (2001) Use-dependent effects of amyloidogenic fragments of (beta)-amyloid precursor protein on synaptic plasticity in rat hippocampus in vivo. J Neurosci 21:1327-1333.

Klyubin I, Wang Q, Reed MN, Irving EA, Upton N, Hofmeister J, Cleary JP, Anwyl R, Rowan MJ (2011) Protection against Abeta-mediated rapid disruption of synaptic plasticity and memory by memantine. Neurobiol Aging 32:614-623.

Kowall NW, McKee AC, Yankner BA, Beal MF (1992) In vivo neurotoxicity of beta-amyloid [beta(1-40)] and the beta(25-35) fragment. Neurobiol Aging 13:537-542.

Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, Viola KL, Klein WL (2007) Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. J Neurosci 27:796-807.

Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL, Lambert MP, Velasco PT, Bigio EH, Finch CE, Krafft GA, Klein WL (2004) Synaptic targeting by Alzheimer's-related amyloid beta oligomers. J Neurosci 24:10191-10200.

LaFerla FM, Green KN, Oddo S (2007) Intracellular amyloid-beta in Alzheimer's disease. Nat Rev Neurosci 8:499-509.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A 95:6448-6453.

Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH (2006) A specific amyloid-beta protein assembly in the brain impairs memory. Nature 440:352-357.

Li M, Chen L, Lee DH, Yu LC, Zhang Y (2007) The role of intracellular amyloid beta in Alzheimer's disease. Prog Neurobiol 83:131-139.

Li S, Hong S, Shepardson NE, Walsh DM, Shankar GM, Selkoe D (2009) Soluble oligomers of amyloid Beta protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. Neuron 62:788-801.

Li S, Jin MB, Koeglsperger T, Shepardson NE, Shankar GM, Selkoe D (2011) Soluble Aβ Oligomers Inhibit Long-Term Potentiation through a Mechanism Involving Excessive Activation of Extrasynaptic NR2B-Containing NMDA Receptors. J Neuroscience 31:6627-6638.

Molnar Z, Soos K, Lengyel I, Penke B, Szegedi V, Budai D (2004) Enhancement of NMDA responses by beta-amyloid peptides in the hippocampus in vivo. Neuroreport 15:1649-1652.

Nagele RG, D'Andrea MR, Anderson WJ, Wang HY (2002) Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease. Neuroscience 110:199-211.

Nimmrich V, Grimm C, Draguhn A, Barghorn S, Lehmann A, Schoemaker H, Hillen H, Gross G, Ebert U, Bruehl C (2008) Amyloid beta oligomers (A beta(1-42) globulomer) suppress spontaneous synaptic activity by inhibition of P/Q-type calcium currents. J Neurosci 28:788-797.

Oddo S, Billings L, Kesslak JP, Cribbs DH, LaFerla FM (2004) Abeta immunotherapy leads to clearance of early, but not late, hyperphosphorylated tau aggregates via the proteasome. Neuron 43:321-332.

Oddo S, Caccamo A, Smith IF, Green KN, LaFerla FM (2006) A dynamic relationship between intracellular and extracellular pools of Abeta. Am J Pathol 168:184-194.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39:409-421.

Parodi J, Sepulveda FJ, Roa J, Opazo C, Inestrosa NC, Aguayo LG (2010) Beta-amyloid causes depletion of synaptic vesicles leading to neurotransmission failure. J Biol Chem 285:2506-2514.

Pellistri F, Bucciantini M, Relini A, Nosi D, Gliozzi A, Robello M, Stefani M (2008) Non-specific interaction of prefibrillar amyloid aggregates with NMDA and AMPA receptors results in Ca2+ increase in primary neuronal cells. J Biol Chem.

Pennuto M, Bonanomi D, Benfenati F, Valtorta F (2003) Synaptophysin I controls the targeting of VAMP2/synaptobrevin II to synaptic vesicles. Mol Biol Cell 14:4909-4919.

Pennuto M, Dunlap D, Contestabile A, Benfenati F, Valtorta F (2002) Fluorescence resonance energy transfer detection of synaptophysin I and vesicle-associated membrane protein 2 interactions during exocytosis from single live synapses. Mol Biol Cell 13:2706-2717.

