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Characterising the effects of genetic risk factors of Alzheimer’s Disease on synaptic transmission: a functional and structural analysis

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Submitted for the degree of Doctor in Philosophy

University of Sussex

September 2019
Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Signature
Acknowledgments

My first, huge thank you goes to my supervisor, Louise Serpell. Thank you for welcoming me in your lab when I came to you that summer in my second year of undergrad, for giving me the opportunity to become a better scientist and for supporting me along the way. You have been the best supervisor I could have ever wished for. You really have a great heart, and I’ll always be grateful. Of course, thank you to my co-supervisor, Kevin Staras. Thank you for welcoming me with a smile every time I knocked at the door, for always having a plan-b when things didn’t go as planned and for your immense help in making sense of things that didn’t really make sense. Thank you to Youssra, who took me under her wing the very first time I stepped into the lab as a clueless undergrad. Thank you to Stephanie Rey, for being the best lab mom I could ever ask for. Devkee (panda), thank you for being the best lab bro ever, for all your huge help and for all the coffee chats. Kate (pickle), thank you for keeping me company in the lonely and cold rig room, and for all the troubleshooting we did together. Thank you to Ana, who shared her PhD journey with me; we made it. Saskia, thank you for all the office chats, and for being there whenever I turned my chair to ask for the craziest advice. And to Cat and Milena, Karen, Mahmoud and Kurtis. I couldn’t have asked for better people to share my journey with. Thank you to Alzheimer’s Society and to all the DTC, without whom I wouldn’t have had this opportunity. Thank you to all the supervisors and PhD students. Lucas, Orla, Rebecca, Alex thank you for being amazing colleagues and great PhD friends. A big thank you to Julian Thorpe, who taught me the joys of Electron Microscopy, and to Pascale Schellenberger, who nurtured that side of my work.

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Characterising the effects of genetic risk factors of Alzheimer's Disease on synaptic transmission: a functional and structural analysis

Abstract

Pathological changes in neural network activity play a key role in Alzheimer's Disease but the underlying processes remain unclear. Key genetic risk factors are correlated with the abnormal increase in production of the Aβ1-42 peptide and the expression of the E4 isoform of the Apolipoprotein E. We began by characterizing how elevated levels of Aβ1-42 affect pre-synaptic activity. Employing a functional approach coupled with a strong EM-based ultrastructure readout in CA3-CA1 hippocampal synapses derived from the AD transgenic mouse model APPSwe/Ind, we show an enhancement in the recycling fraction when compared to WT terminals. Spatial analysis of the retrieved vesicles revealed a preferential localization of the functional pool in APPSwe/Ind synapses around the peri-active zone (AZ) area, suggesting an organizational correlate of functional retrieval deficits. Complementary experiments monitoring glutamate activity using the genetically encoded reporter iGluSnFr showed that Aβ1-42 treatment over the course of 24h was sufficient to bring about a deficit in neurotransmitter release and clearance; the reported impairments were exacerbated after long-term (96h-120h) incubation. By using pharmacological interventions aimed at dampening synaptic over-activity (Levetiracetam) and improving vesicle turn-over (Roscovitine) we were able to partially rescue the reported deficits in neurotransmitter activity. Using the sypHy reporter, 24h incubation with addition of oligomeric Aβ1-42 revealed deficits in vesicle turn-over in ApoE-transgenic mice-derived ApoE3 cultures, while ApoE4 neurons displayed no effect, while ApoE-transgenic mice reported a propensity of ApoE4 animals to maintain an elevated level of functional vesicles at a later age (8 months). Our findings suggest that synapses show hyperactive function in activity-evoked vesicle recruitment with Aβ1-42 treatment but that retrieval pathways become overwhelmed, significantly limiting ongoing signaling. Further investigation on the Aβ1-42 dependent spatial and functional deficits are of vital importance to further elucidate the role of Aβ1-42 in AD and intervene with therapeutic approaches.
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<tr>
<td>AP5</td>
<td>(2R)-amino-5-phosphonovaleric acid</td>
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<td>ApoE</td>
<td>Apolipoprotein E</td>
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<td>E receptor 2</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>APPSwe/Ind</td>
<td>APP with Swedish and Indiana mutations</td>
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<tr>
<td>APs</td>
<td>Action potentials</td>
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<tr>
<td>ara-C</td>
<td>Cytosine arabinoside</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<td>Active zone</td>
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<td>Amyloid-beta</td>
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<td>Beta-secretase 1</td>
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<tr>
<td>BACE2</td>
<td>Beta secretase 2</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CA3</td>
<td>Cornu Ammonis 3</td>
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<td>CamKII</td>
<td>Ca⁺⁺/calmodulin-dependent protein kinase II</td>
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<td>Cell division protein kinase 5</td>
</tr>
<tr>
<td>CNQX</td>
<td>Cyanquinaline</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
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<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporters</td>
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<tr>
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<td>Electron microscopy</td>
</tr>
<tr>
<td>EOAD</td>
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<tr>
<td>ER</td>
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<td>FBS</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
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<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<td>HDL</td>
<td>High-density lipoproteins</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HFIP</td>
<td>Hexafluoroisopropanol</td>
</tr>
<tr>
<td>iGluR</td>
<td>Ionotropic glutamate receptor</td>
</tr>
<tr>
<td>iGluSnFR</td>
<td>intensity-based glutamate sensing fluorescent reporter</td>
</tr>
<tr>
<td>isi</td>
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<tr>
<td>LDLR</td>
<td>Low-density lipoprotein receptor</td>
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<tr>
<td>LEV</td>
<td>Levetiracetam</td>
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<tr>
<td>LOAD</td>
<td>Late onset Alzheimer’s disease</td>
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<tr>
<td>LRP1</td>
<td>Low density lipoprotein receptor-related protein 1</td>
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<tr>
<td>LTD</td>
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<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>Phosphate buffer saline</td>
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<td>non-Photoconverted vesicles</td>
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<td>PFA</td>
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<td>PNS</td>
<td>Peripheral nervous system</td>
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<td>PSD</td>
<td>Post-synaptic density</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>sEPSP</td>
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<td>Very Low Density Lipoprotein Receptor</td>
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<td>Wild type</td>
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CHAPTER 1: INTRODUCTION

1.1 Alzheimer’s Disease: EOAD and LOAD

Alzheimer’s disease is a widely spread neurological pathology characterized by the progressive deterioration of the brain and its functions, firstly brought to public attention by the German psychiatrist and neurophysiologist Alois Alzheimer in 1907. There are two forms of Alzheimer’s Disease (AD): one is genetically determined, called Early Onset Alzheimer’s Disease (EOAD) and accounting for 5%-10% of all the AD; the second is the Late Onset (LOAD) form, far more common (Defina, Moser, Glenn, Lichtenstein, & Fellus, 2013). When referring to AD the terms ‘Familial’ and ‘Sporadic’ are also commonly used in the literature. Familial refers to AD cases in which there is a documented family history of the pathology, with an age of onset of 60-65 years of age, whereas sporadic is used for cases in which the causes are not determined. Familial cases present EOAD due to the genetic component. Specifically, there are three genes in which mutations can lead to EOAD: amyloid precursor protein (APP), presenilin 1 (PSEN-1) and presenilin 2 (PSEN-2) (Sorbi et al., 2001; R E Tanzi et al., 1992; Rudolph E. Tanzi & Bertram, 2005). The APP gene codes for the amyloid precursor protein, a peptide whose proteolytic cleavage leads to the production of the Amyloid Beta protein (Aβ). Aβ is a protein of 40 (Aβ1-40) or 42 (Aβ1-42) amino acids in length which is thought to play an important role in AD onset and progression (Selkoe, 1991). LOAD tends to be of sporadic nature and its underlying causes are not yet fully understood. Sporadic LOAD has been linked with other genetic risk factors that have been shown to be important in AD development, such as PICALM, B1N1 and TREM2 (Scheltens et al., 2016). Currently, the most important risk factor is the gene encoding for the Apolipoprotein E glycoprotein (Bu, 2009).

Abnormal accumulation of amyloid beta (Aβ) in extracellular neuritic plaques (NPs) is a key histopathological hallmark of the disease, together with the aggregation of
hyperphosphorylated Tau in paired helical filaments (PHFs), ultimately deposited in neurofibrillary tangles (NFTs). NFTs load and spreading throughout the brain has been correlated with the severity and progression of the disease.

Aβ is thought to play an important role in neuronal impairment throughout disease progression by affecting different processes such as synaptic plasticity (Parihar & Brewer, 2010), calcium homeostasis (Demuro et al., 2005) and dendritic spines stability (Wei et al., 2010). In particular, Aβ has been shown to have an acute effect on synaptic activity. However, before we can discuss the pathological effects of Aβ, it is important to better understand its mechanisms of processing and production.

### 1.2 Aβ1-42: gene, protein and processing

APP is a 19 exon (Bagyinszky, Youn, An, & Kim, 2014; Y. Yang et al., 2015) gene localized on the long arm of chromosome 21 encoding for a Type-I transmembrane protein (N-terminus of the protein is extra-cellular). Of the 19 exons, only exons 16 and 17 encode for the Aβ sequence. APP, together with APP-like protein 1 and APP-like protein 2, belongs to a family of mammal homologous proteins (shown in Fig. 1.1) characterized by an extracellular sequence displaying Kunitz-type serine protease inhibitor (KPI), E1 and E2 domains (Wasco et al., 1992; Wasco, Peppercorn, & Tanzi, 1993; Zheng & Koo, 2011). The sequence required for the Aβ production, however, is only present in APP. This suggests that the physiological activity of the protein is mainly linked to the other conserved domains, rather than the non-conserved Aβ sequence (Collin, Van Strien, Leunissen, & Martens, 2004; Luo, Tully, & White, 1992). Alternative splicing of the APP mRNAs produces eight isoforms (O'Brien & Wong, 2012), of which APP770, APP751 and APP695 are the most common. Unlike the other two, APP695 lacks the KPI domain but it’s the more common isoform expressed in the brain (de Silva et al., 1997). Interestingly, an increase in the expression of APP751 and APP770 from astrocytes and microglia following brain injury has been reported (Siman, Card, Nelson,
& Davis, 1989; Van den Heuvel et al., 1999). It has been suggested that in the AD brain the balance between KPI and non KPI containing isoforms is affected and KPI-containing isoforms may have a neuro-protective effect (Belyaev et al., 2010).

Figure 1.1. Isoforms of APP. While belonging to the same family of mammal homologous proteins containing the KPI, E1 and E2 domains, the sequence required for Aβ production is only present in APP695, APP751 and APP770. Of these, APP695 is the most common isoform expressed in the brain. Adapted from Chen et al., 2017.

APP is produced in the Endoplasmic reticulum (ER), it then undergoes post-translational modifications in the Golgi/trans-Golgi network (TGN) and is transported to the membrane via TGN-derived vesicles (Greenfield et al., 1999; Hartmann et al., 1997; H. Xu et al., 1997). From the plasma membrane APP can undergo protein cleavage or be internalized in endosomes, where it can be recycled to the surface or targeted to the lysosome for degradation (Caporaso et al., 1994; Nordstedt, Caporaso, Thyberg, Gandy, & Greengard, 1993). Full length APP protein cleavage (depicted in detail in Fig 1.2) occurs as a result of the activity of three proteases: α- β- and γ-secretases. Cleavage of APP by α- and β-secretases leads to the production of two large soluble extracellular fragments, sAPPβ and sAPPα, respectively, as well as two membrane tethered fragments (APP-CTFβ and APP-CTFα). After this initial cleavage, γ-secretase will interact with the APP-
CTFβ leading to the production of Aβ and with APP-CTFα producing p3 (in both cases the APP intracellular domain, AICD, will also be produced).

The γ-secretase activity is brought forth by a protein complex consisting of four components: presenilin (PS), nicastrin, anterior pharynx-defective-1 (APH-1), and presenilin enhancer-2 (PEN2) (Kimberly et al., 2003; Takasugi et al., 2003). The PSEN-1 and PSEN-2 genes, located on chromosome 14 and 1, respectively, are two other important genetic determinants in AD. They contain 12 exons, of which exons 3-12 encode two 450 residue homologous transmembrane proteins, PS1 and PS2 (Cruts, Hendriks, & Van Broeckhoven, 1996; De Strooper et al., 1998; Tomita, 2009). PS1 and PS2 are susceptible to endo-proteolytic cleavage which leads to the formation of N-terminal and C-terminal fragments; these interact with each other forming heterodimers crucial for the proper functioning of the γ-secretase (Wolfe et al., 1999).

The dominant neuronal β-secretase for APP is the β-site APP cleaving enzyme-1 (BACE1). BACE1 gene is localized on the long arm of chromosome 11 and includes 9 exons (Rossner, Sastre, Bourne, & Lichtenthaler, 2006). The promoter is devoid of TATA and CAAT boxes but, like the PSEN-1 and -2 promoter, it possesses a high transcriptionally active GC content (Lee, Tse, Smith, & Landreth, 2012). Since the first 600bp appear to be conserved among species, this region is thought to have an important role in the gene regulation; furthermore, the promoter domain has been shown to have transcription factors binding site, enhancing the heterogeneity of BACE1 activation (Lee et al., 2012).
Figure 1.2. Processing pathways of APP. While the non-amyloidogenic pathway involving the α- and γ- secretases leads to the production of non-toxic species, the amyloidogenic β- and γ- secretases leads to the secretion of the Aβ peptide, whose misfolding and aggregation into neuritic plaques is a key hallmark of AD.

It is known that mutations in the APP gene have specific effects on the mechanisms just described, and most of them have been linked with the development of familial AD (Fig. 1.3). Such mutations include the Swedish mutation (KM570/571NL) that has been shown to increase Aβ load up to eightfold by influencing β-secretase activity, and the London mutation, which alters γ-secretase cleavage. Other mutations (Arctic, Dutch, Iowa) are located in the Aβ domain and lead to increased protein aggregation, while people carrying the Indiana (V617F) mutation display early cognitive impairment leading to the development of AD pathology (Philipson et al., 2010). Transgenic animal models incorporating one or more APP mutations have been important in deepening our understanding of the AD pathology allowing scientists to investigate selected mechanisms and phenotypes.
Figure 1.3 Mutations on the APP gene linked with AD. The Flemish mutation has been linked to a decrease rate of APP cleavage by the $\alpha$-secretase, while the Arctic, Dutch and Iowa mutations are localized in the A$\beta$ domain, mainly causing protein aggregation. The London mutations influence the $\gamma$-secretase cleaving sites and the Swedish mutation exacerbates the cleavage by the $\beta$-secretase. The Indiana mutation has been linked with the development of early cognitive impairment.

1.3 Apolipoprotein E: gene, protein and isoforms

As discussed in section 1.1, while mutations in the APP gene are associated with EOAD, a variety of risk factors have been associated with LOAD, the strongest of which has been shown to be Apolipoprotein E.

The role of apolipoproteins is to act as ligands for specific cell-surface receptors and aid in the transport of lipids in the plasma and the central nervous system (CNS). Apolipoproteins have the ability to bind lipids to form different types of lipoprotein particles, whose role is the transport of phospholipids, cholesterol, cholesterol esters and triacylglycerides (Bu, 2009). Exchangeable apolipoproteins, such as ApoE, are similar in sequence and structure and can associate with lipids in a reversible manner. This dynamic process of apolipoprotein exchange requires a high level of conformational adaptability, suggesting multiple conformations both in their lipid-bound and lipid-free state (Bolanos-Garcia & Miguel, 2003).

Human APOE is encoded by a 3.6 kbp tetra-exonic gene present on chromosome 19 (19q13.2) (Bu, 2009). The APOE gene is present in the general population as three polymorphic alleles: $\varepsilon$2, $\varepsilon$3 and $\varepsilon$4, with a frequency of 8%, 77% and 15%, respectively (Fig. 1.4) (Mahley, 1988). People with a $\varepsilon$4$\varepsilon$4 genotype have a 91% frequency of AD, while it is 47% for $\varepsilon$4 heterozygotes and 20% in non-carriers (Corder et al., 1993). In other terms, the presence of one $\varepsilon$4 allele anticipates the AD risk curve of 5 years earlier,
two ε4 copies move it 10 years earlier and one copy of ε2 shifts it 5 years later (Noguchi, Murakami, & Yamada, 1993; Pastor et al., 2003).

Apolipoprotein E (ApoE) is a 299 amino acid glycoprotein involved in the transport and uptake of lipids (Mahley & Ji, 1999; Rall, Weisgraber, & Mahley, 1982; Zannis, McPherson, Goldberger, Karathanasis, & Breslow, 1984). It regulates intracellular levels of cholesterol and mediates lipid distribution via specific cell surface receptors (Mahley & Rall, 2000). ApoE is synthesized predominantly in the liver and in the brain, and in lesser amounts in the spleen, lungs and kidneys. It is mainly present in the plasma, interstitial fluid, lymph and cerebrospinal fluid (CSF), where it is always associated with lipids. While astrocytes (and microglia), are the main producers of ApoE in the CNS, hepatocytes are the main source of ApoE in the body. In the human and animal brain, ApoE RNA is present in the cerebral cortex, hippocampus, cerebellum, and medulla (Mahley, 1988). Studies show how neurons in the hippocampus and cortex are able to synthesize ApoE in response to injury (Q. Xu et al., 2006). In the peripheral nervous system ApoE has been detected in glia surrounding motor and sensory neurons, as well as in non-myelinating Schwann cells, but not in myelinating ones. In case of injury in the peripheral nervous system (PNS), macrophages recruited at the site secrete ApoE protein (Bellosta et al., 1995; Mazzone, 1996).

The ApoE protein is present as three isoforms ApoE2, ApoE3 and ApoE4. Each of these isoforms differs from one another by one amino acid change, apart from ApoE2 and ApoE4 that differ in two residues (J. Chen, Li, & Wang, 2011; Sivashanmugam & Wang, 2009; Wilson, Wardell, Weisgraber, Mahley, & Agard, 1991). ApoE3, the most common form of this protein, possesses a cysteine at position 112 and an arginine at position 158. ApoE2, linked to premature atherosclerosis and Type III Hyperlipoproteinemia (Mahley, 1988; Mahley & Ji, 1999) due to highly reduced receptor binding, maintains the ApoE3 Cysteine at position 112 but includes another cysteine residue instead of the arginine at
position 158. ApoE4 contains Arginines at both positions 112 and 158, and has been
determined to be a strong risk factor for AD.

The general structure of ApoE, as shown in Fig 1.4, articulates in two domains
connected by a flexible hinge region of 20-30 amino acids (J. Chen et al., 2011). The
secondary structure of ApoE is mainly composed of strongly amphiphatic \( \alpha \)-helices,
which allow ApoE as well as other apolipoproteins to assume different conformations
depending on their lipid-bound state (Segrest et al., 1992). In aqueous solution the full
length protein has the tendency to form tetramers, behavior attributed to the C-terminal
as show by amino acid substitution studies (Yokoyama, Kawai, Tajima, & Yamamoto,
1985). The region formed of residues 136-150, localized within the N-terminal domain,
allows the protein to bind its target receptor, while the region comprised of residues 244-
272 within the C-terminal domain is reserved for binding lipids (Hatters, Peters-Libeu, &
Weisgraber, 2006). The amino terminal (aa 1-191) is composed of four Alpha helices,
and the region that binds to the receptor is localized on Helix 4 (aa 130-164) spanning
residues 136 to 150. On the other hand, studies have shown that truncated ApoE (aa 1-
244) displays reduced binding ability to lipids while inclusion of the 244-272 amino acids
region shows full binding activity, suggesting how the latter region is crucial in normal
lipid binding (Mahley, 1988; Mahley & Rall, 2000). In terms of lipid binding, ApoE appears
to interact with different lipoproteins in an isoform-dependent manner: ApoE2 and ApoE3
prefer binding to High Density Lipoproteins (HDL), while ApoE4 has a preference for
Very Low Density Lipoproteins (VLDL) and Low Density Lipoproteins (LDL) leading to
an increase of cholesterol in the plasma (Hatters et al., 2006).
Aβ and ApoE have been implicated in synaptic dysfunction occurring during AD progression, both in an independent and interactive manner. However, in order to explore what might go wrong during disease progression and how these two proteins could play a role in the pathology, it is important to discuss what is known so far regarding the physiological processes surrounding synaptic activity.

1.4 The Synaptic Vesicle Cycle

Under physiological conditions, neurotransmitter release in chemical synapses is a tightly regulated process involving a great variety of proteins, whose correct functioning is crucial for proper synaptic transmission. Synaptic vesicles biogenesis initially occurs at the Endoplasmic Reticulum (ER), from which a carrier vesicle enriched with SV protein(s) is produced and fuses with the Golgi apparatus (GA) (Watson & Stephens, 2005). Following sorting in the GA, vesicles (or synaptic vesicle precursors) bud off and proceed to be enriched in the lacking SV proteins through endosomal processing, and ultimately be transported along the axon to reach the pre-synaptic compartment.

**Figure 1.4. Schematic structure of the ApoE protein.** ApoE is a 299 aa protein composed of an N-terminal domain, which includes the receptor-binding region and the two polymorphic positions (112 and 158), and the C-terminal region in which the lipid-binding domain is located. The E4 isoform of the protein is thought to undergo a change in its conformation leading to what has been called “domain interaction”, resulting in the interaction between the N-terminal and C-terminal regions. This domain interaction is thought to occur less in the E3 and E2 isoforms. Adapted from Yu tan Hardy 2014.
Here, the vesicle will fuse with the plasma membrane and prompt the segregation of the delivered proteins and other components. The more energetic favourable and protein rich environment of the plasma membrane (Bennett, Calakos, Kreiner, & Scheller, 1992) will transform a SV protein enriched patch to an endocytosed vesicle via tightly regulated clathrin-dependent mechanisms (Haucke, Neher, & Sigrist, 2011). The newly synthesised vesicle is thought to undergo fusion with endosomal structures in the terminal (Hoopmann et al., 2010), before budding off much like from the Golgi and becoming a mature synaptic vesicle. It is unclear at what point of the process neurotransmitter loading takes place. It is important to keep in mind that the pathways and mechanisms described so far remain far from being fully understood and are presented as a guideline to better appreciate the context in which the key components of the SVC originate from and which organelles and cellular structures play a pivotal role.

At this point there is a fusion competent vesicle, filled with neurotransmitter, ready to undergo release. First, we shall discuss the movement of vesicles towards the active zone, their docking and priming for release. Then we shall move onto the mechanism of neurotransmitter release through vesicle exocytosis. Finally, we will discuss vesicle retrieval, the different modes of action and the pathways taken by the endocytosed vesicles in the terminal.

### 1.4.1 Synaptic vesicle exocytosis

Before undergoing exocytosis upon calcium influx, synaptic vesicles need to be filled with neurotransmitter and have to move closer to the active zone, where they will dock and be primed for release. While neurotransmitter re-filling occurs at a certain point after retrieval (depending on the mechanism) for old vesicles, it remains unclear at what point after membrane release newly synthesised vesicles undergo neurotransmitter uptake (Rizzoli, 2014). The movement of synaptic vesicles is correlated with Synapsin, a protein involved in the binding of vesicles to the cell cytoskeleton (Bloom et al., 2003). Synapsin
is present in vertebrates in five different isoforms, with SynapsinIIa being involved in glutamatergic synapses (Song & Augustine, 2015). Synapsin phosphorylation by protein kinase-A following calcium influx has an effect on vesicles motility and their availability to be released (Cesca, Baldelli, Valtorta, & Benfenati, 2010). Other kinases known to be involved in synapsin phosphorylation at different sites are MAPK, CDK5 and CaMKII (Song & Augustine, 2015). Newly formed vesicles do not seem to possess synapsin, and they are therefore much more mobile than their older counterparts (Bloom et al., 2003; Kamin et al., 2010). Once a vesicle is proximal to the active zone, proteins such as piccolo and bassoon are thought to move it in closer proximity with another set of proteins, Rab3-interacting molecules (RIMs) and RIM-binding proteins, whose role is to facilitate vesicle docking and positioning, as well as to maintain a correct active zone organization. Indeed, the clearance of fusion sites from previously exocytosed vesicles and their availability for the following stimulation rounds appears to be one of the key steps in the physiological SVC process (Neher, 2010). Although their specific roles still remain unclear, other proteins are thought to play a part in maintaining vesicles close to the active zone. Munc13 and Calmodulin, for example, are a vesicle priming peptide and a calcium binding protein, respectively (Lipstein et al., 2013). SNAP receptor (SNARE) proteins are key players in membrane fusion and are located both on the vesicle and the plasma membrane itself. Vesicular SNARE (Synaptobrevin, or VAMP) interacts with Snap-25 and syntaxin-1 on the membrane to form partially coiled SNARE complexes (Walter, Wiederhold, Bruns, Fasshauer, & Sorensen, 2010). Other important proteins involved in this step are Complexin, Munc18 and Munc13. While Complexin is thought to stabilize the SNARE complex, Munc18 and Munc13 have been shown to act on syntaxin-1 conformation (Ma, Su, Seven, Xu, & Rizo, 2013).

