Consequences of presynaptic dysfunction in Alzheimer's Disease models

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Henry Taylor

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Abstract

Soluble oligomeric assemblies of amyloid β (Aβo) drive much of Alzheimer's disease (AD) pathology. Among these, changes in synaptic plasticity including attenuated long-term potentiation (LTP), and facilitated long-term depression (LTD), accompanied by postsynaptic weakening, are believed to be significant contributors to disease progression. Much work has been directed at characterising the postsynaptic effects of oligomers, with little or conflicting evidence of their impact at the presynaptic bouton. Some studies investigating presynaptic boutons directly, have suggested that Aβo elevate presynaptic transmitter release. Given that transmitter release precedes postsynaptic processes, an alteration in presynaptic biology could drive postsynaptic pathology. One important downstream process of AD pathology is tau hyperphosphorylation. The relationship between Aβo and tau phosphorylation is unknown. Recent evidence that tau plays a physiological role in AMPAR endocytosis as a result of LTD induction might provide a link between tau phosphorylation and Aβo-induced synaptic changes. Here I probe the presynaptic effects of Aβo and how they impact AD pathogenesis. I confirm that Aβo can enhance both evoked, and non-evoked spontaneous miniature presynaptic release using direct, optical techniques in rat hippocampal cultures. Furthermore, I show that partial reduction in presynaptic function can restore Aβo-induced plasticity deficits in adult mouse acute hippocampal slices. I also demonstrate that these plasticity deficits might underly tau pathology; In rat organotypic slices I show that chronic NMDAR-dependent LTD can cause pathological tau phosphorylation, mimicking the effects of chronic Aβo incubation, which also requires NMDARs. In addition, I show that reduction in presynaptic function prevents Aβo-induced pathological tau phosphorylation, suggesting that the pathological tau phosphorylation that occurs in AD could be a result of repeated LTD-inducing conditions driven by an enhancement of presynaptic release probability. Previously, little work has been done to establish a role for presynaptic changes in AD. I have developed this area within the thesis and have established a link between aberrant Aβo

aggregation and pathological tau phosphorylation that could have an important impact on AD therapy.

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1. Introduction

1.1 Introduction to Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease that accounts for 60-80% of all dementia (Alzheimer's Association, 2020). Already affecting around 5.8 million people in the US (Alzheimer's Association, 2020), its impact on society are only predicted to increase, with studies indicating that over 100 million people will have AD by 2050 (Prince et al., 2013). Early noticeable symptoms include memory loss and language problems (Alzheimer's Association, 2020), however, more recent studies have shown that changes in the brain begin occurring up to 20 years before these symptoms become apparent (Bateman et al., 2012; Jansen et al., 2015; Ossenkoppele et al., 2015).

Although there has recently been some promising evidence for prevention of AD, studies have shown that lowering levels of homocysteine via treatment with vitamin B supplements can improve rate of cognitive decline, and this is reflected in the success of the VITACOG trial (Smith et al., 2018), there is still a real lack of effective therapeutic interventions for AD besides acetylcholinesterase inhibitors (AChIs) (Birks, 2006) and the N-methyl-D-aspartate receptor (NMDAR) blocker memantine (Tariot et al., 2004), both of which merely briefly mask the effects of neuronal loss. Several treatments have shown promise in animal models and early clinical trials, including immunotherapy (Schenk, 2002), however, more recent clinical data show that these treatments are having limited therapeutic effects (Golde et al., 2011). It has been speculated that this could be due to the fact that, by the time these treatments are being applied, the disease has progressed too far to be stopped by such methods. Therefore, it is vital that we continue to strive to understand the early, primary events that drive the further progression of pathology. This will give an insight both into more efficacious therapies, and into an earlier diagnosis that is key to curing the disease.

1.1.1 The Pathology of Alzheimer's Disease

AD neurodegeneration is characterised by loss of synapses and neurons, together with histopathological accumulation of extracellular plaques of aggregated amyloid β (Aβ) and intracellular aggregates, or neurofibrillary tangles (NFTs), of hyperphosphorylated microtubule associated protein tau (MAPT) in brain regions that are associated with higher mental functions such as the neocortex and the hippocampus (Francis et al., 1999) around sites of neurodegeneration (De Strooper and Karran, 2016; Hardy and Selkoe, 2002). Early work found that there was selective loss of the cholinergic enzymes choline acetyltransferase and acetylcholinesterase in the neocortex and hippocampus (Davies and Maloney, 1976) and a loss of presynaptic nicotinic acetylcholine receptors (nAChRs) in cerebral cortex (Whitehouse et al., 1986), and that this was due to a selective, early, degeneration of cholinergic neurons, particularly in the basal forebrain axons of which innervate the cortex (Mobley and Chen, 2019), important for memory (Coyle et al., 1983). Loss of cholinergic innervation could explain much of the cognitive impairment seen in AD patients. Indeed, AD symptoms can be mimicked in the young by impairing cholinergic transmission, and can be transiently improved in AD patients by stimulating or enhancing it (Bartus et al., 1982). This evidence has led to the use of AChIs as the main, frontline pharmacological treatment for AD (Birks, 2006; Lanctôt et al., 2003), however, it is clear that these only briefly improve symptoms, and there is evidence that use of AChIs speeds up the onset of later AD symptoms (Lanctôt et al., 2003). This suggests that there is more to AD pathogenesis than a simple loss of cholinergic neurons.