Podlisny MB, Ostaszewski BL, Squazzo SL, Koo EH, Rydell RE, Teplow DB, Selkoe DJ (1995) Aggregation of secreted amyloid beta-protein into sodium dodecyl sulfate-stable oligomers in cell culture. J Biol Chem 270:9564-9570.

Puangthong U, Hsiung GY (2009) Critical appraisal of the long-term impact of memantine in treatment of moderate to severe Alzheimer's disease. Neuropsychiatr Dis Treat 5:553-561.

Puzzo D, Privitera L, Leznik E, Fa M, Staniszewski A, Palmeri A, Arancio O (2008) Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. J Neurosci 28:14537-14545.

Saavedra L, Mohamed A, Ma V, Kar S, de Chaves EP (2007) Internalization of beta-amyloid peptide by primary neurons in the absence of apolipoprotein E. J Biol Chem 282:35722-35732.

Scholtzova H, Wadghiri YZ, Douadi M, Sigurdsson EM, Li YS, Quartermain D, Banerjee P, Wisniewski T (2008) Memantine leads to behavioral improvement

and amyloid reduction in Alzheimer's-disease-model transgenic mice shown as by micromagnetic resonance imaging. J Neurosci Res.

Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81:741-766.

Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. Science 298:789-791.

Selkoe DJ (2008) Soluble oligomers of the amyloid beta-protein impair synaptic plasticity and behavior. Behav Brain Res 192:106-113.

Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL (2007) Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci 27:2866-2875.

Shrestha BR, Vitolo OV, Joshi P, Lordkipanidze T, Shelanski M, Dunaevsky A (2006) Amyloid beta peptide adversely affects spine number and motility in hippocampal neurons. Mol Cell Neurosci 33:274-282.

Sian AK, Frears ER, El-Agnaf OM, Patel BP, Manca MF, Siligardi G, Hussain R, Austen BM (2000) Oligomerization of beta-amyloid of the Alzheimer's and the Dutch-cerebral-haemorrhage types. Biochem J 349:299-308.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318-324.

Thinakaran G, Koo EH (2008) Amyloid precursor protein trafficking, processing, and function. J Biol Chem 283:29615-29619.

Townsend M, Shankar GM, Mehta T, Walsh DM, Selkoe DJ (2006) Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers. J Physiol 572:477-492.

Zerbinatti CV, Wahrle SE, Kim H, Cam JA, Bales K, Paul SM, Holtzman DM, Bu G (2006) Apolipoprotein E and low density lipoprotein receptor-related protein facilitate intraneuronal Abeta42 accumulation in amyloid model mice. J Biol Chem 281:36180-36186.

Figure legends

Figure 1: Identification of proteins interacting with Aβ by candidate approach. (a) Western blotting with primary antibodies against specific synaptic proteins. All antibodies, detected specific proteins in the starting material at their expected molecular weights (PSD95 at ~80kDa, Synaptophysin at ~38kDa, Vamp2 at ~19kDa and SNAP25 at ~25kDa). Only the primary antibody against Synaptophysin detected a specific protein in the fraction eluted from the Aβ affinity column. (b) Oligomerisation state of Aβ42 compared to Aβ40 detected by western blotting. Monomeric Aβ peptides can be seen as a band at 4kDa. Only Aβ42 displayed visible low molecular weight aggregates.