Changes in the intracellular calcium levels are sensed by synaptotagmin, a calcium sensor localized on the vesicle. Upon calcium binding, synaptotagmin interacts with both the SNARE complex and the plasma membrane (de Wit et al., 2009; Stein,
Radhakrishnan, Riedel, Fasshauer, & Jahn, 2007). It is also thought that synaptotagmin can displace complexin from the SNARE complex. Fusion of the vesicle resembles a collapsing of the structure onto the membrane. At this point, it is unclear whether SV proteins and structures remain localized on the plasma membrane, awaiting to be endocytosed, or rather diffuse throughout indefinitely (Z. Li & Murthy, 2001). While some results suggest the diffusion of a certain amount of SV components, it is likely that the collapsed vesicle remains localized in a membrane patch (Willig, Rizzoli, Westphal, Jahn, & Hell, 2006).

1.4.2 Synaptic vesicle endocytosis

The precise endocytic mechanisms that allow for vesicle retrieval still remain a debated topic. Studies have reported a variety of intermediate structures depending on the locus investigate, the stimulation given, and the technique employed (Rizzoli, 2014; Saheki & De Camilli, 2012). One endocytic mechanism which has been widely agreed on involves clathrin-coated pits to drive vesicle internalization (Fig. 1.5a). On the contrary, strong stimulation appears to elicit a different kind of endocytosis, termed “bulk-endocytosis” (Fig. 1.5c), in which vesicles appear to be retrieved in endocytic masses before regaining their own individuality (Clayton & Cousin, 2009). A third and widely debated process, termed kiss-and-run (Fig. 1.5d), postulated the partial fusion of the vesicle with the membrane before undergoing endocytosis (He & Wu, 2007). Finally, two other endocytic mechanisms that have been proposed are known as fast compensatory endocytosis and ultrafast endocytosis (Fig. 1.5b), with the second process being relatively slower than the first and both occurring after mild stimulation (Gan & Watanabe, 2018; Soykan et al., 2017; Watanabe et al., 2013).

Evidence for clathrin-mediated endocytosis comes from a variety of elegant experiments such as the presence of clathrin-coated intermediates following the blockage of vesicle recycling (Shupliakov et al., 1997) and the inhibition of vesicle turn over when clathrin mediated endocytosis was blocked (S. H. Kim & Ryan, 2009; von Kleist et al., 2011)
initial model for clathrin-mediated endocytosis described the retrieval of the synaptic vesicle from the membrane via clathrin coated pits, which would shed off allowing the vesicle to fuse with sorting endosomes from which new vesicles would be produced (Heuser & Reese, 1973). However, this hypothesis has been revised by considering endosomal structures as intermediates in the endocytic process rather than a step the vesicle has to go through (Takei, Mundigl, Daniell, & De Camilli, 1996). According to this single budding step hypothesis, vesicles can undergo retrieval via clathrin components and be ready for use after shedding off the coating, be endocytosed through clathrin pits in bulk with excess plasma membrane, leaving behind vacuoles after coating shedding, or undergo single-vesicle retrieval and interact with early endosomes, late endosomes and multi vesicular bodies. Proteins playing a key role in this process are clathrin and dynamin, which is important in membrane fission (Ferguson & De Camilli, 2012).

Bulk endocytosis has been shown to occur after strong stimulation. The fusion of a large number of vesicles in a small-time span prompts the formation of membrane infoldings and subsequent fission, giving rise to endosomal intermediates (Wu & Wu, 2007). These temporary organelles, such as vacuoles and cisternae, might be enriched with SV proteins. It still remains unclear how vesicles are retrieved from these intermediate structures. One hypothesis postulates the involvement of the classic coating mechanisms, guiding the budding of vesicles either directly from the vacuoles or after the intermediates may fuse back with the plasma membrane (Clayton, Evans, & Cousin, 2008). However, inhibition (absence) of dynamin did not seem to affect the formation of said structures, despite inhibiting classic clathrin-mediated endocytosis (Ferguson et al., 2007). Other types of non-clathrin related coatings, such as lipid- or protein- mediated types of coating, might be the answer (Glyvuk et al., 2010; Kirchhausen, 2000). It has also been suggested the possibility of lipid- or protein- mediated types of coating (Kirchhausen, 2000). Different modes of endocytosis are present at different synapses, and the preferential use of one modality over another is strictly correlated with the level
and intensity of the stimulation received by the terminal; fast endocytosis tends to occur following brief stimuli, while the slower component is more prevalent upon longer stimulation rounds (Gan & Watanabe, 2018).

**Figure 1.5. Differential modes of endocytosis at the rat hippocampal synapse.** Synaptic vesicle recycling takes place via a variety of mechanisms. (a) Clathrin-mediated endocytosis is thought to be the primary pathway of vesicle recycling, with (b) ultrafast endocytosis taking place with 7-10s time constant and (c) bulk endocytosis occurring following strong stimulation. (d) Kiss-and-run is thought to occur, but it is still a highly debated mechanism. Adapted from (Gan & Watanabe, 2018)

### 1.4.3 Synaptic vesicle pools

Ever since the introduction of the vesicular hypothesis of synaptic transmission (Fatt and Katz, 1952; Del Castillo and Katz, 1954), the mechanisms of action of synaptic vesicles have been subject to investigation. The further notion that there were structural and functional differences among vesicles (Birks and MacIntosh, 1961) led to their arbitrary subdivision in subgroups denominated ‘pools’ in the presynaptic terminal compartment. Different nomenclatures and methods of classification have been proposed in recent
years, depending on the synaptic preparations used, the types of stimulation elicited and vesicles properties investigated, giving rise to a variety of pool types and numbers in the literature (Denker & Rizzoli, 2010).

Recently, Alabi and Tsien (2012) addressed these discrepancies by elaborating a consistent categorization of synaptic vesicle pools; I will hereby refer to this proposal when discussing pools terminology and dynamics. Regardless of the synaptic preparation chosen, it has been identified a group of vesicles that are ready to be released upon stimulation, termed the Ready Releasable Pool (RRP). Upon RRP depletion, synaptic transmission will carry on via recruitment of other vesicles from a second pool called Recycling Pool (RP). Together, RRP and RP constitute the Total Recycling Pool (TRP) of vesicles in the presynaptic compartment, that is the total number of vesicles that can be released. Finally, the remaining vesicles that are not primed for release are part of a third subgroup called the Resting Pool (RtP).

This tripartite pool model derives from a variety of experiments carried out in different synaptic preparations, ranging from Drosophila Neuro Muscular Junctions (NMJs) (Delgado, Maureira, Oliva, Kidokoro, & Labarca, 2000) to cultured hippocampal synapses (Schikorski & Stevens, 1997). However, the data reported has mainly been acquired through high-frequency stimulation levels, with stimuli spanning 10Hz all the way up to 300Hz. This approach was and still is objectively crucial when interrogating the system on the mechanisms and processes underlying the SVC not at the level of single vesicle, but rather focusing on functional groups and subdivisions. At the same time, one has to question whether the conclusions drawn from such experiments hold true under physiological stimulation levels. High frequency and sustained stimulation may be something that the cell can take, but not necessarily something that the neuron wants, thus its behaviour under certain conditions may not only be considered as the answer to a question of mechanical nature, but may also be indicative in regards to safety checkpoints in place that we might not be aware of.
High-frequency stimulation experiments reported a fast release of the RRP in response to the transient rise of Ca\textsuperscript{2+}, followed by a slower exocytosis of the RP and finally the un-primed RtP (Schneggenburger & Neher, 2005). In terms of morphology, the speed in release displayed by the RRP strongly suggests a privileged positioning docked at the pre-synaptic membrane and an already primed status of said vesicles. While the RRP is constituted by docked and primed vesicles, it has been shown that not all vesicles localized at the AZ are part of said pool (Rizzoli & Betz, 2004). On the other hand, the RP and RtP do not appear to hold predefined positions in the terminal and seem to be much more intermixed than when first described. Strong stimulation (>10Hz) in preparations such as cultured hippocampal neurons (Opazo et al., 2010), mouse NMJ (Wyatt & Balice-Gordon, 2008) and calyx of Held (de Lange, de Roos, & Borst, 2003), reported the presence of a resting unreleased RtP, accounting for ~50% of the total amount of vesicles in the terminal (Fig 1.6a).

The situation appeared to be different when a lower, more physiological stimulation was applied (Fig. 1.6b). In the Drosophila NMJ, all vesicles could undergo release, even though a change in the stimulation applied still allowed for a differentiation between RP and RtP (Denker, Krohnert, & Rizzoli, 2009). Stimulation at ~2Hz over a period of 6h-8h in the frog NMJ reported a recycling of almost all vesicles in the terminal at least once (Ceccarelli, Hurlbut, & Mauro, 1972). A similar situation was witnessed in a primary hippocampal culture model, where stimulation at 0.2 Hz led to the release of most vesicles, some of which were reluctant to be released under higher stimulation (Ikeda & Bekkers, 2009). This has led to the hypothesis describing a high level of intermixing between the RP and RtP, wherein an evident differentiation can be reported more clearly only under high stimulation.
Figure 1.6. Synaptic vesicle pools classification under different stimulation paradigms. While strong, unphysiological stimulation has been shown to result in a definite differentiation between the three available synaptic pools (a), during a lower physiological stimulation the net difference between the recycling pool and the reserve pool appears to be less defined, suggesting a more dynamic mechanisms occurring (b). Adapted from (Denker & Rizzoli, 2010)

Taken together, the data discussed so far reminds us that, as we delve into the microscopic environment of neuronal transmission, it is important not to forget that events do not manifest because of consciously taken decisions but rather due to a shift in the balance between the probability of a particular outcome to take place over another. The model steps hypothetically adopted by a synaptic vesicle described in chapters 1.4.1, 1.4.2 and 1.4.3 are dependent on a set of conditions that need to be met at a point in time in order for the organelle to persist in the described path. Every reaction observed is triggered and preceded by any number of actions that determine its outcome. The mechanisms and processes concerning the SVC and overall fitness of the pre-synaptic compartment are present in a dynamic equilibrium, and the information we gather from our experimental investigations is directly linked to the we decide to interrogate the system.
1.5 Synaptic disfunction in Alzheimer’s Disease

1.5.1 The effect of Aβ on synaptic vesicle activity

As detailed in section 1.2, APP reaches the plasma membrane from the ER through the TGN via TGN-derived vesicles. Interestingly, APP has been shown to be prevalent on the membrane of the presynaptic terminal and its non-amyloidogenic fragments have been implicated in various mechanisms such as cell-cell adhesion and synaptic signalling (Sosa et al., 2017). Furthermore, multiple studies showed that the secretases involved in the production of such fragments, as well as the amyloidogenic Aβ1-42, are also highly localized at the synapse (Lundgren et al., 2015; Schedin-Weiss, Caesar, Winblad, Blom, & Tjernberg, 2016). What this may suggest is that APP processing at the synapse is an important mechanism under normal circumstances for its physiological activity, and upon the presence of altering factors (i.e. mutations on the APP and BACE genes) it becomes a site of toxic Aβ1-42 production.

While Aβ-mediated effects on the post-synaptic compartment have been studied in detail (Sheng, Sabatini, & Sudhof, 2012; Dominic M Walsh et al., 2002), pre-synaptic mediated effects still remain to be uncovered. In a recent study employing super-resolution microscopy it was shown that soluble oligomeric Aβ1-42 was localised in the presynaptic compartment and it was absent post-synaptically (Y. Yu, Jans, Winblad, Tjernberg, & Schedin-Weiss, 2018). Synaptic vesicle endocytosis has been found to be affected by Aβ, a deficit which appeared to be dependent on a reduction in the levels of dynamin (Kelly & Ferreira, 2007). Furthermore, a study conducted by Cao and colleagues (2010) using FM dye labelling on transgenic mice expressing the Swedish mutation on the APP gene showed a reduction in vesicle retrieval, as well as decreased levels of key proteins involved in this mechanism, such as dynamin 1, AP180 and synaptophysin (Yamashita, Hige, & Takahashi, 2005). Regarding synaptophysin, Aβ has been found to compete with VAMP2 for its binding, thus possibly leading to a regulation of neurotransmitter release.
(Russell et al., 2012). We will discuss in depth the possible detrimental effects elicited by Aβ in chapter 3.

1.5.2 ApoE and the synapse

Since ApoE has been shown to be such a strong genetic risk factor for LOAD, and it has been associated with changes in spine morphology and synaptic plasticity (J. Kim, Yoon, Basak, & Kim, 2014), it is imperative to better understand the relationship between ApoE isoforms and the SVC. ApoE has been shown to affect synaptic plasticity in an isoform-dependent manner, with ApoE3 promoting neurite outgrowth (Nathan et al., 2002), neuronal sprouting (Teter et al., 1999) and the proper delivery of cholesterol and other lipids following neuronal injury (Mahley & Huang, 2012). However, results on the ApoE4 isoform appear conflicting, with some studies reporting deficits in neurite outgrowth (Teter et al., 2002), others didn’t see any effect (DeMattos, Curtiss, & Williams, 1998).

Initial studies employing ApoE deficient mice reported impairments in synaptic LTP (Veinbergs, Mante, Jung, Van, & Masliah, 1999) as well as cholinergic deficits (Gordon, Grauer, Genis, Sehayek, & Michaelson, 1995). Although initially debated (Anderson et al., 1998), further studies on TR mice expressing human ApoE4 report impairments in synaptic integrity (Cambon, Davies, & Stewart, 2000), LTP (Korwek, Trotter, Ladu, Sullivan, & Weeber, 2009) and changes in the overall hippocampal architecture (Andrews-Zwilling et al., 2010) when compared to TR ApoE3 mice. Behavioural data from ApoE4 TR mice also reports memory impairment following Morris water maze experiments (Andrews-Zwilling et al., 2010). Human and mouse ApoE differ ~30% in structure, and differences have been suggested due to their different promoters (Fagan, Bu, Sun, Daugherty, & Holtzman, 1996). The effects of ApoE on synaptic transmission and its possible interaction with Aβ will be explored in chapter 6.
1.6 Reporters of vesicle activity

Unlike other more accessible physiological structures, the presynaptic terminal and the synaptic vesicles therein contained pose a great deal of difficulty to the experimental investigation. However, advancements in imaging techniques in the past years have provided us with novel tools to utilize in the examination of their structure, function and plastic changes under physiological and pathological conditions (Marvin et al., 2013). Novel Electron Microscopy approaches allowed us to tackle the ultrastructural aspect of the conundrum, providing a better understanding of the architectural and structural components at the presynaptic terminal (Tao et al., 2018). On the other hand, new strategies involving live cell imaging coupled with targeted genetically engineered fluorescent probes provides a different kind of information compared to electron micrographs, shifting the emphasis on the functional aspect of the preparation (Okabe, 2017). It is thus crucial to carefully choose which approaches are more suitable to answer our questions, as well as to understand their strengths and limitations when it comes to data gathering and analysis.

If we would like to closely monitor this complex mechanism of the SVC from beginning to end, we unfortunately start to incur in a series of problems. It is not possible for us to directly follow the fate of each individual vesicle down at their nanometre scale and observe each change in the behaviour and activity they might have in response to a variety of different stimuli. What we have to do is choose to either strictly focus on the functional aspect of the process, forsaking the structural side, or adopt a highly focused ultrastructural approach and gather functional information through indirect means.

1.6.1 Styryl dyes

As previously discussed, the positioning of synaptic vesicles in the terminal is not a random configuration. However, the term configuration has an intrinsic spatial connotation, and as we saw in chapter 1.4.3, the localization of vesicles in the presynaptic terminal is not always directly correlated with the concept of “pools”. Thus, it
would be more correct to talk about different “behaviours” expressed by groups of vesicles under set stimulation paradigms. It is possible to distinguish three different vesicle pools: a RRP, a group of vesicles that are ready to be released upon stimulation, a RP, comprising of those release competent vesicles that are recruited during a strong and/or prolonged stimulation, and finally a RtP, referring to the remaining vesicles not able to be released. While these nomenclatures and subdivisions have obviously been arbitrarily chosen, it is important to keep in mind that while vesicles may appear morphologically similar, in actuality they are functionally distinct.

Styryl dyes, initially characterized by (W. Betz, Mao, & Bewick, 1992), are able to bind to lipid membranes and provide a functional readout of vesicle recycling. Since their identification, a number of dyes with different properties have been employed to study synaptic vesicle kinetic in a variety of preparations, such as primary dissociated cultures, as well as central (W. J. Betz & Bewick, 1992) and peripheral terminals (Ryan et al., 1993).

Specifically, the FM1-43 variant has the ability to emit reactive oxygen species under certain imaging conditions and cause precipitation of compounds such as DAB (Kamin, Revelo, & Rizzoli, 2014). When localized in enclosed organelles, this electron dense precipitate can be ultimately be visualized by using EM techniques (Marra, Burden, Crawford, & Staras, 2014). This allows us to directly gain valuable information on the vesicle ultrastructure and localization in the terminal with respect of their functional recruitment and previous activity.

### 1.6.2 Genetically encoded probes

While the use of FM1-43 dye coupled with EM imaging allows us to directly observe changes in the ultrastructural aspect of the turned over vesicle pool, all the functional information that we can extract from this preparation is purely indirect. Furthermore, what we are left with is how the system looks at a frozen point in time, after vesicle release
and recycling has already taken place. The use of genetically encoded probes applied on a primary hippocampal culture model allows for live imaging of the kinetics of vesicle recycling over multiple rounds of activity. Depending on the probe of choice we can monitor vesicle exocytosis, endocytosis, as well as neurotransmitter release. Below we will explore and validate two genetically encoded reporters and discuss how they can help us gain a better understanding of synaptic mechanisms.

1.6.2.1 sypHy
Initially developed by (Miesenbock, De Angelis, & Rothman, 1998), the use of pH-sensitive variant of GFP (pHluorin) conjugated with a range of proteins targeted to the lumen of synaptic vesicles, such as synaptobrevin (Miesenbock et al., 1998), synaptotagmin (Diril, Wienisch, Jung, Klingauf, & Haucke, 2006), and synaptophysin (Granseth, Odermatt, Royle, & Lagnado, 2006), allows for real-time imaging of exo- and endocytic kinetics over repeated trials. When localised to the interior of synaptic vesicles, the pHluorin fluorescent signal is quenched due to the low pH level (pH ~5.5) in the organelle. When stimulation is applied and release-competent vesicles are recruited to the plasma membrane for neurotransmitter release, the pH level (pH ~7.4) at which the pHluorin is exposed rises due to a continuity of the vesicle lumen with the cytosolic environment. This leads to an increase in the fluorescent emission of the GFP variant and a rise in the recorded signal. As vesicles undergo endocytosis and re-acidification, the endocytic intermediates bud off from the membrane and once again expose the pHluorin to a more acidic environment, effectively quenching the probe once more and driving a decline in the signal. Developed by Granseth et al. (2006), sypHy1x is composed of one super-ecliptic pHluorin conjugated to the second intra-vesicluar loop of synaptophysin (Granseth et al., 2006).

1.6.2.2 iGluSnFr
The use of pHluorin probes tagged to vesicle specific proteins, as in the case of sypHy1x, provides valuable information regarding functional vesicle turnover and changes in
kinetics. However, because the source of signal comes from a change in pH where our probe is exposed to, what we are looking at still maintains a certain structural aspect to it, and it fails to convey information regarding the key player of synaptic transmission: the neurotransmitter itself.

Brain neuronal networks use a variety of neurotransmitters to convey information during transmission. Glutamate is the primary mediator of excitatory signals in the brain, and glutamate transmission has been shown to be impaired in a variety of diseases, such as AD, Parkinson’s Disease and Huntington’s Disease (Parsons et al., 2016). In order to investigate changes in glutamate release and clearance we decided to use an intensity based glutamate sensing fluorescence reporter (iGluSnFr) initially developed by (Marvin et al., 2013), and since then used in a variety of preparations aimed at elucidating pathological changes in glutamate activity (Lewerenz & Maher, 2015). In the absence of glutamate, the probe exhibits a faint fluorescence. Upon glutamate binding, a conformational change leads to a rapid increase in signal intensity (Marvin et al., 2013).
1.7 Aims

Synaptic dysfunction holds a pivotal role in AD progression and has been shown to correlate with the severity of the pathology (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). Here we utilized two experimental model systems, primary hippocampal cultures and acute transverse hippocampal slices from model animals. Cell cultures plated from dissociated primary hippocampal neurons are a widely used model in the field (Grabrucker, Vaida, Bockmann, & Boeckers, 2009) due to their ability to closely resemble the in vivo state and the normal physiology of the tissue of origin, as well as being a great tool to investigate the functional activity at the level of single cells. The level of control, down to the cellular density upon plating, and the possibility of adding exogenous agents further supported this as a suitable model to carry out our experiments (Kaech & Banker, 2006). Coupled with genetically encoded probes (sypHy and iGluSnFr), this model allows us to monitor functional changes at the level of the presynaptic compartment. While primary hippocampal cultures provide a solid and reproducible model to investigate the functional activity of neuronal cells in a physiological environment, one thing that is not maintained from the in vivo environment is the cytoarchitecture and synaptic circuit localization. Hippocampal slices provide a powerful model to investigate structural and architectural characteristics of synaptic connections at the cellular level. Specifically, acute hippocampal slices can be used in conjunction with electrophysiological approaches to explore changes in the ultrastructure of individual synaptic components (Lo, McAllister, & Katz, 1994; Mathis, Furman, & Norris, 2011; Weng, AU - Li, AU - Peng, & AU - Behnisch, 2018).

The main, overarching aim of this work was to explore the role of two key genetic components, Aβ in EOAD and APOE in LOAD, in the patho-physiology of pre-synaptic transmission. By using the aforementioned experimental models and investigational approaches, the main aim can further be broken down into the following:
• To identify the functional deficits elicited by Aβ on the presynaptic compartment at the level of individual glutamate release during sustained stimulation
• To elucidate structural changes of the functional vesicle pool in response to elevated Aβ levels
• To investigate the differential role of the ApoE3 and ApoE4 isoforms in synaptic transmission in the presence or absence of Aβ, as well as their effect on the overall recycling fraction
CHAPTER 2: MATERIALS AND METHODS

2.1 Preparation of Primary hippocampal cultures

2.1.1 Animal Handling

Animal handling was carried out in accordance with Animals (Scientific Procedures) Act 1986. Rat primary hippocampal cultures were prepared from P₀–P₁ rat pups sacrificed using Schedule 1 procedures. Briefly, cervical neck dislocation was performed and death was confirmed by a secondary method.