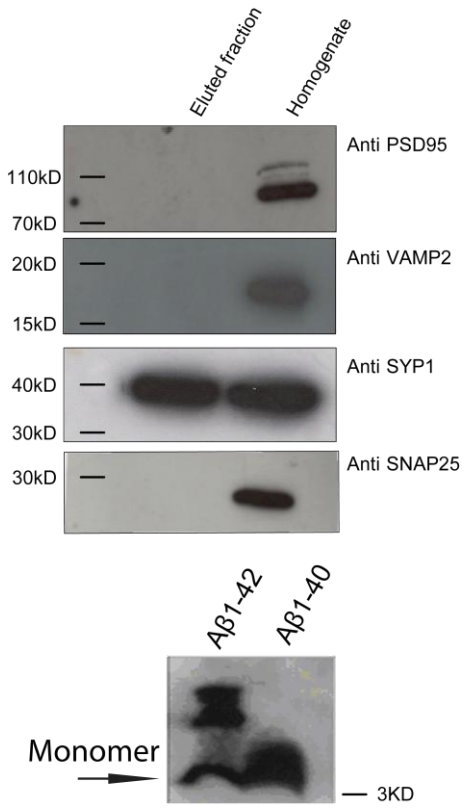

Figure 2: Dynamics of Aβ42 internalisation by sequential immuno-labelling. Mature hippocampal neurons incubated with Aβ42 for 20 min were left to recover for a) 15 min, b) 2hrs and c) 4hrs. Labelling for external (red) and internalised (green) Aβ42 showed extensive overlapping labelling after 15 min recovery period **(a)**. a' high magnification of boxed area in a. a'' and a''' single channel views of a'. Arrows point towards examples of overlapping staining. After 2hrs **(b)** or 4hrs **(c)** recovery period, less overlap was observed. b' and c' high magnifications of boxed areas in b and c respectively. b'', c'' and b''', c''' single channel views of b' and c'. Arrowheads show examples of external and arrows of internalised Aβ42. **d)** Quantification of the extent of co-localisation shows a high degree of co-localisation at 15min recovery (M=0.92±0.07, n=6) compared to 2hrs (M=0.64±0.07, student t-test p=0.0006, n=10) and 4hrs (M=0.70±0.08, student t-test p=0.0021, n=8) of recovery.

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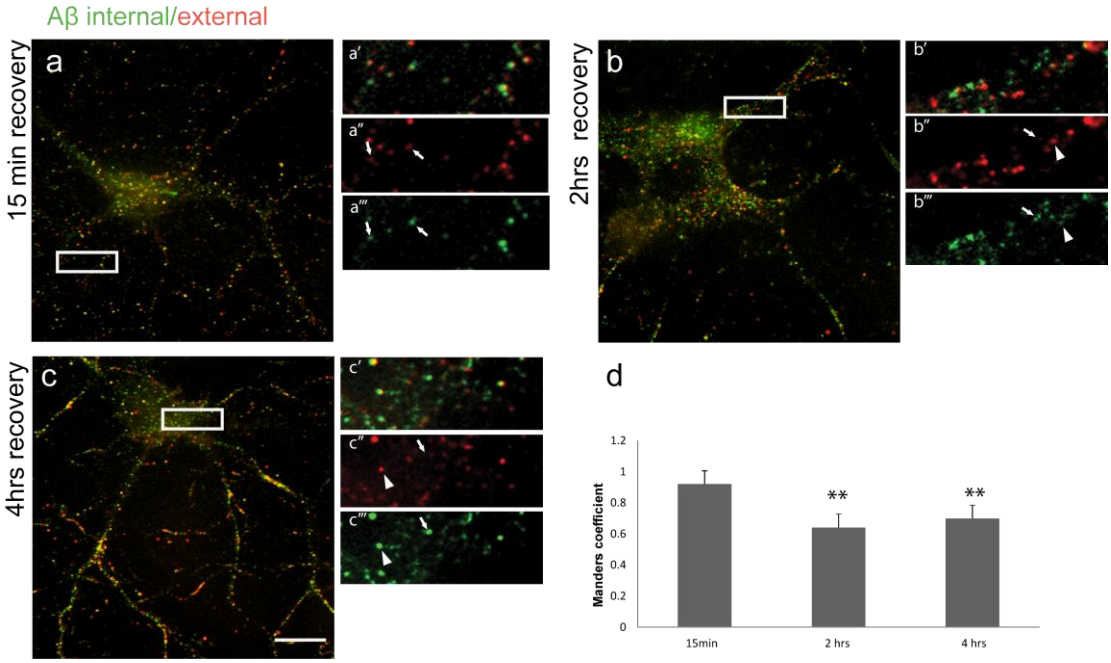