2.1.2 Culture plate preparation

Table 2.1. Reagents used for the preparation of primary hippocampal cultures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanks Balanced Salt Solution (HBSS) (10X)</td>
<td>Gibco™</td>
</tr>
<tr>
<td>Cytosine β-D-arabinofuranoside (ara-C)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Poly-D-lysine hydrobromide (PDL)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>HEPES</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Basal Medium Eagle (BME)</td>
<td>Gibco™</td>
</tr>
<tr>
<td>Glucose (45%)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>Gibco™</td>
</tr>
<tr>
<td>Foetal Bovine Serum (FBS)</td>
<td>Gibco™</td>
</tr>
<tr>
<td>Penicillin-Streptomycin (100X)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>GlutaMAX™ Supplement (100X)</td>
<td>Gibco™</td>
</tr>
<tr>
<td>B27 Supplement (50X)</td>
<td>Gibco™</td>
</tr>
<tr>
<td>Laminin</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>
Table 2.2. Solutions used for the preparation of primary hippocampal cultures

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanks’ Balanced Salt Solution</td>
<td>HBSS 1x, 10 mM HEPES in 10 mL H$_2$O</td>
</tr>
<tr>
<td>Hippocampal Media</td>
<td>BME, 20 mM Glucose, 10 mM HEPES, 1 mM Sodium Pyruvate, 2% FBS, 1% Penicillin-Streptomycin, 1% GlutaMAX, 2% B27</td>
</tr>
<tr>
<td>External Bath Solution (EBS)</td>
<td>137 mM NaCl, 5 mM KCl, 2.5 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM D-Glucose, 5 mM HEPES. Pre-warmed at 37°C</td>
</tr>
<tr>
<td>Cytosine β-D-arabinofuranoside (ara-C)</td>
<td>8.7 μM ARA-C in neuronal growth media</td>
</tr>
<tr>
<td>Poly-D-Lysine (PDL)</td>
<td>20μg/ml in H$_2$O</td>
</tr>
</tbody>
</table>

Ethanol sterilized glass coverslips (12mm, Fisher) were placed in 12 wells of a 24 well plate, filling the remaining wells with sterile water. Poly-D-Lysine (PDL, 20 μg/ml) was added to the wells and coverslips were incubated overnight at 37°C, 5% CO$_2$. The following day, PDL was removed and coverslips were allowed to dry completely before adding a Laminin coating (20μg/ml). Laminin was then aspirated before plating cultures.

2.1.3 Primary rat hippocampal cultures

The brain was removed and placed in a small petri dish filled with ice cold Hanks’ Balanced Salt Solution. The hippocampi were dissected from both hemispheres, carefully removing any meninges that may be present. The dissecting solution was then removed, and the samples were washed three times with 2 ml of Hippocampal media pre-warmed at 37°C. After the last wash, the tissue was gently triturated with a 1 ml
pipette to yield a homogeneous suspension. Cells were plated in 12 wells of 24 well plates, with a density of ~35,000 per well; plates were maintained at 37°C, 5% CO₂ in an incubator until they were ready to be used (DIV 14-18). Due to the composition of the media, both astrocytic and neuronal populations were allowed to proliferate on the coverslips, thus providing a balanced culture both in terms of ratio of cell types and nutrients. To prevent excessive astrocytic growth, cultures were treated at DIV 4-6 with cytosine arabinoside (3.25 μM), ultimately resulting in 3,500-5,000 cells/well (10%-14% overall proportion of astrocyte). Cultures underwent a single half-media change at DIV 7 (upon the addition of the optical reporter of choice depending on the experiment).

2.1.4 ReadyProbes

Cell viability assays were carried out via ReadyProbes (Life Technologies) by adding one drop of each reagent in the culture well. The cell nuclei were stained blue by the NucBlue reagent (excitation: 360nm; emission: 460nm), while cell exhibiting compromised nuclear membranes were stained green by the NucGreen reagent (excitation: 504nm; emission: 523nm). After observing 15 minutes of incubation time, individual coverlips were imaged using a Zeiss CO widefield microscope using the DAPI and FITC filters. 4-5 regions were chosen per each coverslip during imaging.

2.2 Experimental mouse models

2.2.1 Transgenic human ApoE mouse model

The ApoE transgenic mouse model employed targeted replacement (TR) of the mouse ApoE exons 2-4 with the hAPOE correspondent. hAPOE is expressed under the mouse promoter, mRNA levels being the same in various tissues (for transgene construction see Sullivan et al. 1997). Animal colony was established at the University of Sussex. The animals originated from Nobuyo Maeda (University of North Carolina, USA) under a material transfer agreement between Sarah King and Nobuyo Maeda.
2.2.2 APPSwe/Ind mouse model

The AD model used is a double transgenic mouse/human APP695 containing the Swedish (KM570/571NL) and Indiana (V617F) mutations, as well as the tetracycline transactivator (TET) creating a controllable doxycycline-responsive APP transgene expression (for transgene construction see Jankowsky et al. 2005). Mice were expressing the APPswe/ind transgene since birth and displayed higher levels of $A\beta$ protein when compared to matched controls. The animals originated from Joanna Janowsky (Baylor College of Medicine, USA) via Dr Mariana Vargas-Cabellero (University of Southampton) under a material transfer agreement between Joanna Janowsky and Louise Serpell.

2.2.3 Preparation of acute hippocampal slices

<table>
<thead>
<tr>
<th>Compound</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Monosodium Phosphate (NaH$_2$PO$_4$)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium Bicarbonate (NaHCO$_3$)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Magnesium Chloride (MgCl$_2$)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl$_2$)</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

The brain was removed and placed in a 50 ml Falcon tube containing ACSF 1X on ice (mouse genotype and age detailed in specific chapters). In a small petri dish (100 mm x 15 mm, Sigma) containing ACSF solution the brain was cut along the sagittal plane along its midline using a razor blade. After laying each hemisphere down with lobes facing up, a further cut along the horizontal plane was performed, cutting part of the cortex and the cerebellum. This provided a stable base through which the two brain hemispheres were
mounted onto a platform and prepared for vibratome sectioning. Acute transverse hippocampal slices of 300 μM in thickness were cut using a razor blade mounted on a Leica Vibratome and immediately placed in a chamber containing ACSF 1X bubbled with 95% O₂ and 5% CO₂ (Carbogen) at 37°C.

### 2.2.4 Field stimulation and Photoconversion

#### Table 2.4. Reagents used in the photoconversion experiments

<table>
<thead>
<tr>
<th>Compound</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,3’ Diaminobenzidine tetrahydrochloride (DAB)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>FM1-43 FX</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>D-AP5</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>CNQX disodium salt</td>
<td>Tocris Bioscience</td>
</tr>
<tr>
<td>Paraformaldehyde (25%)</td>
<td>Agar Scientific</td>
</tr>
<tr>
<td>Glutaraldehyde (16%)</td>
<td>Agar Scientific</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ammonium Chloride (NH₄Cl)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Osmium Tetroxide (OsO₄) (4%)</td>
<td>Agar Scientific</td>
</tr>
<tr>
<td>Potassium Ferricyanide (C₆N₆FeK₃)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Lead Nitrate (Pb(NO₃)₂)</td>
<td>Agar Scientific</td>
</tr>
<tr>
<td>Aspartic Acid (C₄H₇NO₄)</td>
<td>Agar Scientific</td>
</tr>
<tr>
<td>Sodium Cacodylate</td>
<td>Agar Scientific</td>
</tr>
<tr>
<td>Thiocarbohydrazide</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Uranyl Acetate (UA)</td>
<td>Agar Scientific</td>
</tr>
</tbody>
</table>

#### Table 2.5. Solutions used in the photoconversion experiments

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAB Solution</td>
<td>1 tablet in 10mL of H₂O (1 mg/ml)</td>
</tr>
<tr>
<td>FM1-43 FX</td>
<td>10 mM</td>
</tr>
</tbody>
</table>
Fixative Solution I | 4% Paraformaldehyde, 0.1% Glutaraldehyde in PBS 1x
---|---
Fixative Solution II | 6% gluteraldehyde, 2% paraformaldehyde in PBS 10x and H₂O
Artificial Cerebrospinal Fluid 1X (ACSF) | 125 mM NaCl, 2.5 mM KCl, 25 mM Glucose, 1.25 mM NaH₂PO₄, 26mM NaHCO₃, 1mM MgCl₂, 2mM CaCl₂. Prepared as a 10X solution. Glucose, MgCl₂ and CaCl₂ added fresh when preparing the 1X solution.
Osmium Solution | 3% Potassium Ferrocyanide, 0.3M cacodylate buffer, 4 mM CaCl₂ and 4% Osmium tetroxide

Slices were left for 30 minutes to recover and then transferred to the imaging chamber, perfused with ACSF 1X (bubbled with 95% O₂ and 5% CO₂ (Carbogen)) at the rate of 2-5 ml/min. A U-shaped harp-slice grid was laid on the slice to limit any unwanted movement during the procedure. A bipolar tungsten-stimulating electrode was placed on the CA3 region of the hippocampus while a recording electrode was located in CA1. Briefly, a tungsten wire (0.075 mm) was inserted in both cavities of a theta glass capillary (Harvard Apparatus) and each end was soldered to electric wires in order to connect the probe to the stimulator (Grass stimulator SD9, Astro-Med Inc, USA). The capillary was pulled over a Bunsen burner resulting in a stimulating probe with two tungsten wires exposed (0.2 mm). The recording electrode was produced by pulling a borosilicate capillary glass (Borosilicate Standard Wall with Filament Clark Capillary Glass, 1.5 mm, Harvard Apparatus) with a pipette puller (Narishige) in order to achieve a pipette resistance of approximately 2-4 MΩ during recordings. The recording probe was filled with ACSF 1X and FM1-43FX (20 μM). After positioning both the stimulating and recording electrodes at the aforementioned positions a single pulse at 20 Hz (1 ms) with a voltage between 1V and 2V was delivered to achieve a satisfactory evoked field excitatory postsynaptic potential (at least 0.2 mV amplitude). At this point CNQX (20 μM)
and D-AP5 (50 μM) were added to the perfusing solution in order to reduce recurrent network excitation. CNQX is a competitive α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, while D-AP5 acts as a selective N-methyl-D-aspartate (NMDA) antagonist. FM1–43FX was pressure applied (15-20 p.s.i. positive pressure) to the CA1 region for 3 minutes before applying a stimulation at 20 Hz for 1 minute at the voltage selected during the single pulse stimulation. FM dye injection was continued for 1 more minute at the end of the stimulation to allow for full dye uptake during endocytosis. At this point, FM dye loading in the synaptic terminals was confirmed using confocal fluorescent imaging. The stimulating and recording electrodes were then removed and the slice was left under perfusion with ACSF 1X and blockers (CNQX and D-AP5) for either 1 minute or 20 minutes before removing the U-shaped harp-slice grid and moving the slice into a small petri dish and adding Fixative Solution II. The hippocampal slice underwent microwave enhanced fixation for 12 seconds (700 microwave, calibrated to heat up the solution to 45-50°C after 12 seconds of irradiation) before being washed in Glycine (100 mM in PBS 1X) for 1 hour. After three quick washes in PBS 1X and a 1-minute incubation in Ammonium Chloride (100 mM in PBS 1X), the slice was transferred in a small petri dish (60 mm x 15 mm, Sigma) containing PBS 1X. The dish was then moved back to the imaging chamber and the dye injected region was relocated thanks to the mark left previously by the recording electrode (×40, numerical aperture (NA) 0.8 water-immersion objective, objective power density of ~1,500 mW/cm²). PBS was replaced by DAB solution (1 mg/ml, carbogen bubbled) and blue light (<500 nm from a 100 W mercury lamp) was shone on the target region for approximately 40 minutes. After photoconversion the slice was washed in PBS, 0.15 M Cacodylate Buffer, 0.15M Cacodylate Buffer with 2 mM CaCl₂ and then incubated for 1 hour in Osmium Solution. All of the washes were carried out on ice. Following washes with ultra pure H₂O to remove any trace of osmium, the slice was moved off ice and a filtered warm 1% thiocarbohydrazide solution was added for 20 minutes. The sample was then washed once again in ultra pure H₂O incubated in 2% osmium tetroxide for 30
minutes and finally left overnight in a cold room after washing off the osmium and adding a 1% uranyl acetate solution. After incubation in lead aspartate in a 60°C oven for 30 minutes, the slice underwent sequential dehydration steps in ethanol solutions (20%, 50%, 70%, 90% 5 minutes each, 100% two times 10 minutes each), anhydrous acetone (10 minutes on ice and then replaced with fresh acetone for 10 minutes at RT) and ultimately Durcupan resin (sequential ratios of 25%, 50% and 75% resin in acetone for 2 hours each before moving into 100% resin overnight). The following day the resin was replaced with freshly prepared Durcupan and left to infiltrate for an additional 2 hours before moving the slice in-between two aclar sheets and leaving it to polymerize in a 60°C Oven for 3 days. After complete resin polymerization the slice was removed from the aclar and the region of interest was marked using a razor blade. The resin infiltrated slice was then glued on the top face of a previously polymerized BEEM capsule (Thermofisher Scientific) resin block and left to adhere for the required amount of time. At this point the block was ready for ultra-thin sectioning.

2.3 Electron Microscopy

2.3.1 Ultra-thin Sectioning

Polymerized tissue blocks were mounted on the sample holder of a Leica EM UC7 ultramicrotome. The tissue was located in the block and an area of roughly trapezoidal shape was cut using a razor blade (Astra superior platinum double edge razor blade). In order to remove the superficial layer of resin present on the tissue a glass knife was initially used. Once the tissue was exposed ultra-thin sections of 60nm were cut using a diamond knife (Ultra Diamond Knife - Wet 45° 2.5mm). Slot copper grids (2x0.75 mm, Agar Scientific) were used if serial sections were to be analysed.

2.3.2 Imaging

Sections mounted on grids were imaged using a JEOL JEM1400-Plus Transmission Electron Microscope at 120kV with a Gatan OneView 4K CMOS digital camera.
2.4 Amyloid Beta preparation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilised AB(_{42})</td>
<td>rPeptide</td>
</tr>
<tr>
<td>Zeba 7K Molecular Weight cut-off (MWCO)</td>
<td>Thermo-Scientific</td>
</tr>
<tr>
<td>Hexafluoroisopropanol (HFIP)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide (DMSO)</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

Lyophilised A\(_{β42}\) (CAT# A-1163-02, 0.2 mg from rPeptide, USA, stored at -20\(^\circ\)C) was resuspended by adding 200 \(\mu\)L of HFIP (1mg/ml) to the vial, which was then vortexed for 1 minute before undergoing sonication for 5 minutes (50/60 Hz) in a bath sonicator. Resuspended A\(_{β}\) was then evaporated by passing low flow nitrogen gas until a transparent HFIP film was left at the bottom of the vial. At this point 200 \(\mu\)l of DMSO were added to the vial before vortexing it for 1 minute and sonicating it for 1 minute. Zeba 7K MWCO (ThermoScientific) 2ml columns were equilibrated with HEPES buffer (10mM HEPES, 50mM NaCl, 1.6mM KCl, 2mM MgCl and 3.5mM CaCl\(_2\), pH 7.4) to remove the azide from the column. 200 \(\mu\)L of A\(_{β}\) in DMSO were the added to the equilibrated columns together with 40 \(\mu\)l of HEPES buffer as a stack and centrifuged at 1000g for 2 minutes at 4\(^\circ\)C. The eluted A\(_{β}\) solution was collected in a LoBind Eppendorf tube (1.5 mL) on ice and the protein concentration was immediately measured via a NanoDrop spectrophotometer (280 nm, molar extinction coefficient of 1490 M\(^{-1}\)). A\(_{β}\) was then diluted to 50 \(\mu\)M and left at RT for 2 hours before adding it to the primary cultures (derived from wt rat or ApoE-TR mice, as per protocol described in chapter 2.1). An electron
microscopy grid was also prepared to be imaged to ensure that the peptide added was in the desired oligomeric conformation.

## 2.5 Fluorescence imaging

### 2.5.1 Viral infection

<table>
<thead>
<tr>
<th>Insert</th>
<th>Vector</th>
<th>MOI</th>
<th>DIV infection</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSyn1:SypHy1x</td>
<td>AAV6</td>
<td>200</td>
<td>6/7</td>
<td>University of Helsinki</td>
</tr>
<tr>
<td>pCAG:iGluSnFr</td>
<td>AAV9</td>
<td>1 µL x 1 in 10 dilution (titre unavailable)</td>
<td>6/7</td>
<td>Penn Vector</td>
</tr>
</tbody>
</table>

Adeno Associated Virus (AAV) is a method for gene delivery into postmitotic cells. They were found to enter cells 30 min after infection via clathrin mediated endocytosis and reach the nucleus after 3-4h (Bartlett et al., 2000). AAV6 and AAV9 were used. While AAV6 has been demonstrated to have better neuronal specificity, AAV9 has been shown to have comparable infection rates (Royo et al., 2008).

### 2.5.2 Imaging system

Imaging experiments were performed using an upright microscope BX61WI with a x60 1.0 NA dipping objective and mercury lamp light source. A cooled electron multiplying charge-coupled device camera (EMCCD, Andor Ixon) was used. Emission and excitation filters employed were 480/20 and 520/35, respectively. The room temperature was kept at 23°C during experiments. Binning 4x4, readout speed 13,000 MHz, cooling temperature -77°C.
2.5.3 Field stimulation

A custom-built chamber was used in order to induce field stimulation during recording. Briefly, a glass coverslip was positioned at the bottom of a round glass chamber with two electrodes (platinum wire, 0.5 mm diameter) placed in parallel at ~1 cm distance from each other, connected to a Grass stimulator. Stimulation was delivered using custom-written Micromanager scripts and calibration was performed by altering stimulation frames in relation to the pulses delivered. The number of pulses were initially monitored using an oscilloscope in order to optimize a satisfactory frame interval depending on the pulses desired. From now on when we refer to action potentials (APs) delivered to the culture we mean to denote the number of pulses imparted upon stimulation. Coverslips were transferred from culture media to pre-warmed extracellular bath solution (EBS) in the stimulation chamber. CNQX (20 μM) and AP5 (50 μM) were added to the EBS in order to reduce recurrent network excitation.

2.6 Image Analysis

2.6.1 Electron Micrographs

Images were collected at a magnification of 20,000x, yielding micrographs with a total area of 2.32 μm². We found this to be a suitable magnification resulting in a synaptic terminal per micrograph where synaptic vesicles were adequately distinguishable. In order to carry out the vesicle counting analysis, images were initially imported into Xara (Xara Photo & Graphic Designer v9.2) and the following characteristics were located in each micrograph: active zone, centre of the active zone, empty vesicles (PC-), electron-dense photoconverted vesicles (PC+) and docked vesicles. The latter is a debated topic in the literature, as they are sometimes defined as those vesicles that are in contact with the active zone, while others describe them as the first line of vesicles closer to the release area. Here we arbitrarily decided to count as docked those vesicles whose distance to the active zone is equal to or less than the average diameter of a single vesicle (~40 nm). Images were selected based on the visible presence of an electron
dense post-synaptic density; thus, if an active zone was not clearly distinguishable the micrograph was not taken into consideration for the analysis. Another factor that was taken into consideration was the total amount of vesicles present in the synapse; as synapses of different pool size. It was also crucial to make sure that the synapses analysed underwent vesicle release upon the stimulation provided and did not simply present photoconverted vesicles due to spontaneous release. A baseline of at least 2% electron-dense vesicles was set in order to include the synapse into the analysis. This value was derived from the analysis of pre-synaptic terminals which did not undergo any stimulation prior to photoconversion. Images that satisfied all the aforementioned criteria underwent manual counting of both PC+ and PC- vesicles. After the active zone, the centre of the latter and the docked pool were also localized, the images were exported in a suitable format to be further analysed in Matlab. Here vesicles were automatically counted, and the resulting numbers were double checked against the ones previously obtained. The script, developed by Professor Kevin Staras, also allowed us to extract the absolute distances between each vesicle and the centre of the active zone for all the terminals analysed. Finally, we were able to compute a normalized heat map based on the positioning of PC+ and PC- vesicles in the pre-synaptic compartment.

2.6.2 Fluorescent images

Image stacks were analysed via Igor Pro (ver. 7.0, WaveMetrics) through the use of the SARFIA plugin (Dorostkar, Dreosti, Odermatt, & Lagnado, 2010). Data was stored as single-precision point arrays, termed “waves” on Igor Pro.

2.6.2.1 ROI detection for sypHy

Following image transformation using the Laplace operator, individual ROIs were localized using a threshold calculated as a multiple of the standard deviation (3xSD) was found to be suitable for our experiments) of all the pixel values. This resulted in the formation of the ROI mask, which underwent background fluorescent subtraction to correct for different background fluorescent levels. In the case of sequential recordings,
ROI masks were based upon the first z-stack and applied to the following images. To allow for comparison between different baseline intensities, ΔF/f values were calculated (10 frames prior to stimulation were used as baseline). ROIs had to reach a determined threshold following the fluorescent rise post-stimulation (threshold value was reporter-dependent). ROIs that did not satisfy our criteria were dropped from the analysis.

2.6.2.2 ROI detection for iGluSnFr

Due to the nature of the construct, ROI localization through the use of SD could not be employed for image-stacks from iGluSnFr experiments. ROIs localization was carried out using a customized correlation algorithm written by Professor Tom Baden. After linearly correlating each pixel in the field of view with each other, adjacent pixels with an arbitrarily chosen correlation score of >0.2 were grouped in a single ROI. Correlation was halted when a single ROI reached a total pixel number larger than 10 pixels, or when the correlation score failed to meet the threshold.
CHAPTER 3: THE EFFECTS OF Aβ1-42 ON SYNAPTIC TRANSMISSION

3.1 Introduction

3.1.1 Aβ1-42 and synaptic transmission

The accumulation of Aβ1-42, first in its oligomeric form, and ultimately in diffused extracellular plaques, has been shown to have a pathological effect on a plethora of mechanisms and pathways involved in the physiological synaptic activity. While originally considered the primary toxic element in AD, amyloid plaque load has been shown not to coincide with the severity of cognitive dysfunction, as cognitive deficits can be observed long before plaque deposition (McLean et al., 1999), shifting the focus on the much less understood oligomeric Aβ species. It has been shown how oligomeric Aβ1-42 is capable of promoting oxidative stress (Forloni, 1993) and apoptosis (Loo et al., 1993), as well as changes in the intracellular Ca²⁺ levels and enhanced glutamate toxicity (Mattson et al., 1992). In addition, Aβ1-42 has been shown to exert a post-synaptic mediated effect on plasticity through the disruption of long-term potentiation (LTP) (Dominic M Walsh et al., 2002) as well as the facilitation of long-term depression (LTD) (Y. Li et al., 2017).

On the other hand, presynaptic Aβ1-42 dependent effects have been less thoroughly investigated. Aβ localized in the presynaptic compartment has been suggested to interact with the scaffolding apparatus controlling vesicle trafficking (Russell et al., 2012; Salomone, Caraci, Leggio, Fedotova, & Drago, 2012). Results from animal studies showed an inhibitory effect on vesicle exocytosis under the presence of Aβ oligomers (Moreno et al., 2009). Aβ has also been shown to interact with the correct functioning of the SNARE complex, which is involved in synaptic vesicle fusion at the active zone. Interestingly, no variation was identified when comparing the total vesicle docking in the presence or absence of Aβ oligomers (Y. Yang et al., 2015). Experimental evidence
using primary rat hippocampal cultures showed that while soluble Aβ1-42 oligomeric species appear to have an effect in vesicle endocytosis by slowing down the process, no effect on exocytosis was reported (J. Park, Jang, & Chang, 2013). Low concentrations of soluble oligomeric Aβ coupled with vGlut–pHluorin reporter shows a reduction in the functional recycling pool. Furthermore, no change in the total number of vesicles in the terminal was witnessed (J. Park et al., 2013). Following from this, Aβ has been shown to impact pools organization, although the underlying mechanism remains unknown.

Now, as we discussed in chapter 1.4, a great variety of variables need to be taken into considerations when trying to explore the role of Aβ1-42 in synaptic pathology; Park and colleagues (2013) carried out their procedures using a stimulation as high as 600 APs (20 Hz) which, as we now know is more prone to elicit a specific mode of exocytosis followed by an endocytic mechanisms such as bulk-mediated endocytosis. On the other hand, other methods of stimulation might employ the use of solutions such as high K+ (Kelly & Ferreira, 2007), which wouldn’t allow for precise experimental control. Finally, another crucial variable is the added peptide itself. In fact, what is Aβ1-42’s conformation that we should take into consideration and how does it differ in affecting the SVC? It is possible to distinguish three main pools of Aβ1-42: monomers, oligomers and fibrils. Soluble oligomers encompass a range of assemblies such as dimers, trimers, tetramers and so on. As previously introduced, the developing AD brain presents a variety of soluble Aβ1-42 oligomeric species, which correlate with disease progression and cognitive decline (D M Walsh et al., 1999). On the other hand, amyloid plaque load doesn’t appear to have a direct relationship with synapse failure and neuronal dysfunction, as cognitive deficits can be observed long before plaque deposition. Thus, the synthesis and application of a constant, reproducible and pathologically relevant peptide is of key importance. The preparation of oligomeric Aβ1-42 used throughout our experiments has been well characterized (Marshall et al., 2016), both in terms of its oligomeric structure and its behaviour in a culture environment.
These mechanical and structural observations we discussed so far regarding the effects of \( \text{A} \beta 1-42 \) on the SVC and overall fitness of the pre-synaptic terminal have, of course, functional repercussions in terms of neurotransmitter homeostasis. In particular, glutamatergic transmission has been reported to be pathologically altered in AD.