Figure 3: Presynaptic localisation of Aβ42. Mature hippocampal neurons, incubated with Aβ42 for 20 min were left to recover for 15 min (a and c), or 2hrs (b and d) prior to fixation. Co-labelling for Aβ42 and Syp (a) or Aβ42 and Vamp2 (b) after 15 min recovery reveals distinct labelling patterns between Aβ42 and the presynaptic markers (n=15). a' and c' High magnifications of boxed areas in a and c respectively. a'', c'' and a''', c''' are single channel views of a' and c'. Arrows in a'' and c'' point at Syp and Vamp2 positive synaptic contacts respectively whereas arrows in a''' and c''' show the relevant positions in the green channel. Co-labelling for Aβ42 and Syp (b) or Aβ42 and Vamp2 (d) after 2hrs recovery period shows co-localisation between Aβ and both presynaptic markers (n=39 for each condition). b' and d' high magnification of boxed area in b and d respectively. b'', d'' and b''', d''' single channel views of b' and d'. Arrows in b'' and b''' point at examples of Syp positive synaptic contacts that are also positive for Aβ42. Arrow at d'' and d''' points at Vamp2 positive synaptic contacts that are also labelled for Aβ42. Bracket in d'' shows diffuse Vamp2 staining evident only after the 2hrs recovery period.

Scale bar= 10μm

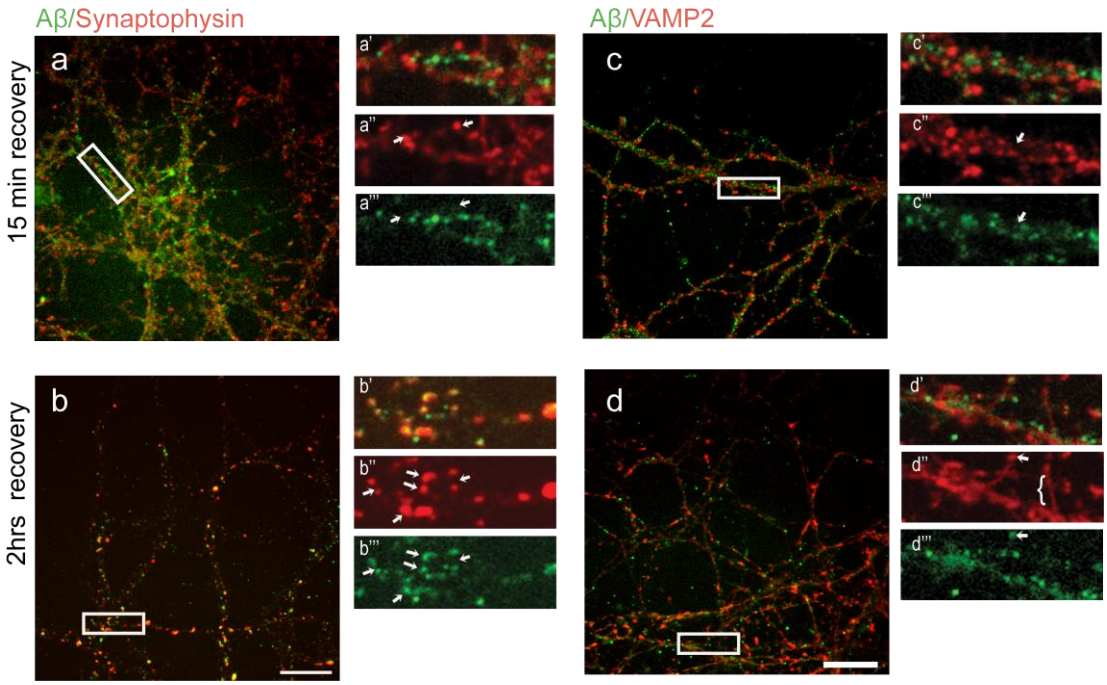

Figure 4: Presynaptic localisation of Aβ42 at glutamatergic synapses. Mature hippocampal neurons, incubated with Aβ42 for 20 min were left to recover for a) 15 min or b) 2hrs prior to fixation. **a)** Co-labelling for Aβ42 and NR2A/B after 15 min recovery period reveals distinct labelling patterns between them (n=20). a' high magnification of boxed area in a. a'' and a''' single channel views of a'. Arrows in a'' point at Aβ42 clusters and arrows in a''' show the relevant positions in the red channel. **b)** Co-labelling for Aβ42 and NR2A/B after 2hrs recovery period shows juxtaposed labelling between them (n=18). b' high magnification of boxed area in b. b'' and b''' single channel views of b'. Arrows in b'' point at Aβ42 clusters and arrows in b''' show the relevant positions in the red channel. **c)** Co-labelling for Aβ42 and NR2A/B after 4hrs recovery period followed by depolarisation by KCl still shows juxtaposed labelling between them (n=10). c' high magnification of boxed area in c. c'' and c''' single channel views of c'. Arrows in c'' point at Aβ42 clusters and arrows in c''' show the relevant positions in the red channel.