### 3.1.2 Glutamate homeostasis and \( \text{A} \beta 1-42 \)

Glutamate is a nonessential amino acid constituting the primary medium of transmission for the major part of excitatory neurons in the CNS. Due to its inability to be transported to the brain, as it cannot cross the BBB, glutamate is primarily produced in neurons and glial cells. When presynaptic terminals undergo \( \text{Ca}^{2+} \)-mediated exocytosis, the glutamate released in the synaptic cleft directly interacts with glutamate-receptors located on the post-synaptic plasma membrane, belonging to either ionotropic (iGluR) or metabotropic glutamate receptor families (mGluR). The NMDA receptors belong to the iGluR family and together with AMPA receptors they are key components in synaptic plasticity, learning and memory processes (Sanz-Clemente, Nicoll, & Roche, 2013). Non physiological levels of glutamate in the synaptic cleft have a detrimental post-synaptic effect on the glutamate receptors and ultimately lead to neuronal death (Karki et al., 2018).

Because of its crucial effects on signalling and potential disruptive results if left unmonitored (Ottersen, Laake, Reichelt, Haug, & Torp, 1996; Wenk, Parsons, & Danysz, 2006), glutamate is readily removed and cleared from the cleft by selected reuptake transporters known as EAAT localised along the astrocytic membrane. Upon uptake, glutamate is converted into L-Glutamine through glutamine synthase, which in turn is taken up by neuronal cells and recycled to form L-glutamate (Shen, Rothman, Behar, & Xu, 2009; Walton & Dodd, 2007). Although this constitutes the primary route through which glutamate is removed from the synaptic cleft, selective glutamate transporters are localized along both glial and neural cells, where glutamate can undergo uptake and be repurposed, as well as to be reused as a neurotransmitter (Walton & Dodd, 2007).
Glutamate refilling of synaptic vesicles occurs through the Cl-dependent activity of vesicular glutamate transporters known as vGluTs (Kalariti, Pissimissis, & Koutsilieris, 2005).

It has been shown how deterioration of glutamatergic transmission is correlated with cognitive deficits and AD progression (Revett, Baker, Jhamandas, & Kar, 2013). In this regard, Aβ1-42 has been implicated in specific steps of the process ranging from physiological glutamine synthase activity (Aksenov, Aksenova, Carney, & Butterfield, 1997) to post-synaptic NMDA blockage relief. However, it still remains unclear how mechanisms are being modified at the pre-synaptic level in terms of vesicle release, turnover and overall terminal homeostasis.
3.2 Treatment with Aβ1-42 for 24 hours affects the kinetics of vesicle endocytosis

Previous experiments carried out in the lab in a similar culture preparation and using the genetically encoded reporter sypHy2x provided us with a solid starting point when it came to deepen our understanding regarding Aβ1-42 dependent effects on vesicle activity. Neurons were infected with the AAV based sypHy1x construct at DIV 4-6 and were imaged at DIV 14-17. Cultures were incubated with 10μM oligomeric Aβ1-42 (concentration calculated at 280nm via nanodrop) or buffer for 24h before imaging with a stimulation of 40 APs (20 Hz) (Fig 3.1. Experiments and data analysis carried out by Dr M.M. Fowler). After normalizing each response to the end of the stimulation frames it is noticeable how Aβ1-42 treated cultures display a decrease in the rate of endocytosis of released vesicles by approximately ~21% when compared to buffer-treated controls (Fig 3.1a.i and Fig 3.1a.ii for heatplots from controls and Aβ1-42-treated cultures, respectively. Fig 3.1b.i and Fig 3.1b.ii display individual exemplar traces). When response amplitudes were divided according to low range and high range, it was clear that while for small responses the Aβ1-42 treated cultures displayed a ~13% decrease in recycling kinetics, the deficit was even more pronounced for large ones (45%) (Fig 3.1c). When comparing responses in between conditions, larger responses from Aβ1-42 treated neurons showed the aforementioned kinetic deficit even when compared to low range Aβ1-42 treated ones.

Overall, this data shows that the endocytic machinery is more affected by Aβ1-42 during larger responses, suggesting a possible mode of impairment driven by Aβ1-42.
Figure 3.1. 24h Aβ1-42 incubation in primary hippocampal cultures leads to a response-dependent impairment in the kinetics of vesicle retrieval and re-acidification. (a.i-a.ii) Heat-plots representing individual responses derived from cultures treated for 24h with either 10μM oligomeric Aβ1-42 (a.ii) or 10μM vAβ1-42 as control (a.ii; vAβ1-42 structure differs from Aβ1-42 by two amino acids, it does not self-assemble and is not toxic to cells, (Marshall et al., 2016)). (b.i-b.ii) Individual exemplar response traces from control (b.i) and Aβ1-42 (b.ii) terminals. (c) Average traces divided by response amplitude, displaying a response-dependent trend in the Aβ1-42 driven kinetic deficit (experiments and data analysis carried out by Dr M.M. Fowler).

3.3 The effect of Aβ1-42 on glutamate release and clearance

The striking impairment in vesicle turn over prompted us to further explore the downstream consequences in terms of the functional aspect of neurotransmitter signalling. In this section we sought to investigate the changes to glutamate release and clearance in the presence of Aβ1-42 by using the optical probe iGluSnFr.

3.3.1 Incubation Aβ1-42 over 24h leads to a decrease in glutamate release and slower clearance kinetics

Cultures were treated with 5μM oligomeric Aβ1-42 or HEPES based buffer (used in the peptide preparation, refer to section 2.4) for 24 h before imaging. 5μM was chosen as our experimental concentration on the basis of previous experiments carried out by Dr Karen Marshall (Marshall et al., 2016) on Aβ1-42 toxicity, showing uptake of the peptide after 2h-12h incubation. The initial protocol devised to assess glutamate release and
clearance followed up from the experiments discussed in section 3.2. However, due to
the nature of the glutamate reporter employed, adjustments had to be made to the
frequency of stimulation in order to avoid probe over-saturation and gain as much
information as possible from the recording (Helassa et al., 2018; Taschenberger,
Woehler, & Neher, 2016). Coverslips were mounted on our custom-built chamber and
incubated in EBS (see chapter 2) supplemented with AP5 and CNQX. An initial stimulus
consisting of 4-5 APs (10 Hz) was used to localize responsive regions. After 1 minute,
neurons were stimulated with 40 APs (10Hz) for 5 consecutive rounds, with a 3.5s
interval in between stimulation (Fig 3.2a). Resulting images were analysed using
IgorPro.7. The initial 4 APs (10Hz) stimulation response was used to create the ROI
mask applied to the following responses. As it is evident from the 1st trial (Fig 3.2b-e),
 glutamate release (quantified as Area Under the Curve, AUC) in neurons subjected to
5μM Aβ1-42 treatment was markedly reduced throughout the 5 stimulation rounds (data
from 4 coverslips. Two-tailed unpaired t-test, p<0.0001) when compared to buffer-treated
controls (data from 2 coverslips). In order to make sure these changes were not driven
by the abnormal activity of individual synapses, we normalized each trace to the average
amplitude of the control condition’s 1st trial. As shown in Fig 3.2c-f, the trend was
maintained. Normalized AUC shows how the rate of decline (mean ± s.e.m , 22.08 ±
0.24%; n=1756) in Aβ1-42-treated neurons from the 1st to the 2nd trial is significantly
higher (p<0.0001), more than double the one recorded in controls (10.11 ± 0.35%;
n=1280). Interestingly, the average percentage decay per round appears to remain
rather constant in Aβ1-42-treated neurons (22.8 ± 0.36%), with no statistical significance
in between trials (Fig 3.2h-i), while controls display a steady increase in decay over the
course of the 5 rounds (~3%). During the 5th round of stimulation, Aβ1-42-treated values
(23.13 ± 0.56%) remained markedly higher than buffer-treated control ones (19.95 ±
0.42%). Normalized traces (Fig 3.3b.i-c.i) and AUC values (Fig 3.3b.ii-c.ii) display a
marked difference in buffer-treated and Aβ1-42-treated cultures (Fig 3.3d-e).
Figure 3.2. Changes in glutamate release in primary hippocampal culture after 24h 5μM Aβ1-42 incubation. (a) Experimental design consisting of an initial low-intensity stimulus of 4-5 APs (10Hz), followed by 60 s rest and five imaging rounds of 40 APs (10Hz). (b) Average traces of buffer treated (black) and 5μM Aβ1-42-treated (red) cultures. (c) Previously depicted traces whose fluorescent intensity was normalized to the control trace’s end of stimulation frames. (d) Heat-map representing individual area under the curve (AUC) values per round of stimulation for the buffer treated condition synapses, self-normalized to the first trial response. (e) AUC values derived from the five stimulation trials of 5μM Aβ1-42 (red) and control terminals (black); a significant difference was reported between the two conditions across all rounds (two-tailed unpaired t-test, p<0.0001). (f) AUC values normalized to the control trace’s value from the first stimulation trial. (g) Heat-map displaying individual AUC values from Aβ1-42-treated terminals responses; data normalized to the first trial response. (h) Graph representing the percentage in decay per individual rounds of glutamate release for Aβ1-42-treated (red) and control cultures (black). The decay in release was significantly different between the two treatment conditions in each round (two-tailed unpaired t-test, p<0.0001). (i) Percentage in the decay of glutamate release expressed as an average across the five stimulation rounds. The difference between Aβ1-42 (red) and buffer treated (black) neurons was reported to be highly significant (two-tailed unpaired t-test, p<0.0001).
Figure 3.3. Normalized average traces and AUC values for Aβ1-42 and buffer-treated cultures. (a.i-a.ii) Heat-plots representing response intensity of Aβ1-42-treated (a.ii) and control terminals (a.i), normalized to the average response peak of the first stimulation trial. (b.i-b.ii) Average traces for buffer (b.i) and Aβ1-42-treated (b.ii) neurons normalized to the average peak response of the first round of stimulation. (c.i-c.ii) Average AUC values separated per condition normalized to the first round release value. (d) Graph superimposing the individual self-normalized traces from Aβ1-42 (red) and control (black) terminals. (e) Normalized AUC for each condition compared, reporting a significant difference per individual stimulation round (two-tailed unpaired t-test, p<0.0001).

Even though a significant difference was maintained between the two conditions throughout the 5 trials, buffer-treated values did report an increasing trend as the rounds progressed, steadily approaching the Aβ1-42 AUC results. This seems to suggest that the drop in glutamate release due to sustained stimulation seen after ~200 APs in the control takes place after only ~40 APs in the Aβ1-42-treated neurons. We therefore decided to investigate whether Aβ1-42 had any effect on glutamate clearance. Single
traces for each condition were normalized to the end of the stimulation frames to allow for glutamate clearance comparison. Average normalized traces for the 1st and 5th rounds are displayed in Fig 3.4a.i-a.ii. Single exponential curves were fitted to the normalized traces, allowing for tau values comparison (Fig 3.4b.i-b.ii). Incubation of Aβ1-42 led to a decrease in the rate of neurotransmitter clearance of 30% after the 1st round of stimulation (40 APs, 10Hz) when compared to buffer-treated neurons. After the 5th trial, Aβ1-42-treated neurons still maintained a significantly higher clearance time. Interestingly, from the 1st to the 5th round the rate of clearance in control cultures decreased by ~46%, while Aβ1-42-treated neurons saw only a ~38% decrease (Fig 3.4).

**Figure 3.4.** Clearance rate analysis of glutamate in Aβ1-42 and buffer-treated cultures. (a.i-a.ii) Response traces normalized to the end of stimulation frames, representing the signal decay of Aβ1-42 (red) and control (black) cultures following stimulation; traces corresponding to round 1 (a.i) and round 5 (a.ii). (b.i-b.ii) Tau values from curve fit for Aβ1-42-treated (red) and control (black) (p<0.0001).
The experimental design employed so far had the limitation of testing neuronal responses to an isolated external stimulation, without any sort of previous background activity being present in the system. As it has been widely reported in the literature, neuronal cells in a physiological environment generate a variety of spontaneous activity patterns, and previous signalling activity has a direct effect on how the neuron will respond at any given moment (Mizuseki & Buzsaki, 2013). Furthermore, even though our stimulation consisting of 40 APs (10 Hz) is aimed at the recruitment of the RRP at the pre-synaptic terminal commonly used when working in a culture system, in a more physiological environment the recorded signalling activity on average doesn’t usually go above ~1/2 Hz (Mizuseki & Buzsaki, 2013). Finally, different modalities of vesicle recycling are employed by the cell depending upon the level of stimulation received. As our previous results suggested an impact by Aβ1-42 on the endocytic machinery, we decided to pre-stimulate both our peptide-treated and control preparation with a set stimulation in order to evoke a baseline activity prior to our recordings. This had the advantages of (i) adjusting our model to mimic what might be taking place in vivo, (ii) providing a functional mechanisms of action to our peptide by creating a continuous cycle of vesicle release and retrieval at a physiological rate and (iii) allowing us to localize a deficit onset point during a gradual increase in stimulation.

A stimulus consisting of 4 APs (10 Hz) was once again initially used to find a responsive region in the coverslip. After that, neurons were stimulated for 5 minutes at ~1 Hz, with 10 APs delivered over 5 seconds followed by 5 seconds of rest, for a total of ~300 APs (1 Hz). After the 5 minutes of baseline stimulation, 30 seconds were observed before beginning the experiment. The protocol consisted of 10 rounds of a 10 APs (10 Hz) stimulation, followed by a set of 2 trials of 40 APs (10 Hz) and 1 round of 60 APs (10 Hz). Each of the first 10 trials had an inter-stimulus interval (i.s.i.) of 3s while the second set of 2 trials had an i.s.i. of 3.5s. The i.s.i. between the 10th trial of 10 APs (10 Hz) and the 1st trial of 40 APs (10 Hz), as well as between the last round of 40 APs (10 Hz) and the
1st at 60 APs (10 Hz), was 13s (Fig 3.5a). Surprisingly, glutamate release (AUC) during the 1st trial of 10 APs after the induced baseline showed no significant difference between the Aβ1-42 treated cultures (data from 4 coverslips) compared to buffer controls (data from 3 coverslips. Fig 3.5c.i). However, a drop of ~20% was observed during the 2nd round in Aβ1-42 treated neurons, while only ~10% was reported in controls (Fig 3.5c.i).

Relative decay in the average release was found to be 9.40% (s.e.m. 0.34%; n=824) for untreated neurons, while it was more than double in Aβ1-42 neurons (18.67 ± 0.29%; n=928) (Fig 3.5c.i). The change in the decay rate was evident when both AUC traces were each normalized to their 1st trial average (Fig 3.6 for the first 10 rounds of stimulation, Fig 3.7 for the two rounds of 40 APs and the single round of 60 APs). The decrease in glutamate release found in Aβ1-42 treated neurons was maintained throughout the 10 rounds of 10 APs (10Hz) (Fig 3.6), with an average decay rate per round of 10% (s.e.m. 0.42%), compared to 8% (s.e.m. 0.45%) in controls (two-tailed unpaired t-test, p<0.0001), as shown in Fig 3.5e. The same trend in terms of decreased release was observed during the following 2 trials of 40 APs (10Hz) (p<0.0001) and single trial of 60 APs (10Hz) (p<0.0001) (Fig 3.5c.ii and Fig 3.5c.iii, respectively. Fig 3.7 for details). However, no significant difference was found in the rate of decay per round during the 40 APs (10Hz) stimulation trials (Fig 3.5f).
Figure 3.5. The effect of previously induced low-level baseline activity on glutamate release. (a) Diagram depicting the protocol used. (b.i-b.iii) Average response traces from Aβ1-42 (red) and control synapses (black) during the trials of 10 APs (Hz) (b.i), 40 APs (10Hz) (b.ii) and 60 APs (10Hz) (b.iii). (c.i-c.iii) Average AUC values representing neurotransmitter release during the trials of 10 APs (Hz) (c.i) (two-tailed unpaired t-test, p<0.0001), 40 APs (10Hz) (c.ii) (p<0.0001) and 60 APs (10Hz) (c.iii) (p<0.0001). (d.i-d.iii) AUC values normalized to the buffer treated first response of the initial stimulation trial of 10 APs (10 Hz). (e-f) Average percentage decay in glutamate release during the 10 APs (10Hz) stimulation rounds (p<0.0001) and 40 APs (Hz) trials (n.s.).
Figure 3.6. Normalized traces and AUC values reported from the initial ten trials of 10 APs (10Hz). (a.i-a.ii) Heatplots depicting individual responses from Aβ1-42 (a.i) and control terminals (a.ii), self-normalized to the average peak reported during the first trial of 10 APs (Hz). (b.i-b.ii) Average traces from Aβ1-42 (b.i) and buffer-treated controls (b.ii) normalized to the average response of the first stimulation round. (c.i-c.ii) Self-normalized AUC values to the initial response from Aβ1-42-treated (c.i) and control (c.ii). (d) Graph representing superimposed average traces from each condition. (e) Normalized AUC values compared per individual stimulation rounds (two-tailed unpaired t-test, p<0.0001).
Figure 3.7. Normalized responses from the 40 APs and 60 APs stimulation rounds. (a.i-a.ii) Heat-plots of the responses derived from the two rounds of 40 APs (10Hz) (Buffer-treated and Aβ1-42, respectively) and (b.i-b.ii) from the single round of 60 APs (10Hz) (Buffer-treated and Aβ1-42, respectively), normalized to the initial average response of the first trial of 10 APs (10Hz). (c.i-c.ii) Normalized traces relative to the responses from the 40 APs (Buffer-treated and Aβ1-42, c.i-c.ii) and 60 APs stimulation trials (Buffer-treated and Aβ1-42, e.i-e.ii), with their respective normalized AUC values (d.i-d.ii, f.i-f.ii). (g-h) Superimposed normalized AUC values for buffer-treated and Aβ1-42 terminals derived from the 40 APs (g) and 60 APs (h) trials (two-tailed unpaired t-test, p<0.0001).

At this point it is possible to compare the Aβ1-42-treated cultures response to 2 rounds of 40 APs stimulation without any previously induced activity and after 340s of ~400 APs at an average of 5.5 Hz. Previous activity led to Aβ1-42-treated neurons displaying a
decrease in the decay of glutamate release between the 1st and 2nd round of ~3% (Fig 3.8a). Clearance rates analysis showed no significant difference between tau values of Aβ1-42-treated neurons with and without being exposed to previous stimulation (Fig 3.8b.i-b.ii).

Figure 3.8. Presence of a baseline activity prior to recording displays an increase in the average decay of glutamate release per round of stimulation. (a) Graph representing the average decay in glutamate release between the 1st and 2nd rounds of 40APs (10Hz) stimulation in cultures incubated with Aβ1-42 for 24h, with or without an additional baseline stimulation prior to imaging (two-tailed unpaired t-test, p<0.0001). (b.i-b.ii) Normalized traces decay for the 1st rounds of 40APs (10Hz) stimulation in cultures incubated with 5μM Aβ1-42 for 24h, with or without an additional baseline stimulation (b.i) and tau scores reporting no significant difference between glutamate clearance times (b.ii).

Taken together, clearance rates of glutamate were not faster after baseline for Aβ1-42 neurons. If anything, clearance rates were unaffected in Aβ1-42 treated neurons. Secondly, Aβ1-42 cultures not only appeared to be relatively unaffected by the addition of a baseline stimulus in terms of decay per round of glutamate release, they actually showed a better trend in maintaining glutamate release over subsequent stimulation when a baseline stimulus was applied. Finally, prolonged stimulation at ~1Hz was not sufficient to incur in the Aβ1-42 driven deficit witnessed without baseline, while a single trial of 10 APs (10Hz) brought about a marked increase in neurotransmitter decay per round.
3.3.2 Deficits in retrieval kinetics were maintained upon long term incubation of 1μM Aβ1-42

The experiments presented so far were carried out after Aβ1-42 incubation at a concentration of 5μM for 24 hours. Based on previous research (Marshall et al., 2016) during this time the peptide had been internalized in the neurons after 2h-12h. However, the response we recorded from the incubated cultures can be considered an acute response to the exogenous peptide. In a more physiological environment, the generation of Aβ1-42, followed by its oligomerization, fibrillation and deposition in plaques occurs over a prolonged period of time. Neuronal activity and communication had the time to adapt and respond to the deficits that Aβ1-42 formation might have brought upon the physiological network. Hippocampal synapses are known to undergo homeostatic adjustments when their transmission properties change, presumably to restore synaptic efficacy towards a set-point value (Gan & Watanabe, 2018). Such adaptive responses have been elegantly demonstrated in examples of chronic synaptic silencing using pharmacological blockers, leading to a significant enlargement of functional vesicle pool sizes (S. H. Kim & Ryan, 2010; Murthy, Schikorski, Stevens, & Zhu, 2001). We hypothesized that the signaling impairments we observe with 24h Aβ1-42 treatment might similarly trigger adaptive responses that would be observable in the longer-term, providing clues as to how synapses are affected in chronic disease. To investigate the longer-term effects of Aβ1-42 incubation in a model culture and its adaptation over an extended period of time we treated the neurons over the course of 96h-120h rather than 24h. In order to do so we had to make sure neurons would survive a longer period of time and still maintain a functional level of activity (Marshall et al., 2016), so that changes in iGluSnFr responses were attributable to the presence of the peptide rather than cell death and decreased viability.
The protocol used to investigate the long-term effect of Aβ1-42 incubation on the kinetics of vesicle retrieval was the same as described in section 3.2, employing the optical reporter sypHy. Cultures were incubated with 1 μM Aβ1-42 for 72h before imaging with a stimulation of 40 APs (20Hz). Traces were normalized to the end of the stimulation frames in order to once again compare the rate of vesicle endocytosis. One-phase decay curves were fitted to the average response traces and Tau scores were compared to elucidate differences in the turned over vesicle rate (Fig. 3.9a). A decrease of ~47% in vesicle retrieval times was found in Aβ1-42 (data from 2 coverslips) synapses when compared to time-matched controls (data from 2 coverslips. Two-tailed unpaired t-test, p<0.0001) (Fig. 3.9b).