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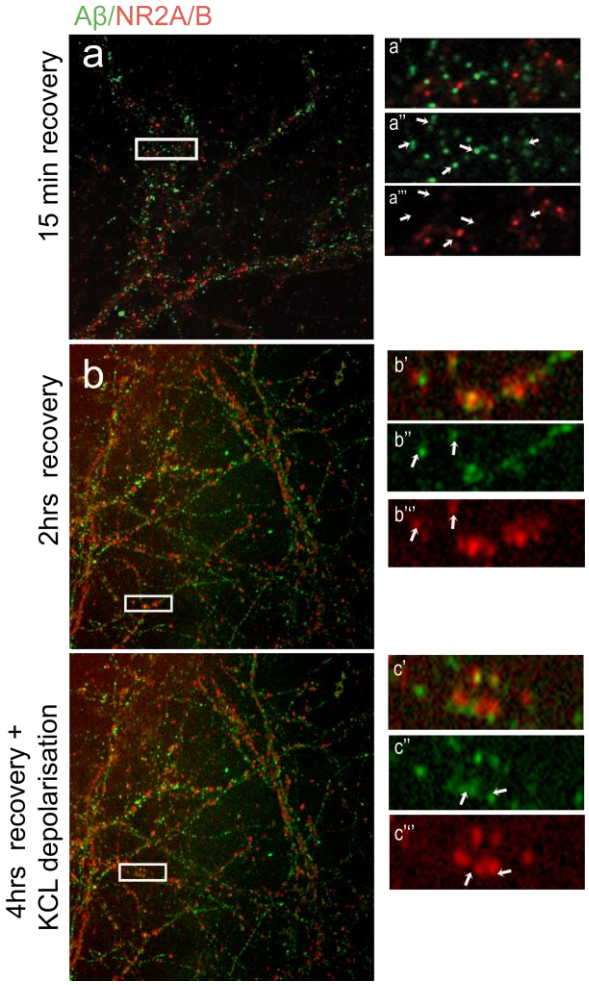
Aβ/NR2A/B
15 min recovery
2hrs recovery
4hrs recovery +
KCL depolarisation
a
a'
a''
a'''
b
b'
b''
b'''
c'
c''
c'''

Figure 5: Disruption of the Syp/Vamp2 complex. **a)** Immunoprecipitation of Vamp2 from hippocampal homogenate assayed by western blotting for Vamp2 and Syp. A band of 19kDa corresponding to Vamp2 and a band of 38kDa, corresponding to Syp were detected in all test conditions. A reduction in immunoprecipitated Syp is evident in the presence of aggregated Aβ42 (lane 3) when compared to control conditions (scrambled Aβ, lane 4) suggesting a disruption to the Vamp2-Syp complex. Lane 6 shows absence of non-specific binding on the beads. **b)** Co-labelling for Syp and Vamp2 in untreated hippocampal neurons display a significant degree of co-localisation. **c)** Co-labelling of Syp and Vamp2 in hippocampal neurons incubated with Aβ42 and allowed to recover for 15 min shows extensive co-localisation as well. **d)** After 2hrs recovery period, Vamp2 staining is more diffuse with extensive stretches of Vamp2 immunoreactivity as indicated by arrows in c. c' high magnification of boxed area in c. c'' and c''' single channel images of c'. Bracket in c'' shows diffuse Vamp2 labelling. **e)** Co-labelling for Syp and Vamp in hippocampal neurons, incubated with scrambled peptide and allowed to recover for 2h is similar to control. **f)** Quantification of co-localisation between Synaptophysin and Vamp2. Aβ42 treated cells left to recover for two hour showed decreased level of co-localisation (0.85±0.09, n=24, student t-test p=0.0003) compared to control, untreated cells (0.961±0.03, n=24). Neurons left to recover for 15 min (0.99±0.02, n=24) or incubated with the scrambled peptide (0.99±0.01, n =18) did not display differences compared to control.