Figure 3.9. Aβ1-42 treated cultures maintain a decrease in the rate of endocytosis of released vesicles. (a) Average traces derived from 40 APs (20Hz) stimulation of Aβ1-42 (red) and buffer treated (black) cultures, normalized to the end of the stimulation frames. (b) Normalized tau scores reporting a significant decrease in vesicle turn over times in Aβ1-42 treated terminals (two-tailed unpaired t-test, p<0.0001). (Primary data collected by Dr Arjuna Ratnayaka, analysed by Luca Biasetti).
3.3.3 Long term incubation of 1μM Aβ1-42 exacerbates deficits in glutamate release and clearance

The protocol used was the same as previously described in 3.3.1. Glutamate release was assessed in cultured neurons treated with 1μM Aβ1-42 for 96h-120h and buffer treated controls as AUC during stimulation interval frames. After baseline stimulation, Aβ1-42-treated neurons (data from 4 coverslips) displayed a marked decrease in neurotransmitter release during the 1st round of 10 APs (10 Hz), 68% of the amount released in controls (data from 4 coverslips, two-tailed unpaired t-test, p<0.0001) (Fig 3.10c.i). Average release in Aβ1-42 treated neurons was consistently lower throughout the 10 stimulation rounds (Fig 3.10b.i-c.i). This trend was also maintained in the 2 subsequent trials of 40 APs (10Hz) (p<0.0001) (Fig 3.10c.ii), and 1 of 60 APs (10Hz) (p<0.0001) (Fig 3.10c.iii). Average decay in glutamate release per round during the 10 trials of 10 APs was found to be higher in Aβ1-42-treated cultures (mean ± s.e.m, 8.97 ± 0.64%; n=570), compared to buffer treated neurons (7.54 ± 0.39%; n=845) (p<0.0001) (Fig 3.10d). No significant difference in decay per round was found in the 2 trials of 40 APs in controls and Aβ1-42-treated terminals (14.18 ± 0.66% and 15.68 ± 1.26%, respectively) (Fig 3.10e).
Figure 3.10. Long-term incubation of 1μM Aβ1-42 exacerbates deficits in glutamate release. (a.i-a.ii) Heat-plots depicting individual responses of Aβ1-42 (a.ii) and control terminals (a.i) during the 10 Aps (10Hz), 40 Aps (10Hz) and 60 Aps (10Hz) stimulation rounds. (b.i-b.iii) Average traces of Aβ1-42 (red) and control cultures (black) for the 10 Aps (10Hz) (b.i), 40 Aps (10Hz) (b.ii) and 60 Aps (10Hz) (b.iii) stimulation rounds. (c.i-c.iii) AUC values during each stimulation set, reporting a notably lower glutamate release by Aβ1-42 throughout (two-tailed unpaired t-test, p<0.0001). (d) Average decay in glutamate release during the first ten 10 Aps (10Hz) trials, Aβ1-42-treated terminals displayed a markedly higher decay in glutamate release per round compared to controls (p<0.0001). (e) No significant difference was found between the average decay in neurotransmitter release during the two 40 Aps (10Hz).
Glutamate clearance was slower in treated neurons from the 1st to the 10th round of 10 APs (10 Hz) (p<0.0001), as shown by the data normalized to the end of the stimulation frames (Fig 3.11.a.i-a.ii, respectively). Aβ1-42 treated neurons showed an increase in clearance times of ~30% compared to controls during the 1st round, which was interestingly reduced to ~27% in the final 10th round. Slower clearance kinetics were still present in the 1st trial of 40 APs (~23%) (p<0.0001) (Fig 3.11b.i), while the difference was not significant anymore for the following rounds of stimulation (Fig 3.11b.ii-c).

Figure 3.11. Glutamate clearance is impaired upon long-term incubation with Aβ1-42. (a.i-a.ii) Clearance times were significantly increased by Aβ1-42 incubation during the 1st and 10th stimulation rounds of 10 APs (10Hz); (b.i-b.ii) the trend was maintained during the 1st round of 40 APs (10Hz) but not during the 2nd. (c) No significant difference was reported during the 60 APs (10Hz) trial in terms of glutamate clearance.
Thus, longer incubation with a lower Aβ1-42 concentration led to a decline in neurotransmitter release since the 1st round of 10 APs (~98% of control release at 24h, Fig 3.5, ~68% at 4d, Fig 3.10, respectively). However, it also led to a decrease in the average glutamate release per round during the first 10 trials (Fig. 3.10c.i-c.ii-c.iii), as well as a decrease in the average decay per trial (Fig 3.10d-e). Glutamate clearance was also markedly impaired (Fig 3.11).

3.4 Long-term Aβ1-42 incubation brings about changes in the size of the functional vesicle pool

The data presented in the last paragraph aimed at investigating homeostatic adaptive changes in regard to neurotransmitter release and clearance upon long-term Aβ1-42 incubation, and how this may compare to an early acute response. The stimuli used in the protocols so far were focused on gaining information on the system’s ability to maintain functional neurotransmitter transmission in the presence of Aβ1-42.

Next, we were interested in testing how the overall capability of vesicular release in the system may be affected in the long-term upon Aβ1-42 incubation. To investigate this idea, we used the ability of Bafilomycin A1 to act as a blocker of vesicle reacidification by inhibiting v-ATPase activity, effectively trapping them in an alkaline state after stimulation-induced release. By subsequently adding NH₄Cl, we prompted the release of the whole vesicle population present in the terminal, reporting a readout of the TP.

A 40APs (20Hz) stimulus was used to identify a responsive region in cultures infected with sypHy2x and incubated with Aβ1-42 1μM for 40h. After observing 2 minutes rest, neurons were incubated for 1 minute with 1μM Bafilomicyn prior to stimulation with a saturating stimulus of 600 APs (20Hz) aimed at the release of the TrP. Following the end of the stimulation protocol, cultures were exposed to EBS containing NH₄Cl, allowing for the neutralization of the pH of the remaining vesicle pool in the terminal. At 40h, buffer-
treated synapses had a total functional pool size of ~37% (data from 2 coverslips) while 1 μM Aβ1-42-treated neurons were, on average, higher 43% (data from 3 coverslips. Fig 3.12a.i-b.i). However, these results were not statistically significant.

We decided to prolong the peptide incubation time to 96-120h. Cultures were once again tested for a response positive region by using a 40APs (20Hz) stimulus, before incubation of Bafilomycin as described in the previous paragraph. A 1200APs (20Hz) saturating stimulus was employed to prompt the TrP release and NH₄Cl was used to gather the full TrP readout. As we predicted, synapses incubated with Aβ1-42 revealed an even more pronounced upscaling of the functional pool (49%). Data from 8 coverslips at 10h, 1 coverslip at 96h) when compared to buffer-treated control (32%. Data from 2 coverslips at 120h, 2 coverslips at 96h) (two-tailed unpaired t-test, p=0.048) (Fig 3.12a.ii-b.ii).
Figure 3.12. Aβ1-42 incubation reports a time-dependent re-scaling of the functional vesicle pool in culture. (a.i-a.ii) Average traces from Aβ1-42 (red) and buffer-treated cultures (black) normalized to the fluorescence level achieved upon NH4Cl addition following Bafilomycin treatment; cultures were incubated with the peptide for either 40h (a.i) or 96h-120h (a.ii). (b.i-b.ii) Graphs representing the difference in the total functional recycling fraction in the two conditions depending the incubation length (40h, n.s.; 96h-120h, two-tailed unpaired t-test, p=0.048). (Primary data collected by Dr Arjuna Ratnayaka, analysed by Luca Biasetti).
3.5 Discussion

Elucidating the Aβ1-42-driven changes to presynaptic mechanisms of vesicle exocytosis and retrieval remains a key focus required to shed light in AD. While the exact mechanisms of toxicity are yet to be fully described, the body of research carried out so far provides a variety of potentially affected sites of action.

The research presented aimed at gaining a better understanding of the potential changes brought upon by Aβ1-42 on the presynaptic mechanisms. Firstly, we investigated how the physiological properties were altered at the level of vesicle exocytosis and turnover after short- or long-term Aβ1-42 incubation. We uncovered a deficit in vesicle endocytosis kinetics, which prompted us to enquire how a potential decrease in the speed of vesicle turnover may impact transmission at the level of neurotransmitter release, specifically, glutamate. At a 24h Aβ1-42 incubation time point we reported deficits in both glutamate release and clearance. This behaviour was exacerbated upon 96h-120h incubation with Aβ1-42. Finally, we looked at Aβ1-42-driven modification at the level of total functional pool size. Notably, we reported a long-term homeostatic rescaling of the TrP, where Aβ1-42-treated cultures displayed an increase in the functional pool compared to a constant size in buffer controls.

3.5.1 Aβ1-42-driven endocytic kinetic deficits persist upon long-term peptide incubation

Previous research carried out in the lab reported a decrease in vesicle turnover rate in Aβ1-42 treated neurons of ~21% in comparison to buffer controls upon 24h peptide incubation. This has been corroborated in the literature in recent years (Kelly & Ferreira, 2007; J. Park et al., 2013), and constitutes a starting point for our investigation. A decrease in endocytic kinetics can be seen as (i) a potential disruption point in the SVC with selective downstream effects on short- and long-term physiological transmission and (ii) a symptomatic result of upstream mechanistic impairments brought upon by the
presence of the peptide. In this regard, it is important to keep in mind that albeit *in vitro*, the model we are working on exhibits a physiological level of plasticity (and other properties) at the level of single cells, all the way to neuronal networks, thus being capable of homeostatic adaptation and changes when its basal properties are altered by exogenous stimuli (such as the addition of Aβ1-42). Recycling deficits have been witnessed at an early time-point of Aβ1-42 exposure, when the response from the cellular network can be considered as an reaction to the addition of the peptide (despite the fact that oligomeric Aβ1-42 is already being internalized in primary hippocampal cultures after 2h-12h of incubation). However, when Aβ1-42 exposure is prolonged and the system is given the opportunity to deal with the non-physiological variable in order to maintain its proper functions, the deficit in vesicle turnover is still present, suggesting a possible primary role in the Aβ1-42-driven toxicity cascade over time. What can be the downstream effects on the otherwise physiological transmission? Vesicle retrieval is a process encompassing a plethora of mechanisms employed by the cell depending on the intensity of the release stimulus, the rate of transmission required, and many other variables. Regardless, some constants have to be maintained and thus can be key loci for a hypothetical Aβ1-42-driven toxic cascade.

From kiss-and-run mediated neurotransmitter release in which only a percentage of the vesicle fuses with the membrane, to fully fledged vesicle collapse, presynaptic membrane fusion is an unchangeable constant in the SVC. Thus, as a certain number of vesicles undergo this part of the process, the rate at which vesicle “detachment” occurs is crucial to maintain a sustainable level of volumetric stress on the plasma membrane both in terms of already fused organelles, but also in case of a subsequent round of vesicle release.

Slower turnover rates can also mean a net decrease in vesicles availability in the terminal following a set stimulation round. However, whether this may lead to a proper
mechanistic impairment is dependent on when and if said retrieved vesicles will be
needed again for synaptic transmission. In this case, both the rate, intensity and sustain
over time of the release stimulus will play a crucial role. If, for example, the release of
the RRP is elicited by a stimulation, and release is required to be maintained over an
extended period of time, the retrieval and availability of the initially exocytosed vesicles
will probably not have a big impact overall, if we presume the presence of other already
primed vesicles in the terminal. On the other hand, if an appreciable vesicle pool is
exhausted upon stimulation, their prompt and consistent turn over may be required to
face sustained transmission.

Lastly, it is important to contextualize the impairments discussed so far. A mechanistic
deficit at any point in the SVC ultimately leads to a functional deficit in synaptic
transmission.

3.5.2 Glutamate release and clearance are affected by Aβ1-42 in a time
dependent manner

We concluded the previous paragraph introducing the key concept that a mechanistic
deficit is directly correlated to a functional impairment in the physiological synaptic
transmission. We reported a statistical decrease in glutamate release upon Aβ1-42
incubation after 24h when cultures were prompted to the RRP release over a sustained
period of time. This functional result is in line with the previously discussed mechanistic
deficit of vesicle retrieval and availability. When a low baseline stimulus was introduced
prior to stimulation, the drop in neurotransmitter release became more appreciable only
following a higher intensity stimulation round. This can be explained by considering how
different levels of stimulation elicit different kinds of mechanistic responses. The fact that
this level of activity did not reveal as robust a change as the one reported with a 40APs
stimulation can be attributed to two reasons: (i) the deficit in vesicle turnover is not
present when this specific kind of retrieval is implemented, or (ii) the endocytic rate is
indeed affected but the recycled vesicles are not required to maintain transmission.
However, as the stimulation intensity is increased over subsequent rounds, the Aβ1-42-driven deficit emerges and its indirect effect on glutamate release is recorded.

Now, it is interesting to see how the same protocol applied to cultures incubated with Aβ1-42 for a longer period of time reports different results. In fact, a baseline stimulation becomes sufficient to elicit a lowering of neurotransmitter release from the first round of higher sustained transmission. Objectively, a decrease in the reported neurotransmitter is directly related to the overall quantity released upon vesicle fusion. Meaning, if we postulate that the amount of glutamate per vesicle is maintained constant, the only way to explain the reported data is (i) a decrease in the number of vesicle released, (ii) a reduction in the rate of successful fusion events, or (iii) a decrease in the possibility given to vesicles to undergo membrane fusion and ultimately release). The amount of vesicle fusion events that can take place at a given point in time can be limited either by functional variables (overall number of fusion-competent vesicles) as well as by structural variables (membrane availability). Upon a single stimulation round, we expect the vesicle pool in the terminal to be in its basal state, meaning that enough time has passed from a hypothetical previous round of synaptic transmission, and any change brought forth by said stimulus has already been adjusted to. In case of sustained stimulation trials, each one of the sequential rounds of release and recycling has an impact on the following one, thus predictions can be made when a component of the SVC is mechanically altered. In our case, the slower rate of vesicle turn over could hypothetically have the following effects: (i) lower vesicle availability in the terminal over sustained release, (ii) decrease the “free” area on the plasma membrane wherein exocytosis (fusion) can take place and (iii) structurally hinder vesicle movement due to accumulation of endocytic intermediates. However, what does this mean in terms of neurotransmitter release? If any of these mechanistic impairments were to take place, it would be possible to report deficits in release in case of sustained stimulation.
What we noted when cultures were stimulated with 40APs (10Hz) was a decrease in glutamate release from round 1, thus shifting our attention away from a deficit in recycling (which has yet to occur) and more towards an impairment in either vesicle availability or successful fusion events. The intriguing finding was a constant value in the decay rate in between stimulation rounds. While buffer-treated cultures saw a gradual decrease in glutamate release, Aβ1-42-treated neurons expressed their deficits right away, and more importantly did not display accumulated deficits as the trials persisted. In this case, a defect in recycling does not appear to have a consistently degrading effect on transmission.

When a low intensity baseline was introduced prior to recording, glutamate release during the 1st trial didn’t appear to be strongly affected by the presence of Aβ1-42. Let us discuss what events may have taken place in light of the points raised so far in this chapter. By applying a baseline stimulation we are eliciting a continuous cycle of vesicle release and recycling, wherein deficits brought forth by Aβ1-42, whether known or unknown to us, will inevitably have an effect on our recording rounds. We know from the sypHy data that Aβ1-42 can cause a slower rate of vesicle endocytosis, and our previous experiment using iGluSnFr reported a decrease in glutamate release independent of an impairment in vesicle turnover. However, both these experiments were carried out by using 40 APs protocols stimulation (20Hz and 10Hz, respectively). The fact that a non-significant difference in release was recorded during the 1st round of 10APs (10Hz), and that a proper decrease was reported only from the 2nd trial, suggests that whatever deficit taking place did not come functionally into play until the stimulation frequency reached a certain threshold (or no deficit was present at all during baseline activity). Specifically, the release deficit was not elicited with this level of stimulation, while the recycling impairment was present and played a role in the decrease in release.

Apart from neurotransmitter release, the use of iGluSnFr allowed us to track rate of glutamate clearance. 40APs stimulation reported a decrease in clearance in Aβ1-42
neurons compared to control. Now, glutamate undergoes reuptake into the presynaptic terminal via specialized transmembrane channels. Thus, a possible acute Aβ1-42-driven impairment of glutamate reuptake may correlate with a structural and/or functional deficit in the glutamate channels.

When Aβ1-42 incubation times were extended beyond the previously tested 24h, it was possible to witness the transition between an acute response to a more homeostatic adaptation taking place in the preparation. At 72h Aβ1-42 treatment, neurons still maintained the recycling deficits as seen by using sypHy readouts. This was interesting to see at a longer incubation time point, suggesting a primary role played by the turnover impairment in the Aβ1-42-driven toxicity effects.

Even more interesting were the results reported from the long-term glutamate experiments. While using the same protocol, the introduction of a baseline activity was now sufficient to bring forth a decrease in glutamate release right from the 1st stimulation trial of 10APs (10Hz). What might have changed at this point in time? (i) deficits that were not present during baseline stimulation at 24h did now appear at 96h-120h incubation and caused the drop in neurotransmitter release, (ii) deficits were always present at both time points but now they were enough to cause a disruption in the sequential exo-endocytosis cycling, (iii) disruption in the glutamate clearance and recycling were not present and now they appeared, (iv) disruptions in neurotransmitter recycling were always present and only now started to play a key role, (v) a rescaling of the functional pool took place (a decrease in the vesicles available leads to a decrease in glutamate released. However, if we take into consideration the other known deficits, the slowed turn over, an increase in the size of the TrP can have a detrimental effect because even though the cell responds to a request of increased neurotransmitter release by upscaling the functional pool, the deficit in endocytosis may lead to a blockage effect wherein the more vesicles are released the more stress is put upon the recycling machinery, thus have an even more detrimental result).
3.5.3 Long-term Aβ1-42 drives functional pool rescaling

When we interrogated the system in regards to its total functional vesicle availability at a prolonged Aβ1-42 incubation time points, we had the chance to report on the homeostatic adaptation taking place in treated cultures. Data from the experiments employing bafilomycin and ammonium chloride at 40h incubation point allowed us to gauge a functional readout of the TrP available in the terminal in Aβ1-42 and buffer-treated synapses. We reported no significant difference between the two conditions. When the experiment was repeated at the 96-120h incubation timepoint, the increase in vesicle availability in the peptide treated neurons became significantly different. From this data it seems that cultures undergo a rescaling of the functional vesicle pool when incubated with Aβ1-42 depending on the exposure time; while an initial change is already seen at 40h, when neurons have been exposed for 96h-120h the upscaling is statistically significant.

Working in a culture system allows for extensive variable manipulation in a model closely resembling a physiological environment in terms of functional activity and synaptic behaviour. While we proposed a 96h-120h Aβ1-42 incubation as a possible long term time point where we could assess not only an acute but also an adaptive response from the cell to the presence of the peptide, it is obvious how the amount of variables, events and complexity of the situation in an in vivo system cannot be properly replicated. First of all, the process of Aβ1-42 production is intrinsic in an in vivo environment, while in our model the peptide application is exogenous and already prepared, meaning the is no involvement (and possible downstream and long-term effects) on cellular organelles, transport system and membrane stability. Secondly, and probably the biggest variable, time. So far we have been using expressions such as “short-term” and “long-term”; in a physiological system decades of abnormal Aβ1-42 production, misfolding and downstream effects take place, and cells and neuronal networks are exposed to such events. Finally, the choice of intensity and continuity of the APs elicited in our preparation
was strictly dependent on the mechanistic questions we wanted to assess. On the other hand, physiological network activity usually never exceeds ~1Hz on average (Mizuseki & Buzsaki, 2013).

As previously mentioned, Aβ1-42 was exogenously added to our preparation after neurons had reached network maturation. While in the proper pathological environment, Aβ1-42 generation and production cycle took place over an extended period of time, our system was interrogated at the time point which peptide formation was already achieved. Aβ1-42 formation, oligomerization and so on, may as well directly or indirectly affect the SVC at a more prenatal level, when vesicular proteins are initially transported in the terminal towards the plasma membrane, and vesicles are formed and primed for functional release. When interrogating the system over a longer Aβ1-42 incubation period, once again what we are looking at is merely the possible homeostatic adaptation to the peptide which now has been able to directly affect the system in multiple ways. However, the continuous peptide production and delocalization is not completely included in this model. In other words, Aβ1-42 is a secreted protein and in a pathophysiological environment it undergoes delocalization upon production from the membrane-localised APP; thus while the movement and extracellular localization is taken into consideration in our model, we cannot account from “where” the peptide is delocalized from (membrane APP), as Aβ1-42 is added exogenously. While this allows us to reduce the number of variables in play, focusing primarily on the structural and mechanistic effects that Aβ1-42 can have on the preparation, we might be missing a key player in the dynamic changes and activity brought upon by the production of the peptide.
CHAPTER 4: DEFECTS IN FUNCTIONAL SV ORGANIZATION IN APPSwe/Ind AD MICE LEAD TO A PREFERENTIAL LOCALIZATION OF THE RECYCLED POOL IN THE TERMINAL

4.1 Introduction

In the previous chapter we explored the functional deficits in the synaptic vesicle cycle and the consequent glutamatergic transmission resulting from the incubation of primary hippocampal cultures with oligomeric Aβ1-42 over time periods ranging from 24h to 4 days. We reported a time-dependent impairment in glutamate release and clearance in terminals treated with Aβ1-42 and considered how this functional deficit is accompanied by a mechanistic disruption in the physiological vesicle retrieval processes. We concluded the chapter by focusing on the structurally higher hierarchical level of vesicle pools, presenting evidence supporting a possibly homeostatically-driven change at the level of the total functional recycling fraction. This leaves us with a number of points to address: in particular, how can we shed more light on the possible structural deficits and, can we do this in a more physiologically relevant preparation (discussed at the end of the last chapter).

To tackle these questions, we decided to move from a primary culture preparation to an in vivo chronic model of AD, in order to test whether the synaptic defects we see with extended Aβ1-42 incubation times in vitro are indicative of similar events occurring in a physiological environment, where Aβ1-42 production, activity and effects are intrinsic characteristics in the developmental progression of the animal. We used an established transgenic mouse model, APPSwe/Ind (Jankowsky et al., 2005), which is characterized by strongly enhanced Aβ production (Sri et al., 2019). The AD mouse model used employs a tet-off system wherein transcription activation of the APPSwe/Ind transgene is regulated via the tetracycline transactivator (TTA) binding to the TetO/CMV promoter. In the presence of doxycycline, the TTA is no longer able to bind the promoter region
and mRNA transcription arrests. Aβ and APP expression in the model was thoroughly characterized at different ages and transgene activation time points (Sri et al., 2019). For the sake of the current project, we will focus on the characterization of developmental-onset mice, that is those animals whose transgene expression began shortly after birth upon activation of the CaMKIIα promoter (Bayer, Cappai, Masters, Beyreuther, & Multhaup, 1999). Aβ and APP concentrations were quantified via the use of SDS-soluble lysates (Fowler et al., 2014). Developmental-onset mice showed significantly higher Aβ and APP concentrations when compared to wild-type mice (Sri et al., 2019). At 12 weeks of age, mice showed a poor performance in the Morris water maze and the results were in line with other developmental models of APP (Roberson et al., 2007; Webster, Bachstetter, Nelson, Schmitt, & Van Eldik, 2014).

In this chapter we propose to further investigate the underlying causes of Aβ1-42-driven vesicle pool re-scaling by coupling strong electrophysiological stimulation paradigms with established synaptic ultrastructural read-outs (Marra et al., 2014, 2012; Rey et al., 2015), allowing us to gather crucial detail in terms of pool turn-over, vesicle localization and inter-vesicle information which could not be extracted from our previous preparation.

### 4.2 Organizational fate of the turned-over vesicle pool

Using acute hippocampal brain slices from APPSwe/Ind or WT animals (APPSwe/Ind N of animals=2, Age= 2 months; WT. N of animals=2, Age= 2 months), CA3→CA1 synapses were loaded with the activity-dependent fluorescent dye FM1-43 (W. J. Betz & Bewick, 1992; Gaffield & Betz, 2006; Ryan et al., 1993) as upstream Schaffer collaterals were electrically stimulated to recruit the total available recycling pool (Fig. 4.1a) (Marra et al., 2014; Ratnayaka, Marra, Branco, & Staras, 2011; Rey et al., 2015). In a second step, the FM-dye internalized into recycled vesicles was photoilluminated to drive the polymerization of diaminobenzidine (DAB), forming an electron-dense osmiophilic precipitate visible at ultrastructural level (Fig 4.1b) (Denker et al., 2009;
Henkel, Simpson, Ridge, & Betz, 1996; Rizzoli & Betz, 2004). This allows the ready discrimination of recycled and resting vesicles based on the dark luminal appearance of the former and we used this classifying approach to quantify the size of the recycling pool as the proportion of total vesicles in each photoconverted terminal (Fig. 4.1c).

Electron micrographs were collected from each condition (Fig. 4.1d) and individual images were selected using the criteria described in section 2.6.1.