Scale bar= 10μm.

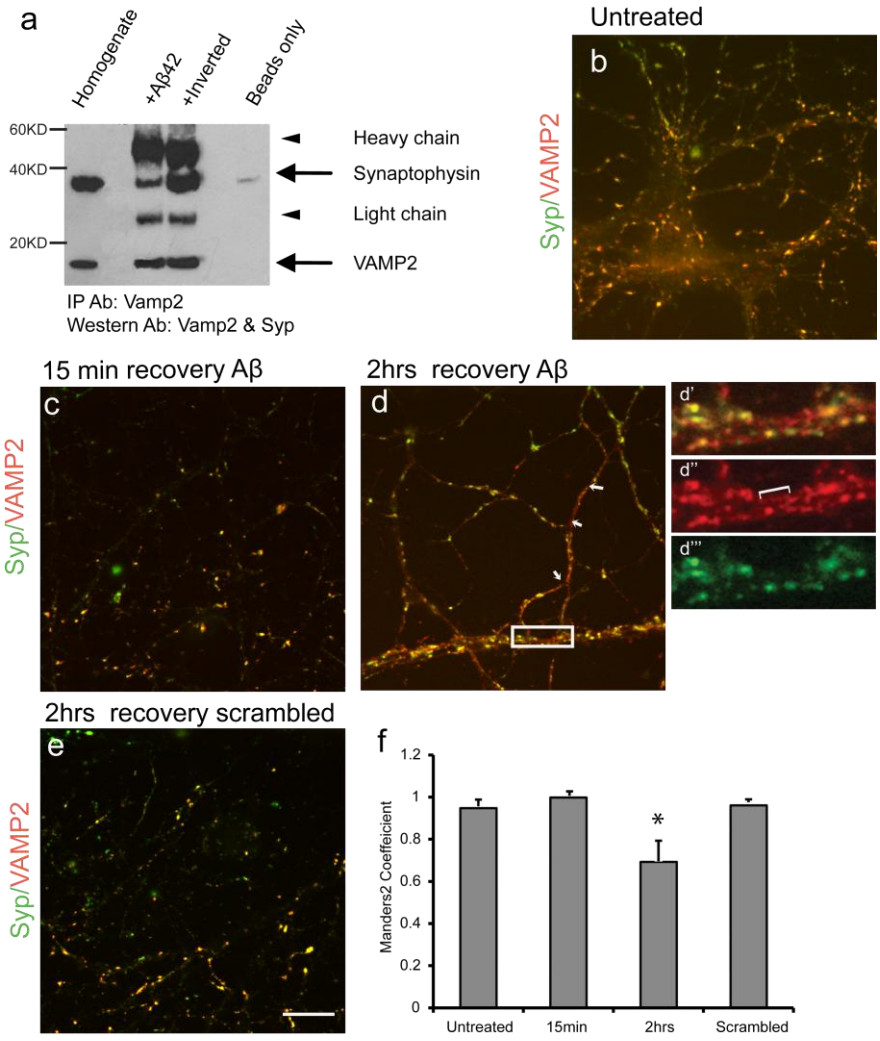
a
Homogenate
+Aβ42
+Inverted
Beads only
60KD
40KD
20KD
Heavy chain
Synaptophysin
Light chain
VAMP2
IP Ab: Vamp2
Western Ab: Vamp2 & Syp
Untreated
b
Syp/VAMP2
15 min recovery Aβ
c
Syp/VAMP2
2hrs recovery Aβ
d
d'
d''
d'''
2hrs recovery scrambled
e
Syp/VAMP2
f
Manders2 Coeffeicient
1.2
1
0.8
0.6
0.4
0.2
0
*
Untreated
15min
2hrs
Scrambled

Figure 6: Induction of primed SVs in hippocampal neurons to fuse to the presynaptic membrane labelled with Syp (green) is visualised by the internalisation of FM1-43FX dye (red). 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). a' and b' high magnifications of boxed areas in a and b respectively. a'' and a''' single channel views of a'. b'' and b''' single channel views of b'. Arrows in a'' and b'' point at synaptic contacts labelled with Syp and arrows in a''' and b''' show the relevant positions in the red channel. **c)** Induction of docked SV fusion visualised by internalisation of FM1-43FX in neurons exposed to Aβ42 followed by 2hrs of recovery results in a significant increase in internalised dye (red) at synaptic contacts marked by Syp labelling (green) (n=20). c' high magnification of boxed area in c). c'' and c''' single channel views of c'. Arrows in c'' point at synaptic contacts labelled with Syp and arrows in c''' point at the relevant positions in the red channel.

Scale bar=10μm

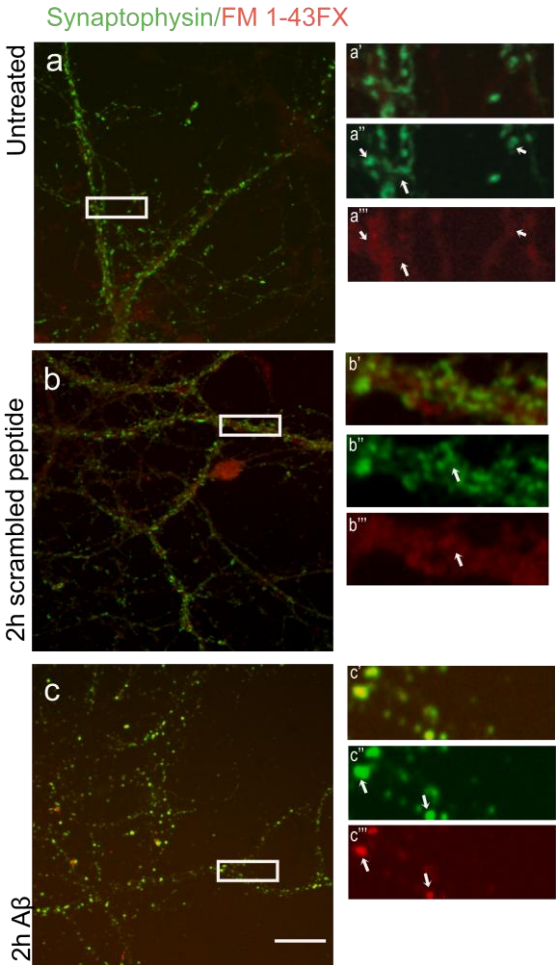
Synaptophysin/FM 1-43FX
Untreated
2h scrambled peptide
2h Aβ
a
a'
a''
a'''
b
b'
b''
b'''
c
c'
c''
c'''

Figure 7: (a) fEPSPs recorded in the CA1 in response to a 50V stimulus of the Schaffer collateral commissural pathway showed that the effect of Aβ in fEPSPs is concentration dependent. Only mid-range concentrations of 0.3nM and 1nM were able to increase the fEPSP slope (p=0.028 and p=0.048 respectively n=5 for each concentration tested). (b) Aβ42 increases the stimulation response compared to the response recorded in control, untreated slices over a range of stimuli. Results are presented as the percentage of the maximum fEPSP from control recordings (n≥5). Inlet in shows the trace of EPSP slopes between a control slice and a slice treated with Aβ42 stimulated at 50V. (c) Chelation of Aβ42 using 6E10, a specific antibody for the peptide, abrogates the enhancement at all input values (n=5). (d) Scrambled Aβ did not induce increased responses compared to controls. (e) In control slices HFS potentiated the EPSP evoked for at least 120 minutes. The increase at 60 minutes after HFS was significant (students t-test p=0.0006 n=5). (f) Aβ42 treatment blocks the induction of long term potentiation (LTP) in hippocampal slices. After a 45 minute bath application of 1nM Aβ42, HFS did not potentiate the EPSP slope.