**Figure 4.1 Ultrastructural investigation of the functional vesicle pool in acute transverse hippocampal slice.** (a) Diagram portraying the experimental approach employed; a stimulation of ~1200 APs was applied in the CA3 region of acute hippocampal slices while FM1-43 dye was being puff-applied in the CA1 region via a recording pipette. (b) Schematic representation of dye loading in the functional boutons, wherein vesicles recruited to the synaptic membrane take up the FM1-43 dye upon turnover. The addition of DAB in the presence of string blue light leads to the creation of a dense osmophilic precipitate in the recycled organelles. (c) (left) Exemplar close-up electron micrograph of electron dense PC+ vesicles and empty PC- ones juxtaposed to their schematic representation; (right) Average cross-section density profile of PC+ and PC- vesicles. (d) Exemplar high-magnification (20k) electron micrographs depicting presynaptic terminals (light blue), docked vesicle region (red), AZ (yellow) and postsynaptic structures (light orange) in WT and APPSwe/Ind mice.
4.2.1 APPSwe/Ind mice display an increase in the functional pool fraction

Consistent with the measurements discussed in chapter 3, an initial quantitative analysis of the PC+ fraction in each terminal was carried out to compare changes in the size of the turned over vesicle pool upon stimulation in 2-month APPSwe/Ind transgenic mice in respect to age-matched controls. The results were in line with our chronic Aβ1-42 treatment data *in vitro*, revealing a highly significantly larger functional pool size in the transgenic model (mean ± s.e.m., 34 ± 3 %, n=61) when compared to wt (10 ± 1 %, n=43) (p<0.0001). Cumulative distribution analysis of the recycling fractions reported a broad distribution range in the APPSwe/Ind mice, while control synapses prevalently localized in the lower percentage ranges. Taken together, this initial analysis of the data reports a broader spread of values in APPSwe/Ind versus WT (Fig 4.2a), supporting our hypothesis that the sustained presence of elevated Aβ1-42 drives a homeostatic expansion in the functional vesicle pool.
Figure 4.2. Quantitative analysis of the total functional vesicle pool in WT and APPSwe/Ind presynaptic terminals. (a) Column diagram displaying the individual recycling fraction values in WT and APPSwe/Ind animals (mean ± s.e.m., WT: 9.86 ± 1.34 %, n=43; APPSwe/Ind: 33.96 ± 3.20 %, n=61; two-tailed unpaired t-test, p<0.0001). (b) Histogram representing the cumulative frequency distribution of the total recycling fractions per condition. (c.i-c.ii) Graph plotting recycling fractions for APPSwe/Ind (R²: 0.1652) (c.i) and WT (R²: 0.1820) (c.ii) synapses against the total vesicle number, representing an inverse relationship between the number of vesicles contained in the terminal and the percentage of functional ones.

4.2.2 Recycling vesicle pool localization in the terminal in APPSwe/Ind mice

We used the nanoscale detail offered by our functional-ultrastructural method to investigate whether the APPAswe/Ind versus WT background influenced the physical positions of vesicles within the terminal architecture. Based on representative middle sections for each synapse, we first mapped the coordinates of all recycled and resting vesicles, as well as the AZ. Fig 4.3a displays each individual representation for the 43 synaptic sections derived from WT mice, where red crosses correspond to PC+ vesicles, blue ones to PC-, and the 0 on the X axis represents the AZ. To directly summarize the way that vesicles were organized in the terminal, we constructed mean density plots by overlaying these vesicle coordinates for each synapse. Because individual samples were
sectioned at a random plane of orientation, thus not representing the exact vesicle localization in the terminal with respect to the midline, vesicle positions were calculated to assume lateral symmetry around the midline (Fig 4.3c). Following symmetrical correction, coordinates for each vesicle were then normalized with respect to the vesicle cluster boundaries and active zone center, as shown in Fig 4.3b. These normalized maps for all synapses in one condition were overlaid and used to build a 10 x 10 grid density matrix (Fig 4.3d), where coordinates for individual synapses were superimposed, which was then smoothed with a Gaussian filter and color-coded (Fig 4.4). These heatplots were normalized to a given pool class so that total intensity sums to one (Fig 4.4a.i), which is useful for exploring positional characteristics, but also intensity-corrected so that the intensity of one pool class is scaled to the control values (Fig 4.4a.ii), which represents the density of vesicles as well. For WT, resting vesicles formed an even density cloud, but in the case of the recycling pool, this cloud is shifted towards the AZ, as shown in Fig 4.4a.i-a.ii.
Figure 4.3. Schematic representation of vesicles' spatial localization analysis steps. (a) Set of diagrams depicting PC+ (red) and PC- (blue) vesicles positioning in the terminal mapped with respect to the AZ, derived from individual EM synaptic sections (WT n=43, APPSwe/Ind n=61; terminal sections represented here are derived from the WT micrographs, the same analytical procedure was applied to APPSwe/Ind sections). (b) Individual sections were normalized with respect to their own terminal boundaries and AZs. (c) In order to account for the fact that terminals were cut at a random plane of sectioning prior to imaging, lateral symmetry around the midline was applied. (d) Individual normalized terminal sections were superimposed in a single density matrix, maintaining information regarding PC+ (red) and PC- (blue) vesicles.
Figure 4.4. Spatial localization of PC+ and PC- vesicles in WT and APPSwe/Ind terminals. (a) i. Heat-maps of PC+ and PC- vesicles derived from each condition normalized according to individual vesicle pool, providing a qualitative readout of vesicle positioning with respect to the AZ; ii. Intensity corrected heat-maps depicting a quantitative representation of individual fractions relative to control values. (b) Schematic diagram displaying PC+ (black) and PC- (empty) vesicles (grey border) and their absolute distance (red and blue, respectively) to the AZ (brown). (c) Cumulative fraction of the absolute distances of PC+ and PC- pools to the AZ for each condition. (d) Average distances for recycling and resting pool fractions, depicting a non-significant positioning between the same pool within WT and APPSwe/Ind, while reporting a significant change in pool localization between PC+ and PC- (mean ± s.e.m; n=925 APPSwe_PC+: 187.4 ± 3.82 nm, n=2101 APPSwe_PC-: 220.1 ± 2.83 nm, two-tailed unpaired t-test, p<0.0001; n=2016 WT_PC+: 186.9 ± 8.10 nm, n=2364 WT_PC-: 217.9 ± 2.53 nm, two-tailed unpaired t-test, p<0.0004)

To provide direct quantification of our findings, we carried out analysis based on measuring the Euclidean distances between each vesicle and the closest point on the active zone and plotted cumulative frequency distribution plots and distance distribution plots for each pool class (Fig 4.4c). Aligned with the density maps, we found that
recycling vesicles in WT synapses favoured positions in the terminal that were biased towards the active zone (left shift in cumulative plot versus resting). The normalized heatplots are informative because they depict the position of vesicles within a terminal, independent of size, but one concern is that this normalizing step could distort the representation of vesicle positions. To address this potential issue, we also generated raw coordinate plots which plot absolute coordinate positions for each vesicle (Fig 4.5e.i-h.ii). Versions of these heatmaps without and with lateral symmetry removed reveal a similar outcome; recycling vesicles favour positions near to the AZ. This bias in recycling versus resting vesicles is consistent with the previous observations by (Marra et al., 2012; Rey et al., 2015) that recycled vesicles return to preferential locations in the pool architecture with respect to the AZ.

Next, we carried out the same analysis in APPSwe/Ind samples. Strikingly, although the fraction of recycled vesicles was much higher in the APPSwe/Ind slices, the relative distribution of vesicles was almost identical to those in WT synapses with recycled vesicles at sites closer to the active zone. Thus, in broad terms, the population of recycled vesicles occupy approximately the same relative space with the cross-section of the terminal. To provide a more detailed analysis we also took the raw position maps for WT and APPSwe/Ind and normalized their intensity values. This allowed us to then calculate the difference in recycled vesicle maps between WT and APPSwe/Ind. The subtracted image shows that our AD model has additional signal in a cloud around the active zone, which might indicate a tendency for local aggregation at this site (Fig. 4.6b).
4.2.4 Recycled vesicles in APPSwe/Ind synapses display a clustering tendency at specific regions in the terminal

To address this further, we next considered how recycled vesicles might be organized with respect to each other, by carrying out a cluster analysis. This could indicate whether vesicles tend to aggregate following their retrieval which could point to possible differences in post-endocytic trafficking pathways between WT and APPSwe/Ind. To do this, we measured the PC+ vesicle fractions in concentric circular regions of interest of increasing size (20 nm radial distance steps) surrounding each individual PC+ vesicle (Fig 4.7a). All values for these circular bins were expressed as a fraction of PC+ vesicles in the whole synapse. This normalization allowed us to examine changes in clustering independent of the wholesale change in the functionally-recycled pool fraction observed under different conditions. In WT animals, overall analysis showed limited clustering (Fig 4.7b). However, by contrast, local clustering was a significant feature of APPSwe/Ind synapses (Fig 4.7c) – in other words, recycled vesicles tended to show a higher propensity of aggregation in the terminal.
Next, we examined where this aggregation tendency arose from – for example, whether it is expressed across the whole terminal or in specific regions. To address this, we looked at the degree of clustering relative to the specific position in the synapse by dividing the population of vesicles into four compartments (back: 390-800 nm, core: 260-390 nm, fronto-peripheral: 130-260 nm, active zone: 0-130 nm; regions provided are based on the distances from the AZ) and then repeating the clustering analysis selectively on these regions. This revealed that vesicles tended to aggregate at the back (back: 390-800nm) but also in a region adjacent (AZ: 0-130nm) to the AZ (Fig 4.7d.i-d.ii-d.iii-d.iv).
Figure 4.7. Clustering analysis of the recycling pool fraction with respect to individual PC+ vesicles. (a) schematic representation of the analytical approach taken to extract information regarding the possible propensity of PC+ vesicle to occupy spatial positions in the terminal dependent of each other. A single PC+ vesicle was chosen as the epicentre and the number of recycled vesicles surrounding it in a 20 nm circle was quantified. Radial increments of 20 nm were applied each time and the quantitative PC+ analysis was repeated sequentially until all the vesicles in the terminal were included. This analysis was carried out for every PC+ vesicle in every terminal (APPSwe/Ind n=61, WT n=43). (b-c) Clustering plots for APPSwe/Ind (c) and WT (b) PC+ vesicles; clustering propensity was reported to be non-significant in WT terminals, while in APPSwe/Ind synapses PC+ vesicles displayed a significant difference in positioning with respect to each other at 60 nm (p=0.008), 80 nm (p=0.012) and 100 nm (p=0.04) radial distances (one-sample t-test). Further analysis led to the arbitrary subdivision of the terminal in 4 portions: a back region (390-900 nm), a core area (260-390 nm), a fronto-peripheral region (130-260 nm) and the active zone area (0-130 nm). Clustering analysis was repeated on PC+ isolated from the aforementioned regions. (d.i-d.iv) Upon region segmentation, recycling vesicles in APPSwe/Ind terminals showed a significantly higher clustering modality in the back region between 100 and 160 nm radial increments (p=0.026, 0.019, 0.033, 0.036, corresponding to the 100, 120, 140 and 160 nm regions).
4.2.3 Turned over vesicles preferentially localize at peri-active sites

So far, the data reported displayed i) an increase in the functional vesicle pool fraction in the transgenic animals when compared to controls and ii) a preferential spatial localization towards the frontal compartment of the pre-synaptic terminal in our model. Following from these results, we proposed to quantitatively investigate the recycling pool fraction of the docked vesicle pool. As expected, analysis of the docked pool itself revealed more functional vesicles in APPSwe/Ind (mean ± s.e.m., 53.59 ± 4.07%, n=46), while WT only reported an average of 32.04 ± 5.46% (n=19) (Fig 4.8b). However, when corrected for the overall differences in functional pool size in APPSwe/Ind versus control, this effect was not significant and did not account for the segregation in vesicles towards the front of the synapse. We hypothesized that the distribution of vesicles at sites lateral to the AZ might be an alternative basis for the spatial bias we observed. This lateral region, referred to as the peri-active zone (peri-AZ) (Fig 4.8c), has particular relevance because it is the proposed site for vesicle retrieval following fusion (Neher, 2010). Consistent with our hypothesis, analysis of these sites revealed a significantly higher fraction of functional vesicles than could be accounted for by the higher overall recycling pool fraction (Fig 4.8d).
Figure 4.8. Quantification analysis of PC+ and PC- vesicles localized in the AZ and peri-AZ area. (a) Histogram depicting the relative frequency distribution of the docked recycling fraction per number of synapses in WT and APPSwe/Ind mice. While WT slices displayed a peak synapse number at around 20% of the PC+ pool fraction, APPSwe/Ind slices reported a higher number of terminals with a recycling docked fraction of 50% and above. (b) Graph reporting individual docked recycling fraction values in WT and APPSwe/Ind animals (mean ± s.e.m., WT: 32.04 ± 5.46%, n=19; APPSwe/Ind: 53.59 ± 4.07%, n=46; two-tailed unpaired t-test, p=0.0038). (c) Schematic representation of the docked pool (red) and the per-AZ (blue) localization in the terminal. (d) Graph displaying individual values of the recycling pool fraction divided by the arbitrary left and right peri-AZ regions adjacent to the AZ in WT and APPSwe/Ind synapses (mean ± s.e.m; n=29 L_APPSwe/Ind: 78.44 ± 4.81%, n=25 R_APPSwe/Ind: 75.66 ± 5.52%; n=2 L_WT: 41.66 ± 8.33%, n=5 R_WT: 43.33 ± 4.08%. Two-tailed unpaired t-test between the total recycling fraction of L_APPSwe+ R_APPSwe/Ind and L_WT+ R_WT, p=0.0013). (e-f) WT (e) (R²: 0.6935) and APPSwe/Ind (f) (R²: 0.2075) PC+ fractions plotted against the total docked vesicle pool, once again supporting the trend of a lower percentage of recycled vesicles present in larger synapses and vice versa.
4.3 Discussion

Here we employed a powerful function-ultarstructural approach that allowed us to explore deficits in vesicle pools at the level of their nanoscale organization in the terminal. The relevance of this approach stems from recent work suggesting that the size and physical positioning of functional vesicle pools in the terminal is a key determinant of synaptic efficacy (Marra et al., 2014; H. Park, Li, & Tsien, 2012; Rey et al., 2015), and therefore a potentially important substrate the disease may affect. Based on a purely quantitative assessment of the functional pool capability between APPSwe/Ind mice and age-matched controls (2 months of age), we show that mice expressing increased levels of Aβ1-42 since birth display a net increase in the TrP available for release. This result shows clear alignment with our data reported at the end of chapter 3, where we demonstrated that a longer Aβ1-42 incubation in primary hippocampal cultures led to an upscaling of the functional pool size. Notably, a similar outcome takes place in a model ex vivo system where Aβ is chronically raised for a significant period in development.

4.3.1 Functional pool up-scaling as a homeostatic response

The re-scaling of the recycling fraction as a type of adaptive response has been previously presented in the literature upon chronic synaptic silencing following the use of pharmacological blockers. Increased synaptic strength has been reported in hippocampal neurons following a 3 days treatment with TTX (1 μM) (S. H. Kim & Ryan, 2010), while in a different study an increase of 10.5% in the recycling fraction was also witnessed upon 3 days TTX (0.5 μM) incubation (Welzel et al., 2011). Pre-synaptic enhancement correlated with Aβ1-42 has been investigated by Abramov et al. (2009), where Aβ1-42 overexpression was indirectly mimicked by actively impairing peptide degradation through inhibition of an Aβ1-42-degrading peptidase, neprilysin. The study concluded that an Aβ1-42 dependent loss of synaptic terminals drives a compensatory increase in the area of the remaining active terminals. On the other hand, in another
study employing pre-synaptic injection of intra-axonal oligomeric Aβ1-42, synaptic transmission was reported to be inhibited together with a decrease in the docked vesicle pool (Moreno et al., 2009). However, as the authors also point out, these results are attributed to an acute response to Aβ1-42 injection, and should not be compared to synaptic responses derived from long-term Aβ1-42 effects.

4.3.2 Up-scaling as a result of Aβ1-42-driven increase in vesicle mobility

As we previously discussed, Aβ1-42 oligomers have been implicated in the impairment of LTP (Lei et al., 2016) and in the facilitation of LTD (S. Li et al., 2009), thus indirectly modulating AMPA and NMDA receptors as well as overall calcium homeostasis (Green & LaFerla, 2008). Aβ1-42 has also been found to induce mitochondrial calcium release as well as extracellular calcium influx (D. Park & Chang, 2018) and internal calcium release from the ER (Ferreiro, Oliveira, & Pereira, 2008). This pathological alteration of calcium levels in the pre-synaptic terminal has been shown to directly impact inter-synaptic vesicle trafficking through synapsin I hyperphosphorylation (D. Park & Chang, 2018). In physiological conditions, synapsin I phosphorylation through protein kinases such as CDK5 leads to a decrease in binding to SVs, effectively increasing SVs mobility (Cesca et al., 2010).

Whether the up-scaling of the released pool can be considered a physiological adaptation of the system to face the increase in neurotransmitter demand due to Aβ1-42-driven impairments or just another point of the SVC altered by the presence of Aβ1-42, it is still open to debate.

Another hypothesis that was tested was the spatial fate of the functional vesicle pool following recycling. In chapter 3, we postulated how a deficit in vesicle turn over may lead to a structural impairment in terms of membrane availability for vesicle fusions, as well as a potential reduction in spatial freedom provided to new vesicles in the long term. Here we reported how, following retrieval, vesicles undergo preferential localization in
the region close to the AZ, specifically around the peri-active area. This may suggest that a deficit in vesicle turnover not only affects the following rounds of release in terms of vesicle availability, but also in terms of the area available. The notion that a possible rate-limiting step in the SVC could be constituted by the accessibility of set release sites has been a debated topic in the literature (Neher, 2010). Vesicle load upon fusion on the pre-synaptic membrane can be considered a balance between (i) the size of the active zone and its organization when it comes to vesicle residues translocation towards the peri-active area, (ii) the duration of the release process and (iii) and the efficiency through which they are retrieved (Cano & Tabares, 2016). In this regard, the excess in membrane region in small central synapses, such as the ones located in CA1, needs to be readily removed upon sustained stimulation (Roos & Kelly, 1999), due to a higher ready-to-go vesicle availability compared to larger synapses, coupled with a reduced volume of the peri-AZ area. Regardless of their fate upon retrieval, vesicles budding off the peri-AZ are required to move away from the retrieval region in order to allow for more vesicles to leave the membrane, especially during sustained levels of stimulation. We reported a statistically elevated level of inter-vesicle clustering in the back-most region of the terminal as well as a noticeable increment in turned-over vesicle grouping in the fronto-peripheral area in APPSwe/Ind synapses. This propensity of recycled vesicles to localize in close proximity with one another in a pathological setting, together with a preferential localization close to the peri-AZ may constitute an important point of disruption in the otherwise physiological activity of the SVC.

Taken together, this suggests that a subset of vesicles recycled by intense activity appear to aggregate at sites adjacent to the AZ in Aβ1-42-treated synapses versus WT. Aligned with our *in vitro* functional data, our findings are consistent with the idea that Aβ1-42 acts to overwhelm the vesicle retrieval and re-use pathway, providing a mechanistic basis for deficits in ongoing transmission.
CHAPTER 5: LEVETIRACETAM AND ROSCOVITINE PARTIALLY RESCUE THE Aβ1-42-DRIVEN DEFICIT IN GLUTAMATE RELEASE

5.1 Introduction

The Aβ1-42-driven deficits discussed so far could, in theory, be modulated by directly acting on two specific points of the SVC: (i) the availability of vesicles capable of undergoing release and (ii) the rate at which they are endocytosed back in the synaptic terminal. (i) The first point can initially be derived based on the data we reported in regards to glutamate release; if we presuppose that vesicle release kinetics and neurotransmitter re-filling remain constant and independent of Aβ1-42 presence, then overall common result of a time-dependent decrease in glutamate release in Aβ1-42-treated cultures could be attributed to a change in the vesicles available and their mobility state in the terminal. The data we report in vitro through the use of sypHy, which was also corroborated by our results ex vivo on APPSwe/Ind hippocampal slices, further support this hypothesis that a change in the functional pool scaling can and is indeed taking place. Changing this parameter by either promoting further upscaling of the releasable pool fraction or limiting the number of vesicles capable of being released could shed light on whether the impairment witnessed upon Aβ1-42 treatment is of a more functional (due to an Aβ1-42-related increase in neurotransmitter demand) or structural nature (due to an overwhelming load of vesicles clogging the SVC machinery), respectively. (ii) The latter point is directly related to the consistent sets of data we derived through the use of sypHy at 24h to 72h Aβ1-42-incubation time points in primary hippocampal cultures, indicating a noticeable slowing of the vesicle retrieval kinetics. As we discussed in chapter 3, a deficit of this sort in the retrieval machinery can be expected to have direct structural consequences on membrane availability for further fusion
rounds, on the area that can be accessed by vesicles waiting to be releases, and more importantly on vesicle recycling during sustained transmission.

To examine these possibilities further, we next considered modulators that might alleviate these aspects of dysfunction. In particular, we focused on two compounds, the anti-epileptic, Levetiracetam (LEV), and the cyclin-dependent kinase 5 (CDK5) inhibitor, Roscovitine, both with reported therapeutic value in AD models (Shukla, Skuntz, & Pant, 2012), but where understanding of their mechanisms of action has remained enigmatic.

LEV is an anti-epileptic drug commonly prescribed to treat partial and generalized seizures (De Smedt, Raedt, Vonck, & Boon, 2007; Kaminski, Gillard, & Klitgaard, 2012). Experimental evidence has shown that LEV is able to enter the recycling vesicles upon synaptic activity and interact with its receptor SV2A, ultimately reducing neurotransmitter release (Meehan, Yang, McAdams, Yuan, & Rothman, 2011). While the exact mode of action remains to be elucidated, studies have reported a decrease in vesicle release probability upon treatment with LEV, as well as a decrease in neurotransmitter release (X.-F. Yang & Rothman, 2009). Other modes of actions of the drug have been shown to include the reversal of the inhibition of GABA and glycine-gated currents by negative allosteric modulators as well as the partial inhibition of the N-type calcium channels (Abou-Khalil, 2008).

Roscovitine is a selective CDK5 antagonist. CDK5 is an enzyme important in neuronal development and post-synaptic signal integration. Inhibition of CDK5 has been shown to increase pre-synaptic release, and Roscovitine-dependent inhibition of CDK5 has been found to potentiate glutamate release (Chergui, Svenningsson, & Greengard, 2004). Furthermore, modulation of CDK5 activity has also been implicated in vesicle endocytosis performance, although there is disagreement in the literature regarding its exact effect on vesicle turnover (Nguyen & Bibb, 2003). Inhibition of CDK5 via the use of Roscovitine has been shown to correlate with a decrease in vesicle retrieval,
suggesting an important role of CDK5 in sustained stimulation (Tan et al., 2003). This result has been supported by Evans and colleagues (2007), who showed that prompt CDK5-mediated re-phosphorylation of a group of phospho-proteins known as dephosphins, a group of phosphoproteins essential for synaptic vesicle endocytosis, following stimulation allows for maintained rounds of vesicles recycling. On the other hand, Tomizawa et al. (2003) reported an increase in the intensity of fluorescent puncta upon Roscovitine treatment in primary hippocampal neurons in their styryl dye-based study. This controversy has been elegantly deconvoluted by Nguyen and Bibb (2003), who attributed the increase in vesicle turnover witnessed by Tomizawa and colleagues to be the result of a limited investigation of a single endocytic round; this suggests that the inhibition of CDK5 has detrimental effects on subsequent retrieval rounds due to the lack of dephosphins phosphorylation. However, the mechanisms of action of both LEV and Roscovitine remain to be completely elucidated.
5.2 LEV-treatment after short-term 5μM Aβ1-42 incubation leads to a potentiation in glutamate release

LEV (300 μM) was added to neuronal cultures incubated with 5μM Aβ1-42 for 24h, 3h before imaging. Following the same protocol described in 4.3.1, a low stimulus of 4APs (10Hz) was used to determine the location of a responsive region. After observing 1 minute with no stimulation, cells were stimulated with 5 consecutive rounds of 40 APs (10Hz). The i.s.i. between each round was 3.5s. Once again, single traces were normalized to the average amplitude of the control’s 1st trial to make sure the effect seen on glutamate release was not driven by a minute number of responses. The resulting normalized average traces were consistent with the average fluorescent traces. The effect of LEV treatment in terms of average glutamate release (expressed as AUC) was quite surprising. During the 1st trial of stimulation, Aβ1-42 incubated cultures treated with LEV displayed a higher neurotransmitter release compared to both the untreated Aβ1-42-treated cells as well as the buffer-treated ones (Fig 5.1c). In fact, their response was ~118% that of controls. However, while the initial glutamate release was increased compared to the other two conditions, the release decay per round was the same as the Aβ1-42-treated neurons (Fig 5.1e). Notably, the average decay was significantly lower when compared to Aβ1-42-treated neurons (two-tailed unpaired t-test, p=0.0092) (Fig 5.1f).