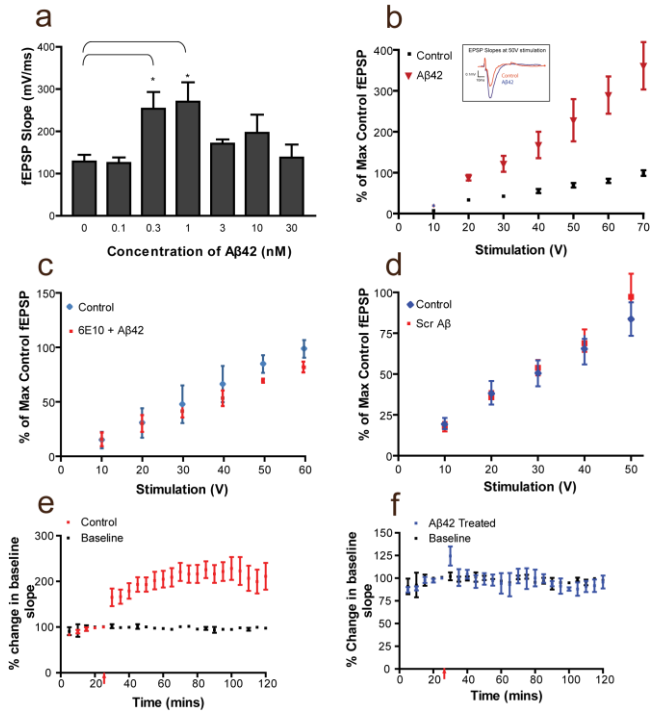

Figure 8: ELISA assay from fractionated conditioned media from 7PA2 cells and control CHO cells detected Aβ oligomers in the conditioned medium of 7PA2 cells but not of CHO control cells (a). Hippocampal slices were incubated with medium from fractions 12, 24 and 36 of the CHO control cells as well as the 7PA2 cells. fEPSPs were recorded in response to Schaffer collateral commissural pathway stimulation at a range of stimulus from 10V through to 60V (b and d). Incubation with cell-derived Aβ, 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 50V in slices exposed to 7PA conditioned medium (c) and to control CHO conditioned medium (e) show that cell derived Aβ increased fEPSP (student's t-test p=0.003 n=5), whereas the control medium had no affect at 50V (n=5) (e).

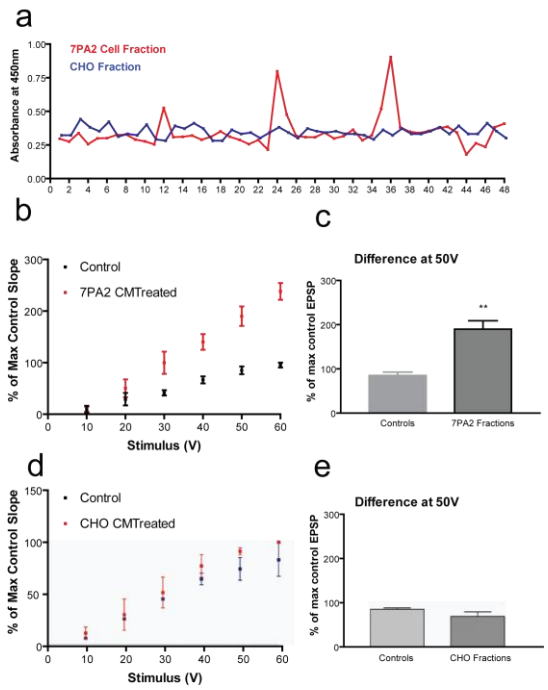

Figure 9: A proposed model explaining the cellular and molecular mechanisms by which Aβ42 affects synaptic activity: Aβ42 is taken up by glutamatergic neurons and reaches the presynaptic terminal. At the presynaptic terminal, it disrupts the complex between Syp and Vamp2 (A), increasing the number of primed SVs (B). Consequently, Aβ42 increases the amount of neurotransmitter release, positively modulating synaptic plasticity at low concentrations (C). At high concentrations (or chronic exposure) aberrant neurotransmitter release rapidly depletes the SV pool disrupting LTP. In addition, aberrant glutamate release induces NMDARs signalling resulting in impairment of LTP, LTD induction, neuronal loss (excitotoxicity) and cognitive impairment seen in AD. Furthermore, in a concentration and time dependent manner, Aβ could be increasingly secreted from the presynaptic terminal directly affecting potential targets on the post-synaptic membrane (C). Finally the SVs are endocytosed where they could either be reused or recycled (D).