Glutamate clearance in neurons treated with LEV was exactly the same as Aβ1-42 cultures in terms of tau values from the 1st to the 5th round (Fig 5.1g,i-g.ii,h.i-h.ii). While LEV is commonly used to decrease vesicle probability of exocytosis as well as neurotransmitter release, incubation with the drug brought about the opposite effect in our preparation. Glutamate release was the only thing that was affected as the decrease in response per trial as well as neurotransmitter clearance was entirely unaffected when compared to Aβ1-42-treated neurons. Taken together, LEV simply raised the level of
glutamate release at the beginning of our stimulation, showing no effect on the characteristic deficits in the average decay per round and clearance found in Aβ1-42 treated neurons.
Figure 5.1. The effect of LEV on glutamate release and clearance in 24h Aβ1-42 treated cultures. (a) Average response traces of buffer-treated (black), Aβ1-42-treated (red) and Aβ1-42+LEV treated (grey) cultures. (b) Traces normalized to the average response amplitude of the 1st stimulation round of Aβ1-42-treated (red) and Aβ1-42+LEV treated (grey) cultures. (c) AUC values for individual stimulation rounds; Aβ1-42+LEV treated (grey) cultures maintained a markedly higher glutamate release per round compared to Aβ1-42-treated ones (red) (two-tailed unpaired t-test, p<0.0001). (d) AUC values for Aβ1-42-treated (red) and Aβ1-42+LEV treated (grey) cultures normalized to the 1st stimulation round, showing no significant difference between the two conditions. (e) Average decay in glutamate release divided per individual rounds, reporting a non-significant difference between Aβ1-42-treated (red) and Aβ1-42+LEV-treated (grey) cultures in most of the trials (rounds 1,3,4: n.s.; round 2: p=0.0092). (f) Decay per round averaged throughout the 5 trials, once again showing no difference between Aβ1-42-treated (red) and Aβ1-42+LEV treated (grey) cultures. (g.i-g.ii) Normalized glutamate clearance traces for the 1st (g.i) and 5th (g.ii) stimulation rounds derived from Aβ1-42-treated (red) and Aβ1-42+LEV treated (grey) cultures. (h.i-h.ii) Tau scores reporting no significant difference in the glutamate clearance times between Aβ1-42-treated (red) and Aβ1-42+LEV treated (grey) cultures in the 1st (h.i) and 5th (h.ii) trials.

5.3 Pre-stimulated Aβ1-42-treated cultures report a decrease in glutamate release decay per round following LEV application

LEV was also used in conjunction with the second stimulation protocol described in section 3.3.1. The introduction of a baseline activity (~1Hz) prior to culture imaging in cultures incubated with 5μM Aβ1-42 for 24h did not bring about the decrease in neurotransmitter release witnessed during the 40 APs (Hz) stimulation, at least not until one trial of 10 APs (10Hz) was given. Treatment with LEV showed a similar level of glutamate release during the aforementioned 1st stimulation round (Fig. 5.2b.i). However, in contrast to what was seen in Aβ1-42-treated neurons, no decrease in neurotransmitter release was reported during the 2nd round, as well as during each of the following stimulation trials at 10 APs, 40 APs and 60 APs (Fig. 5.2b.ii-b.iii). Interestingly, the average decay in glutamate release per round was significantly lower than Aβ1-42 treated neurons (p<0.0001) and was found to be lower than controls (Fig. 5.2c.i-c.ii).

Data from these experiments suggests that the addition of a baseline level of activity prior to recording allowed LEV to rescue the deficit in glutamate release and average
release decay per round shown in Aβ1-42 cultures, displaying level of responses equal if not improved when compared to the control condition.

Figure 5.2. Glutamate release is increased upon LEV addition to Aβ1-42 treated cultures in the presence of a baseline stimulation prior to imaging. (a.i-a.iii) Average traces of buffer-treated (black), Aβ1-42-treated (red) and Aβ1-42+LEV treated (grey) cultures derived from the 10 APs (10Hz) (a.i), 40 APs (10Hz) (a.ii) and 60 APs (10Hz) (a.iii) stimulation rounds. (b.i-b.iii) AUC values for the 10 APs (10Hz) (b.i), 40 APs (10Hz) (b.ii) and 60 APs (10Hz) (b.iii) stimulation rounds; Aβ1-42+LEV treated (grey) cultures maintained a higher glutamate release each stimulation round compared to Aβ1-42-treated ones (red) (two-tailed unpaired t-test, p<0.0001). (c.i-c.ii) Average glutamate decay per round during the 10 APs (10Hz) (c.i) and 40 APs (c.ii) (10Hz) stimulations; decay in release was markedly reduced in Aβ1-42+LEV treated cultures (p<0.0001).
5.4 Roscovitine and LEV partially rescue Aβ1-42-mediated decrease in glutamate release in a similar fashion

Finally, we decided to investigate the effects of LEV on neurons treated with 1μM Aβ1-42 over the course of 96h – 120h. The addition of LEV significantly reduced the decline in glutamate release reported in Aβ1-42 neurons during the 1st trial of stimulation of 10 APs (10Hz). The AUC per round in LEV treated cultures was maintained significantly higher than Aβ1-42 treated neurons for the remaining trials of 10 APs, as well as for the 2 rounds of 40 APs and the single trial of 60 APs (Fig 5.3b.i-b.ii-b.iii). While the average decay per round during the 10 APs trials was significantly lower than Aβ1-42 cultures and comparable with buffer-treated neurons (Fig 5.3c), the decay recorded between the 1st and 2nd rounds of 40 APs was not significantly different (Fig 5.3d).

Glutamate clearance was not affected by the presence of LEV in the culture prior to stimulation. However, the trend in the LEV trace decay for the 1st and 10th trials of 10 APs is worth examining in further detail. In fact, it is possible to distinguish an initial tendency of the decaying LEV curve to follow the trend displayed by control recordings. During the first 150ms, both the LEV-treated, and the control curves display a common overlap. Between 150ms and 500ms from the end of the stimulation, it is possible to notice a shift in the trend of the LEV curve where it seems to slow down before ultimately joining the Aβ1-42 curve from the 500ms mark onwards (Fig 5.3f).

Roscovitine was added to Aβ1-42 treated cultures at a concentration of 100μM, 30 minutes before cell imaging. Average glutamate release was improved throughout all the stimulation trials upon addition of Roscovitine when compared to Aβ1-42-treated neurons. Average neurotransmitter release decay per round in between the 10 APs trials was also reported to be lower than in Aβ1-42 incubated neurons and not significantly different from controls (Fig 5.3c). Interestingly, while we did not report a difference in glutamate decay per round between Aβ1-42 and buffer-treated neurons during the 2
trials of 40 APs, the addition of ROS significantly lowered the average decay in neurotransmitter release during this set of stimulation (p<0.0001) (Fig 5.3d). Glutamate clearance kinetics displayed a degree of variability but remained relatively inconsistent when tau scores were compared among conditions, after fitting an exponential line of best fit to the normalized response (Fig 5.3e). However, the ROS line decay follows the trend displayed by the control up until 150ms post-stimulation, before slowing down between 150ms and 500ms, to then overlap with the Aβ1-42 line (Fig 5.3f).
Figure 5.3. Treatment with either LEV or Roscovitine partially rescues long-term Aβ1-42-mediated deficits in glutamate release. Average traces of buffer-treated (black), Aβ1-42-treated (red), Aβ1-42+LEV treated (grey) and Aβ1-42+Roscovitine treated (yellow) cultures derived from the 10 APs (10Hz) (a.i), 40 APs (10Hz) (a.ii) and 60 APs (10Hz) (a.iii) stimulation rounds. (b.i-b.iii) AUC values for the 10 APs (10Hz) (b.i), 40 APs (10Hz) (b.ii) and 60 APs (10Hz) (b.iii) stimulation rounds; Aβ1-42+LEV treated (grey) and Aβ1-42+Roscovitine treated (yellow) cultures maintained a higher glutamate release each stimulation round compared to Aβ1-42-treated ones (red) (two-tailed unpaired t-test, p<0.0001). (c-d) Average glutamate decay per round during the 10 APs (10Hz) (c) and 40 APs (d) (10Hz) stimulations; decay in release was markedly reduced in Aβ1-42+LEV treated and Aβ1-42+Roscovitine treated cultures (p<0.0001) during the 10 APs (10Hz) stimulation. No significant difference in the average glutamate release decay was found between Aβ1-42+LEV treated and Aβ1-42 treated cultures during the 40 APs (10Hz) rounds, while neurons treated with Roscovitine showed a significant decrease in neurotransmitter release decay (p<0.0001). (e) Tau scores of buffer-treated (black), Aβ1-42-treated (red), Aβ1-42+LEV treated (grey) and Aβ1-42+Roscovitine treated (yellow) cultures divided for the 1st and 10th trial response from the 10 APs (10Hz) (1-2), 1st and 2nd trial response from the 40 APs (10Hz) (3-4) and individual trial response for the 60 APs (10Hz). (f) Average response traces from the 1st round of 10 Aps (10Hz) normalized to the end frames of stimulation.
5.5 Discussion

The decrease in glutamate clearance reported in chapter 4 is in line with evidence in the literature showing an impairment in the reuptake by EAAT channels on the astrocytic membranes by the presence of Aβ1-42 (Matos, Augusto, Oliveira, & Agostinho, 2008). Furthermore, the fact that no change was detected in glutamate-disassociation from the probe upon treatment with LEV at 24h is consistent with what is known so far regarding the mode of action of the drug. However, what is surprising is the end result of LEV treatment on neurotransmitter release. In fact, while LEV has been reported to have a negative effect on vesicle release (Meehan et al., 2011), here we reported a significant increase in the glutamate released when compared to cultures treated only with Aβ1-42. Although this may initially seem in contradiction with the known literature, it is important to analyse how we could reconcile both sets of results. The mode of action of LEV described so far involves the drug’s localization inside the lumen of the synaptic vesicle, where it would interact with its receptor SV2A. In order to achieve such positioning, the protocol we employed that takes advantage of the induction of a baseline activity prior to stimulation is the most competent by allowing LEV to enter the synaptic vesicle lumen and exert its effect (Meehan et al., 2011). However, interesting changes were also reported when using the direct stimulation protocol of five trials of 40 APs (10Hz). In this case, the only reported difference between Aβ1-42 and LEV-treated Aβ1-42 cultures was an increase in the glutamate release response. The heightened release was also significantly increased when compared to buffer-treated controls. However, no changes were witnessed in terms of the decay in the rate of neurotransmitter release per round. Taken together, this data suggests a temporary rescue effect elicited by LEV purely by focusing on the exocytic deficit which, in the long term, is not sufficient to rescue the Aβ1-42-induced response phenotype. It still remains puzzling how the only LEV dependent effect ultimately results in an increase in glutamate release. One possible explanation is that by in fact reducing the number of vesicles released, the reported
impairment in turnover and retrieval kinetics will not have the expected impact on the overall transmission both functionally and structurally. At the same time the heightened release is reported on the 1st stimulation round, when no prior induced activity was taking place.

The effect displayed in Aβ1-42 + LEV treated cultures when a baseline activity was introduced is slightly different. During the 1st trial, no significant difference can be reported between Aβ1-42 + LEV-treated neurons, Aβ1-42-treated neurons and buffer-treated controls. This is in contrast with the increase in the LEV response seen when using the previously described protocol. However, this time the decay in glutamate release per round is also affected by the addition of LEV; specifically, there is a decrease in neurotransmitter release decay, possibly accounting for the sustained increase in glutamate transmission not only due to an initially higher starting point, but also because of a decrease in loss per round of stimulation. Once again, no difference was be reported between the glutamate clearance rates between Aβ1-42 neurons and Aβ1-42 + LEV treated cultures. Thus, the recycling deficit remains unsolved.

We have previously discussed the presence of a secondary Aβ1-42-related impairment in regard to vesicle release. One possibility is LEV acting through this part of the SVC pathway and providing the cell with the tools needed for sustained transmission by reducing the functional pool availability. Hypothetically, the exocytosis-dependent deficit is resolved by a decrease in vesicles recruited to undergo release, and at the same time the stress on the endocytic machinery is reduced: the impairment is not dealt with directly, but ends up having a minimal effect overall.

Over a longer Aβ1-42 incubation time period, the positive effects elicited by treatment with LEV persist when compared to Aβ1-42-treated cultures. However, they are actually significantly lower than buffer-treated controls, albeit higher than Aβ1-42-treated ones. This could be due to the low response reported in Aβ1-42 alone, suggesting a rather
proportional effect carried out by LEV. In other words, the addition of LEV does not rescue the deficits under every circumstance, but more so depending on the severity of the impairment expressed. If, like the neural cells incubated at 24h, the initial release is somewhat levelled with control cultures, the treatment with LEV allows for a full phenotypic rescue, bringing back response levels at times more performing that buffer cultures. On the other hand, if a preparation incubated over a longer period of time with the peptide, displaying a markedly reduced response from the beginning (indicating a higher degree of impairment), LEV will still have a positive response, but it won’t be able to fully restore culture activity to a control level. Although in our experiments we compared the effect of LEV added on cultures following Aβ1-42 incubation, it could be interesting to use a second condition where LEV alone is applied to buffer treated controls; this would provide further information regarding the ratio of change elicited by the treatment with the drug.

Cultures incubated with Aβ1-42 over a 96h-120h period of time were also subjected to treatment with another vesicle-related drug, Roscovitine. By acting as an antagonist of the protein CDK5, Roscovitine has an effect on a variety of mechanisms and key processes in the SVC. The drug has primarily been connected with an increase in the number of functional vesicles available for release, by inhibiting CDK5 activity and freeing more organelles from the tightly regulated presynaptic cytoskeletal apparatus. Glutamate release in Roscovitine-treated synapses was enhanced when compared to Aβ1-42-treated cultures and maintained levels of response higher than LEV-treated preparations. The data reported from Roscovitine experiments is consistent with the expected effect of the drug; an increased vesicle mobilization would in fact solve an apparent impairment in release due to an insufficient number of primed organelles. Furthermore, by acting on CDK5, Roscovitine has also been implicated in having an impact in the rate of vesicle turn over, which remained unaffected following LEV usage. Thus, by having this effect on both of the reported Aβ1-42-driven deficits, it could be
possible to explain the stronger effect of Roscovitine in rescuing the pathological phenotype when compared to LEV alone. However, despite a significant ameliorating effect elicited by the drug, the variables affected (release, response etc.) are still not back to their physiological levels.

In chapter 3 we proposed different modes of clearance and reuptake for glutamate; while the primary pathway involves the EAAT transporters on the plasma membrane of astrocytic cells (Zhou & Danbolt, 2014), neurons can also mop up the glutamate release upon transmission and re-use the amino acid for vesicle refilling, among other things (Danbolt, 2001). While the clearance rates were and remained relatively slower in $\text{A}\beta 1-42$ incubated cultures and $\text{A}\beta 1-42 + \text{LEV}$ neurons at 24h, with and without a baseline activity, at longer time point incubation the effects of both LEV and Roscovitine appeared to be significantly different. In fact, during the initial 150ms of the clearance recordings, the behaviour of LEV and Roscovitine treated $\text{A}\beta 1-42$ cultures seemed to be remarkably similar to buffer treated controls. Somehow, the $\text{A}\beta 1-42$-driven impairment of glutamate reuptake was temporarily countered. This, however, only lasted for a very short period of time, after which the drug-treated traces changed their decay trend and continued along a path similar to $\text{A}\beta 1-42$ synapses. Unfortunately, it is not yet clear the exact mechanism through which $\text{A}\beta 1-42$ affects neurotransmitter reuptake.

The last topic left to discuss is the functional pool upscaling process undertaken by presynaptic terminals after extended $\text{A}\beta 1-42$ incubation. LEV is acting to reduce the functional size of an already over expanded TrP down to physiological levels or perhaps even lower. On the other hand, Roscovitine enhances vesicle motility, effectively exacerbating the adaptive response triggered in $\text{A}\beta 1-42$ synapses by peptide incubation. However, the experiments aimed at the RRP and TrP readouts alone appear to report contradicting results to glutamatergic ones. In fact, we did not witness a net increase in neurotransmitter release in $\text{A}\beta 1-42$ treated cultures at the same time points in which an
expansion of the functional pool has been shown to take place. Vesicle readout was performed by monitoring the change pH level of the organelle core, which usually takes place upon fusion with the membrane and release. One possible hypothesis is that more vesicles do indeed undergo fusion, accounting for the rise in pH: however, in the case of specific vesicles, no glutamate is released, leading to a so called "phantom release". In this case, the homeostatic response of the cell to the glutamate demand upon Aβ1-42 incubation is matched by a non-functional (partial) upscaling of the release-competent pool. The adaptive reaction is of purely structural nature, as the terminal cannot keep up with the refilling of the newly mobilized vesicles (perhaps, due to the aforementioned deficit in glutamate reuptake). A second hypothesis could rely on a series of fusion events not correlated with vesicles per se, but with intermediate structures mobilized from the terminal. Similar to the previous case, a change in pH would be reported but an increase in glutamate release would not take place.
CHAPTER 6: APOE AFFECTS SYNAPTIC TRANSMISSION AT THE LEVEL OF THE TOTAL RECYCLING POOL IN AN AGE-DEPENDENT MANNER

6.1 Introduction

In chapter 1, we discussed the genetic component associated with EOAD, which is directly correlated with APP processing and the production of the 42 aa fragment of the Aβ protein. On the other hand, LOAD is usually not correlated with a family history of the disease but has been associated with a broad set of genetic risk factors, the strongest of which, as we explored in section 1.3, is ApoE. However, the exact mechanisms through which the presence of the E4 isoform of ApoE would exacerbate and catalyse the AD phenotype are yet to be fully elucidated.

ApoE has been shown to act in an isoform-dependent manner on the overall fitness of the synapse, with E3 facilitating neurite outgrowth (Fagan et al., 1996), lipid redistribution following injury (Q. Xu et al., 2006) and being associated with a higher spine density compared to E4 (Ji et al., 2003). Furthermore, E4 has been shown to be less efficient than the E3 isoform in cholesterol transport (Rapp, Gmeiner, & Huttinger, 2006).

ApoE has also been shown to interact with Aβ in an isoform dependent manner (Kanekiyo, Xu, & Bu, 2014). The Aβ toll on the brain for AD patients can be interpreted as an overproduction of the protein, as an inability of the body to clear the polypeptide, or both. Despite the apparent direct or indirect connection between Aβ processing and ApoE (Kanekiyo et al., 2014), the connection between the two has yet to be established.

In terms of receptor interaction, it has been shown that ApoE LRP1 receptor is able to bind soluble APP as well as its neuronal isoform via the FE65 (APBB1) adaptor protein through the NPxY motif on APP (Holtzman, Herz, & Bu, 2012). This leads to an increased rate of APP processing and Aβ production. However, LRP1 is also involved in Aβ
clearance, thus counterbalancing the Aβ processing activity. Aβ clearance is an extremely important mechanism in the human brain and can be carried out via two main processes: receptor-mediated or endopeptidase–mediated. The first one involves the brain parenchymal cells and takes advantage of the interstitial fluid drainage pathway or the blood brain barrier (BBB), while the second one is carried out through proteolytic degradation. Receptor-mediated clearance is thought to involve LRP1, LDLR and VLDLR, which are present in Aβ plaques and can bind Aβ directly or indirectly via ApoE chaperone activity. Aβ-ApoE binding, which occurs via the apolipoprotein C-terminal domain, affects ApoE physiological functions and is isoform dependent: E3 has higher affinity than E4, leading to an increased level of Aβ clearance (Hone et al., 2005). While receptor-mediated Aβ clearance is a good way of reducing the Aβ toll on the brain via internalization and targeting to the lysosome, an increased intracellular level of the peptide can be neurotoxic (Billings et al., 2005). The current position on E4 and Aβ states that the apolipoprotein acts synergistically with Aβ oligomers to exert neurotoxicity in the brain at a greater extent when compared to ApoE3 (Yu, Tan, & Hardy, 2014). Furthermore, Aβ deposition appears to be ApoE isoform dependent, being the highest with E4, decreasing with E3 and reaching low levels in the presence of E2 (Youmans et al., 2012). An APOE gene dose dependence of Aβ deposition has also been documented in recent studies with mutant human APP mice expressing knick-in hAPOE (Bien-Ly et al., 2012). ApoE mediated Aβ clearance mechanisms are yet to be elucidated, but so far ApoE is the best characterized chaperone of Aβ. ApoE4 tends to be more easily cleaved by proteolytic enzymes when compared to ApoE3, and this leads to the fragmentation of ApoE4 and the formation of neurotoxic species (Brecht et al., 2004).

The data presented so far outlines ApoE4 as an important player in synaptic vulnerability. Here we decided to explore the ApoE-dependent effects on synaptic transmission in a primary hippocampal culture model derived from TR ApoE mice of known genotype (all mice used were homozygous for either the ε3 or ε4 allele). Following from our results in
chapter 3, we investigated the effects of Aβ1-42 on either genotype in terms of exo- and endocytic vesicle kinetics in vitro, in order to explore whether the synaptic deficits we previously uncovered were maintained, or even exacerbated now that ApoE was introduced as a variable. Complementary ex vivo experiments on said TR model allowed us to monitor an age-dependent ApoE effect on the pre-synaptic functional pool activity. This also allowed us to indirectly compare the status of the turned over fraction in our ApoE TR model to the results discussed in chapter 4, where we explored the effects of increased Aβ1-42 production on the functional pool.
6.2 Preferential expression of ApoE3 and ApoE4 in primary hippocampal cultures does not alter synaptic exocytosis and endocytosis

We set out to investigate whether there was an isoform-dependent effect on the function of pre-synaptic terminals in primary hippocampal cultures expressing either the E3 or E4 isoform of the ApoE protein. This was done by infecting primary hippocampal cultures with the AAV based sypHy construct (described extensively in previous chapters) at DIV 4-6 and conduct imaging at DIV 14-17. Coverslips were mounted on our custom-built chamber and incubated in EBS supplemented with NMDA and AMPA receptors blockers, AP5 and CNQX, respectively (see chapter 2). Responsive regions were identified using a modified shorter protocol of 40 APs (20Hz). After observing 2 minutes for recovery, cultures were imaged and stimulated at 40 APs (20Hz). No difference was reported when average responses were compared between the two ApoE genotypes (Fig. 6.1a). Furthermore, cumulative distribution of maximum amplitude values derived from individual traces following baseline- and bleach-correction revealed no significant difference between terminals expressing either the E3 or E4 protein isoform (E3, n=224; E4, n=260) (Fig. 6.1b). We further investigated whether there was a difference on the endocytic kinetics following our stimulation. Individual response traces were normalized to the stimulation end frames (Fig 6.1c) and single exponential curves were fitted to the beginning of the slow endocytic component, allowing for tau values comparison. Average tau scores were normalized to the E4 genotype responses. Although cultures expressing the E4 isoform initially appear to display a decrease in endocytic retrieval rates, no significant difference was reported between the two genotypes (E3, n=224; E4, n=260) (Fig. 6.1d).
Figure 6.1. Synaptic vesicles exocytosis and endocytosis in primary cultures derived from ε3ε3 and ε4ε4 TR mice. (a) Average responses to 40 APs (20Hz) in ε3ε3 (black) and ε4ε4 (red) cultures. (b) Cumulative frequency distribution of individual response sizes. Inset represents the variability in the maximum response amplitude. (c) Average response traces from ε3ε3 (black) and ε4ε4 (red) cultures normalized to the initial frames corresponding to the slow endocytic component. (d) Tau scores derived from single exponential fits, normalized to the ε4ε4 (red) values. Data from 3 coverslips for ε3ε3 genotype and from 4 coverslips for ε4ε4 genotype.

6.3 ε4ε4 TR mice display an age dependent rescaling of the functional vesicle pool

Although our results from primary hippocampal cultures do not seem to indicate a difference in either exo- or endocytic kinetics between E3 and E4 expressing terminal, we decided to further investigate the matter by shifting our experimental model to the ApoE transgenic mouse lines from which our cultures were derived. While primary hippocampal cultures provide a solid and reproducible model to investigate the functional activity of neuronal cells in a physiological environment, one thing that is not maintained from the in vivo model is the cytoarchitecture and synaptic circuit localization.
Hippocampal slices provide a powerful model to investigate structural and architectural characteristics of synaptic connections at the cellular level. Specifically, acute hippocampal slices can be used in conjunction with electrophysiological approaches to explore changes in the ultrastructure of individual synaptic components (Lo et al., 1994; Mathis et al., 2011; Weng et al., 2018). The approach employed here follows the same experimental protocol described in chapter 4.2. Briefly, acute hippocampal slices were derived from either homozygous E3 or E4 transgenic mice of 3 or 8 months of age (data from 2 mice for each genotype at 3 months and 8 months of age). CA3→CA1 synapses were stimulated at 1200 APs (20Hz) and FM1-43 dye was pressure-applied in the CA1 region, localizing on the synaptic plasma membrane during vesicle turnover. Slices underwent microwave-enhanced fixation at two different time points, either 1 minute or 20 minutes following the end of the stimulation and dye loading. Samples were then incubated with DAB and photoilluminated to drive its precipitation, effectively creating an electron-dense precipitate which would later allow us to discriminate between the recycling and resting fraction during EM imaging.

At 3 months of age, we did not report a significant difference in the total recycling pool between the two genotypes when slices where fixed at either 1 minute (mean ± s.e.m, E3 28.39 ± 4.30%; E4 18.08 ± 0.88%) or 20 minutes (E3 39.50 ± 3.01%; E4 30.41 ± 2.86%) following stimulation, showing a similar level of vesicle turnover. However, a small difference was found when the recycling fraction was compared between the two time points in the same genotypes. In fact, both E3 (1 minute, 28.39 ± 4.30%; 20 minutes, 39.50 ± 3.01%; two-tailed unpaired t-test, p=0.0444) and E4 (1 minute, 18.08 ± 0.88%; 20 minutes, 30.41 ± 2.86%; two-tailed unpaired t-test, p=0.0182) reported an increase in the total turned over pool at 20 minutes compared to 1 minute (Fig. 6.2a-b).

When the same experiments were repeated in slices derived from 8-month-old mice, the results were quite different. Comparison between the two genotypes revealed the total
recycling fraction was markedly elevated in E4 samples at both 1 minute (E3 8.19 ± 1.19%; E4 15.04 ± 1.90%, p<0.0001) and 20 minutes fixation times (E3 8.03 ± 0.88%; E4 45.58 ± 4.05%, p<0.0001). There was no significant difference in terms of turned over vesicles between 1 minute and 20 minutes in E3 terminals (1 minute, 8.19 ± 1.19%; 20 minutes, 8.03 ± 0.88%). In contrast, E4 slices showed a striking difference, with a net increase in the functional fraction at 20 minutes fixation compared to 1 minute (1 minute, 15.04 ± 1.90%; 20 minutes, 45.58 ± 4.05%; p<0.0001) (Fig. 6.2c-d).

Figure 6.2. Quantitative analysis of the total recycling pool in ε3ε3 and ε4ε4 TR mice at 3 months and 8 months of age. (a) Total recycling fraction quantification in 3-month-old ε3ε3 and ε4ε4 TR mice following a 1200 APs (20Hz) saturating stimulus, with sample fixation carried out at 1 minute or 20 minutes following the end of stimulation. (b) Cumulative probability distribution of the total recycling fraction for ε3ε3 and ε4ε4 TR mice at 3 months of age. (c) Total recycling fraction quantification in 8-month-old ε3ε3 and ε4ε4 TR mice. (d) Cumulative probability distribution of the total recycling fraction for ε3ε3 and ε4ε4 TR mice at 8 months of age.
If we further compare the data gathered from the E4 mice at 3 months of age, we notice how there is no significant difference in the percentage of the recycling fraction at the 1 minute fixation (3-month-old, 18.08 ± 0.88%; 8-month-old, 15.04 ± 1.90%, n.s.). However, vesicle turnover at the 20 minutes time point was relatively higher in the 8-month-old mice compared to the 3-month-old ones (3-month-old, 30.41 ± 2.86%; 8-month-old, 45.58 ± 4.05%, p=0.0028) (Fig. 6.3a-b).

Figure 6.3. Comparison between the total recycling fraction in e4e4 mice at 3 and 8 months of age. (a) Total recycling fraction quantification in 3-month-old and 8-month-old e4e4 TR mice following a 1200 APs (20Hz) saturating stimulus, with sample fixation carried out at 1 minute or 20 minutes following the end of stimulation. (b) Cumulative probability distribution of the total recycling fraction for e4e4 TR mice at 3 and 8 months of age.

6.4 Aβ1-42 incubation displays a lower degree of cytotoxicity in ApoE4-expressing cultures compared to ApoE3 ones

Once we explored the differential effects of E3 and E4 on synaptic transmission both in vitro and ex vivo, we decided to investigate how these results might be affected in the presence of Aβ1-42. We decided to investigate the underlying cytotoxic effects that Aβ1-42 might be eliciting in our preparation. Cultures were incubated with 5μM oligomeric Aβ1-42 or buffer-treated for 24h and 72h, before carrying out a ReadyProbes assay in order to gather a read out of cell viability in each genotype. This allowed us to have a
measure of cell death by calculating the percentage of green labelled cells. After 24h $\text{A}\beta 1-42$ incubation at 5$\mu$M, we reported a $\sim 35\%$ increase in cell death in $\varepsilon 3:3$ cultures (mean ± s.e.m; buffer-treated, 31 ± 2%, cells(n)=999; $\text{A}\beta 1-42$-treated, 42 ± 3%, cells(n)=1158; results from 2 independent experiments; two-tailed unpaired t-test, $p=0.0021$), while $\varepsilon 4:4$ neurons did not seem to show any change at all in terms of cytotoxicity (buffer-treated, 24 ± 2%, cells(n)=1545; $\text{A}\beta 1-42$-treated, 23 ± 2%, cells(n)=1419; results from 2 independent experiments) (Fig 6.4a-b). Following 72h 5$\mu$M $\text{A}\beta 1-42$ incubation, cytotoxicity in $\varepsilon 3:3$ cultures almost doubled (buffer-treated, 33 ± 2%, cells(n)=1019; $\text{A}\beta 1-42$-treated, 63 ± 4%, cells(n)=1383; results from 2 independent experiments; two-tailed unpaired t-test, $p<0.0001$), while $\varepsilon 4:4$ cells reported only a $\sim 45\%$ increase in cytotoxicity (buffer-treated, 22 ± 2%, cells(n)=1243; $\text{A}\beta 1-42$-treated, 32 ± 3%, cells(n)=1072; results from 2 independent experiments; two-tailed unpaired t-test, $p=0.0077$) (Fig 6.4a-b).

Figure 6.4. 5$\mu$M oligomeric $\text{A}\beta 1-42$ ReadyProbes cell viability assay. Cytotoxicity assays using ReadyProbes were carried out in $\varepsilon 3:3$ (a) and $\varepsilon 4:4$ (b) cultures upon addition of 5$\mu$M oligomeric $\text{A}\beta 1-42$, at DIV 13-14.
6.5 Aβ1-42 incubation elicits changes in the endocytic kinetics in ApoE3-expressing cultures, while ApoE4 remain unaffected

Based on the data reported so far, there does not seem to be an apparent functional difference between E3 and E4 primary hippocampal synapses in terms of vesicle exo- and endo-kinetics under physiological conditions. Next we explored whether there would be any change in behaviour upon Aβ1-42 treatment. Cultures were incubated with 5µM oligomeric Aβ1-42 or buffer for 24h before imaging with a stimulation of 40 APs (20 Hz). Cumulative distribution of the individual maximum amplitudes derived from E3 cultures revealed that, upon Aβ1-42 incubation, terminals tended to display larger responses compared to buffer-treated controls (E3, n=224; E3+Aβ, n=604; two-tailed unpaired t-test, p=0.0001) (Fig. 6.5a). Interestingly, the presence of Aβ1-42 in E4 cultures appeared to have an opposite effect, as seen in the cumulative distribution of individual amplitude responses (E4, n=260; E4+Aβ, n=456; p=0.0097) (Fig. 6.5b). Although small, there was a significant difference between Aβ1-42 and buffer-treated E4 controls. When the average traces were normalized to the beginning of the slow endocytic components, we proceeded to compare the tau scores derived from the fitted exponential curves. Aβ1-42 treated E3 cultures displayed a marked reduction in the turnover kinetics when compared to buffer-treated neurons, with a decrease of ~35% in endocytic rates (E3, n=224; E3+Aβ, n=604; p=0.0002) (Fig. 6.5c-d). On the other hand, Aβ1-42 incubation did not result in any statistically significant effect in E4 cultures (n.s.) in terms of vesicle retrieval (Fig. 6.5e-f).
Figure 6.5. Aβ1-42 effects on endocytic kinetics in primary cultures derived from ε3ε3 and ε4ε4 TR mice. (a-b) Cumulative frequency distribution of individual response sizes from ε3ε3 (a) and ε4ε4 (b) cultures in Aβ1-42 (red) and buffer-treated controls (black). Inset represents the variability in the maximum response amplitude. (c) Average response traces from buffer treated (black) and ε3ε3+Aβ1-42 (red) cultures normalized to the initial frames corresponding to the slow endocytic component. (d) Tau scores derived from single exponential fits, normalized to the ε3ε3+Aβ1-42 (red) values. (e) Average response traces from buffer treated (black) and ε4ε4+Aβ1-42 (red). (f) Tau scores derived from single exponential fits, normalized to the ε4ε4+Aβ1-42 (red) values.
6.6 Discussion

6.6.1 ε3ε3 and ε4ε4 report similar vesicle exo- and endo- kinetics in TR mice-derived primary hippocampal cultures

The data derived from our primary culture model suggests the idea that, in the absence of other secondary factors, cells expressing either the ApoE3 or ApoE4 protein isoform tend to display similar performances in terms of synaptic transmission. Now, data gathered from dissociated hippocampal cultures has suggested the possibility of an ApoE-dependent change in the vesicle release process due to the localization of ApoEr2 and VLDR on the presynaptic membrane, which have the ability to alter the resting Ca²⁺ state through a reelin-mediated mechanism (Bal et al., 2013). However, an ApoE isoform-dependent effect has yet to be demonstrated. In support of our data, synaptic transmission was reported to be unaffected in the CA1 region of the hippocampus, although these experiments were carried out in TR mice (Korwek et al., 2009). It is important to keep in mind that ApoE is primarily produced by astrocytes, and neuronal ApoE production mainly occurs in a situation of neuronal injury or stress. Thus, the absence of a genotype dependent effect we see in our primary cultures could be due to a lack of involvement of the ApoE protein in key synaptic processes over time in our model. Secondly, the stimulation applied here was of moderate intensity and was primarily aimed at the recruitment of the RRP. If an ApoE-dependent deficit were to be present in regards to sustained transmission or recruitment of vesicles from the TrP, we may not have been able to uncover it.

6.6.2 ε4ε4 TR mice display an age-dependent increase in the recycling vesicle fraction compared to ε3ε3 TR mice

An in vivo model expressing differential ApoE genotypes would provide a better insight on possible changes in synaptic transmission, allowing for age related changes to be investigated. Furthermore, it would allow us to explore changes at the level of the total
functional fraction. Data from our TR ApoE mouse model shows that, at 3 months of age, there is no significant difference between ε3ε3 and ε4ε4 mice in terms of the total recycling pool following a saturating stimulus. Furthermore, the quantitative analysis carried out at both time points shows that, in both genotypes, there is a slight increase in turned over vesicles after 20 minutes were observed prior to sample fixation when compared to 1 minute only. This tells us that (i) there doesn’t seem to be a genotype-dependent difference in terms of the total functional pool available in the terminal and the endocytic mechanisms taking place during vesicle turnover, and (ii) the discrepancy in pool fraction witnessed at 20 minutes compared to 1 minute, although an interesting point of discussion, remains a constant occurrence between the two genotypes. This slight but significant difference could be attributed to a number of factors. Depending on the mode of action, vesicle retrieval has been estimated to span from 1 s following ultra-fast endocytosis (Watanabe et al., 2013) up to 20 s in the case of endosomal-mediated recycling (Balaji, Armbruster, & Ryan, 2008), thus a possible impairment in the retrieval machinery that would lead to an increase in the process by minutes, or tens of minutes, seems to be highly improbable. Furthermore, the trend is not genotype-dependent. However, what could be occurring is either (i) a delocalization of a percentage of the turned over vesicles, with them being initially moved away from the terminal, thus eluding our analysis, or (ii) the recycled vesicles were indeed present in the terminal but, at the 1 minute time point, some of them still did not regain their individual form from endosomal intermediates. (i) It has been documented how, while certain pools are synapse-specific, some synaptic vesicles are able to move in between adjacent synaptic terminals (Staras et al., 2010). In this regard, it could then be possible to attribute the increase in recycling fraction at 20 minutes to turned over vesicles belonging to the terminal under analysis or, possibly, deriving from an adjacent pre-synaptic compartment. (ii) As we previously discussed in chapter 1.7, vesicle endocytosis is a complex mechanism that encompasses a variety of modalities both functionally and mechanically. While low levels of stimulation have been shown to elicit clathrin-mediated and ultrafast endocytosis,
stronger stimuli have been associated with bulk- and endosomal-mediated endocytosis (Clayton & Cousin, 2009). Thus, it could be argued that at the 1 minute time point a certain percentage of vesicles was still part of an endosomal intermediate and was still in the process of budding off from the organelle.

Contrary to the results in 3-month-old mice, when the same experiments were carried out in animals of 8 months of age ε4ε4 carriers reported a net increase in the total functional pool when compared to ε3ε3 mice, both at 1 and 20 minutes fixation. Furthermore, we witnessed an even greater disparity between the two time points in ε4ε4 mice at 8 months compared to 3-month-old ones. Taken together, the data at this later age point not only maintains and exacerbates the heightened recycling fraction at 20 minutes post-stimulation, but also suggests a baseline increase in the total functional pool availability in ε4ε4 mice. This could be explained in terms of an E4-dependent increase in the resting Ca\textsuperscript{2+} levels in ε4ε4 mice, a feature which has been shown to occur in AD conditions (Berridge, 2011) and the E4 isoform has been associated with Ca\textsuperscript{2+} dysregulation (Veinbergs, Everson, Sagara, & Masliah, 2002). Contrary to our results, excitatory synaptic transmission has been found to be reduced in TR ε4ε4 mice; however, this was carried out in the amygdala region of the murine brain and was reliant on spontaneous postsynaptic excitatory potentials (EPSP) readouts (Wang et al., 2005). A discrepancy in the structural process occurring in the pre-synaptic compartment and the functional aspect of fusion events might provide an explanation to this apparent contradiction. On the other hand, a slight decrease, although not significant, in vesicle recycling was witnessed in 3-month-old mice. However, it is important to remember that the data discussed so far is derived from an N=2 from each genotype at 3 months and 8 months of age, thus further experiments are needed to corroborate the results reported.
6.6.3 Aβ1-42 incubation leads to an ApoE isoform-dependent effect on synaptic transmission

After exploring individual ApoE-dependent effects on pre-synaptic activity, we decided to further investigate how the presence of Aβ1-42 might alter or trigger changes in its physiological processes. 24h acute incubation with Aβ1-42 in primary hippocampal cultures derived from ε3ε3 TR mice displayed a notable reduction in endocytic kinetics following RRP mobilization. This result is in line with similar experiments previously carried out in preparations derived from WT rat hippocampus (Marshall et al., 2016), suggesting a close behaviour of ε3ε3-expressing cultures and WT in response to Aβ1-42. Furthermore, this supports the use of ε3ε3-derived preparations as the archetype of physiological activity when working with ε4ε4 ones. On the other hand, ε4ε4 cultures did not appear to exhibit any perturbation to exo- and endocytic kinetics in response to Aβ1-42. The only difference reported was a slight decrease in the overall maximum response amplitudes when compared to ε3ε3 ones. The lack of an Aβ1-42-dependent effect in ε4ε4 cultures was quite striking, especially due to the data supporting an impairment of E4 in Aβ trafficking, degradation and clearance (Jiang et al., 2014). At the same time, this decrease in Aβ uptake and processing might exert a beneficial effect in the short-term, wherein at 24h the peptide has yet to exert any influence on pre-synaptic processes, as seen in the ε3ε3 cultures. In support of this hypothesis, cell viability assays showed no difference in terms of Aβ1-42-driven cytotoxicity in ε4ε4 at the 24h time point, while ε3ε3 neurons reported a significant increase in cell death. It would be useful to further investigate these results by monitoring the kinetics of Aβ1-42 uptake in ε4ε4 cultures and see how they might differ from ε3ε3, as well as an in depth analysis on changes in ApoE expression levels.

Taken together, the data presented here suggest that, while SVC kinetics do not appear to be affected by E4 expression in the short-term when compared to E3, as the network
matures and ages we witness an expansion of the functional pool, and a possible preferential endosomal-mediated mode of vesicle turnover that ultimately could have an effect on sustained transmission. Interestingly, the short-term incubation with Aβ1-42 does not elicit any deficit in the RRP release and turnover in E4 expressing cultures. Further studies exploring the synaptic behavioural changes in the long term, as well as the effect elicited by various degrees of sustained stimulation, are needed to provide a more complete analysis of the Aβ-dependent effects of the E4 isoform on altered synaptic transmission.
CHAPTER 7: GENERAL DISCUSSION

7.1 Proposed Mechanism of Aβ1-42-mediated toxicity on synaptic transmission

The results of Chapters 3 described the results of the acute effects of Aβ1-42 on neuronal cultures. The incubation of hippocampal neurons with oligomeric Aβ1-42 for 24h resulted in a deficit in the retrieval kinetics revealed following sypHy experiments (Fig 7.1a). Furthermore, a retrieval-independent impairment in glutamate release was also shown (Fig 7.1b). As the cell undergoes synaptic transmission, these two deficits continue to build up but may not manifest in a significant detrimental effect on physiological activity in the short term. In fact, the introduction of a physiological baseline activity prior to imaging did not seem to uncover a deficit in glutamate release. However, as time passes and the impaired function continues, the cell (and neuronal network) may undergo a homeostatic change and adaptation, in order to appease the neurotransmitter demand and counter the decrease in release. This is done by upscaling the size of the functional vesicle pool and mobilizing a greater number of vesicles (Fig 7.1c).

However, the result of this adaptation leads to further deficit because: (1) the decrease in release is not only due to low vesicle release, but also because of a problem with the recycling machinery (Fig 7.1d). Thus, while this strategy could initially work for the cell, in the long term it actually has the opposite outcome, leading to an even greater exacerbation of the deficit in vesicle turnover. (2) there is also a decreased rate of glutamate clearance from the synaptic cleft (Fig 7.1e). This could potentially lead to a reduced availability of glutamate in the terminal (Fig 7.1f), that may result in a problem in vesicle refilling (Fig 7.1g). In other words, despite the increase in the number of vesicles available, not all of them will be glutamate-filled, ending up with a 2-step problem: vesicles are obstructed in their passage towards fusion sites (Fig 7.1h), and
even if they were to undergo successful fusion, glutamate is not released (Fig 7.1i). The deficits are not directly dealt with and the strategies devised by the cell actually lead to a positive feedback loop exacerbating the issue. Our results suggest that a mechanical impairment at a certain point during vesicle turnover might be one of the driving factors behind pre-synaptic Aβ1-42-mediated toxicity.

In Chapter 4, the longer term effect of elevated Aβ explored utilising a transgenic mouse model of Alzheimer's disease. The hypothesis above further supported by the data derived from the ex vivo experiments on 3 month old APPswe/Ind mice, which showed: (i) an increase in the total functional vesicle pool available when compared to WT, and (ii) a preferential localization of the retrieved vesicles in the peri-AZ area in APPswe/Ind mice, as well as a significantly higher clustering of the turned over vesicles in the 390-800nm and 130-260nm terminal regions from the AZ. This tells us that a remodelling and upscaling of the functional pool is not an in vitro-exclusive result but it also takes place in vivo in a physiological situation over a longer time frame.

7.2 Pharmacological modulation of specific SVC components provide insight in Aβ1-42-mediated deficits

The results of Chapter 5 explored how the detrimental effects of Aβ may be modulated pharmacologically. Notably, in the case of both LEV and Roscovitine, the Aβ1-42-induced deficits seem to be partially rescued. LEV's impact in the preparation can be exemplified down to a mechanical decrease in synaptic vesicles apt to undergo release (Fig 7.1j). As we already discussed in the section 5.5, the quantitative restriction in vesicles exocytosis appears to be a beneficial effect in the long-term turnover and release, decreasing the pressure on the endocytic process. Thus, the effect witnessed by the addition of LEV could, in theory, be due to an effective decrease in the over expanded TrP by eliminating fusion of glutamate-deficient upregulated vesicles, once again ameliorating the situation in a structural manner. On the other hand, Roscovitine-
induced vesicle mobilization leads to the increased availability of neurotransmitter-filled vesicles (Fig 7.1k-l), in contrast to the “phantom mobilization” created by the adaptive response, ultimately triggering a qualitative amelioration. The increase in vesicle release expected with the addition of Roscovitine does not take place because of a higher degree of structural mobilization due to inhibition of CDK5. In fact, vesicles are already highly mobilized due to Aβ1-42. Roscovitine acts on the recycling part of the mechanisms (Fig 7.1m), facilitating vesicle turnover and thus structurally countering the adverse effect derived from the heightened mobilization.

Figure 7.1 Aβ1-42-mediated deficits on synaptic transmission and possible pharmacological interventions. The primary Aβ1-42-mediated effects on vesicle kinetics appear to affect the rate of endocytosis (b), as well as neurotransmitter release (a) and clearance from the synaptic cleft (e). Long-term effects also include an upscaling of the functional vesicle pool (c), as well as deficits in the recycling machinery (d) and in the uptake of glutamate (e), leading to a decreased availability of neurotransmitter in the terminal (f) to be reused in transmission (g). This might account for no glutamate release upon successful fusion events (i). Modulation of specific checkpoints of the SVC (j-k-l-m) suggest that a structural impairment at the level of the peri-AZ (h) could be one of the crucial factors in Aβ1-42-driven synaptic toxicity.

7.3 The role of ApoE in Aβ1-42-mediated and independent modes of alteration in synaptic transmission

In Chapter 6, we explored the impact of the major risk factor for late onset AD (ApoE4) on neuronal synaptic function. The role of ApoE in the mechanisms described above is complex, and our results do not fully allow us to propose an exact mode of action. In
terms of ApoE-dependent effects on synaptic transmission, our in vitro data suggests that there is no specific difference between ε3ε3 and ε4ε4 cultures in terms of specific deficit of synaptic transmission. Of course, this data was derived from experiments carried out on 14 DIV hippocampal cultures, presumably not under stress (Bu, 2009) and in which the actual concentration of ApoE was not known (i.e. if there was an isoform-dependent difference). On the other hand, ex vivo longitudinal data using acute slice from ApoE TR mice showed an age-dependent increase in the total recycling pool in ε4ε4 animals at 8 months of age; however, this tells us nothing about any possible change in the SVC kinetics. Further experiments employing a wider variety of ages as well as samples per time point would allow us to reach a more defined conclusion.

We then wanted to explore whether there was a different vulnerability of ApoE4 cultures to Aβ1-42 toxicity and Aβ dependent synaptic effects described in Chapter 3. However, ε3ε3 cultures showed a very similar response to Aβ as those shown previously for WT rat hippocampal neurons, displaying a slowed endocytic behaviour remarkably similar to the previously reported results from WT preparations in the presence of Aβ1-42, while ε4ε4 neurons did not show any specific deficit in synaptic transmission. While this might appear counter intuitive at first glance, the cytotoxicity data showed that ε4ε4 cultures are slower to respond to the toxic effect of Aβ1-42. Although these findings require further exploration, it is possible that this could be due to ε4ε4 slowed metabolism of the peptide. However, longer Aβ1-42 incubation time points as well as different stimulation protocols are needed to further explore these results.

Taken together, ApoE4 might confer an initial protection from Aβ1-42-mediated deficits in synaptic transmission while, in the long term, might lead to the exacerbation of these impairments both in an Aβ1-42-dependent and independent manner.
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