As well as a dysfunction in cholinergic transmission, there is also disruption to glutamatergic synapses (Wang and Reddy, 2017). These synapses make up a large part of pyramidal neuronal connections in the neocortex and the hippocampus and are highly studied in the context of synaptic plasticity (Luscher and Malenka, 2012), a proposed mechanism for learning and memory

(Bear and Malenka, 1994). Loss of hippocampal CA1 neurons correlates well with learning and memory deficits in AD patients (Padurariu et al., 2012), and the hippocampus is a structure heavily involved in these processes (Bartsch et al., 2011; Jarrard, 1993). Since there is a correlation between glutamatergic synaptic loss and learning and memory deficits (Francis, 2003), much of the work on AD has looked at disruption to glutamatergic synapse function.

As well as neurodegeneration, AD is characterised by specific protein aggregates of Aβ (in the form of extracellular plaques) and MAPT (as hyperphosphorylated tau tangles). These protein aggregates are a key feature of AD diagnosis which is often performed post-mortem. The distinctive presence of these two pathologically aggregated proteins has driven the direction of a significant, perhaps predominant fraction of AD research and has sparked much debate. It is clear that both proteins play an important role in AD pathogenesis, but the nature of that role is still poorly understood.

1.1.1.1 Microtubule Associated Protein Tau in Physiology and Pathology

In normal physiology, MAPT is primarily associated with microtubule (MT) stabilisation, particularly in the axons of neurons, where tau is most abundant (Aronov et al., 2001; Hirokawa et al., 1996; Kanai and Hirokawa, 1995). MTs are important in neurons for establishing cell structure, and also for transport of numerous constituents of the cell, including signalling molecules and organelles, along axons. Axonal transport is essential for normal cell function, and dysfunction of it or the processes surrounding it are implicated in many neurodegenerative diseases (Roy et al., 2005). In its role in stabilising MTs, tau binds to the MTs reversibly at a MT-binding domain composed of repeats of a highly conserved tubulin-binding motif (Lee et al., 1989) (Figure 1.1). The binding of tau with MTs, therefore, exists in a dynamic equilibrium between bound and unbound. It appears that this constant binding and detachment of tau from the MTs is required for axonal transport. The position of this equilibrium is altered by the variable phosphorylation of

tau by several kinases and phosphatases, allowing for the tuning of this association (Ballatore et al., 2007).

In addition to its role in MT stabilisation in axons, endogenous tau has also been found more recently at the dendrite in rodent neuronal cultures and rodent slice preparations (Ittner et al., 2010; Kimura et al., 2014; Mondragon-Rodriguez et al., 2012; Swanson et al., 2017; Zempel et al., 2013). Here, the role of tau is less well known, but it has been shown to interact with a variety of other structures and enzymes, including the Src-family tyrosine kinase Fyn and PSD-95 (Lopes et al., 2016; Mondragon-Rodriguez et al., 2012) with which it has been shown to form a complex that facilitates NMDAR-mediated excitotoxicity (Ittner et al., 2010), and SynGAP1 and extracellular signal-regulated kinase (ERK), which are also involved in NMDAR-dependent excitotoxicity (Bi et al., 2017). The promiscuous nature of tau with binding to other proteins also hints at its propensity for misfolding and aggregation (Ballatore et al., 2007).

Many of the functions, both physiological and pathological, as well as the location of tau depend on both the isoform of tau, and post-translational modifications. There are six isoforms of human tau, encoded for by the *MAPT* gene containing 16 exons on chromosome 17. The different isoforms range in length from 352 residues to 441 residues, and each contain three or four repeat regions towards the C-terminus, thought to be the primary site for MT binding, alongside a proline-rich region and a C-terminus flanking region (Figure 1.1) (Regan et al., 2017). Isoforms of tau containing three repeat (3R) and four repeat (4R) regions exist in a mixture during normal, healthy states, however changes to the relative levels of 3R and 4R present can play a role in different pathological states (Barron et al., 2020; Schoch et al., 2016). Tau can undergo extensive post-translational modifications, including at 77 serine/threonine and 4 tyrosine phosphorylation sites (Noble et al., 2013), as well as undergoing acetylation, methylation, and ubiquitination (Mandelkow and Mandelkow, 2012), all of which have been implicated in disease.

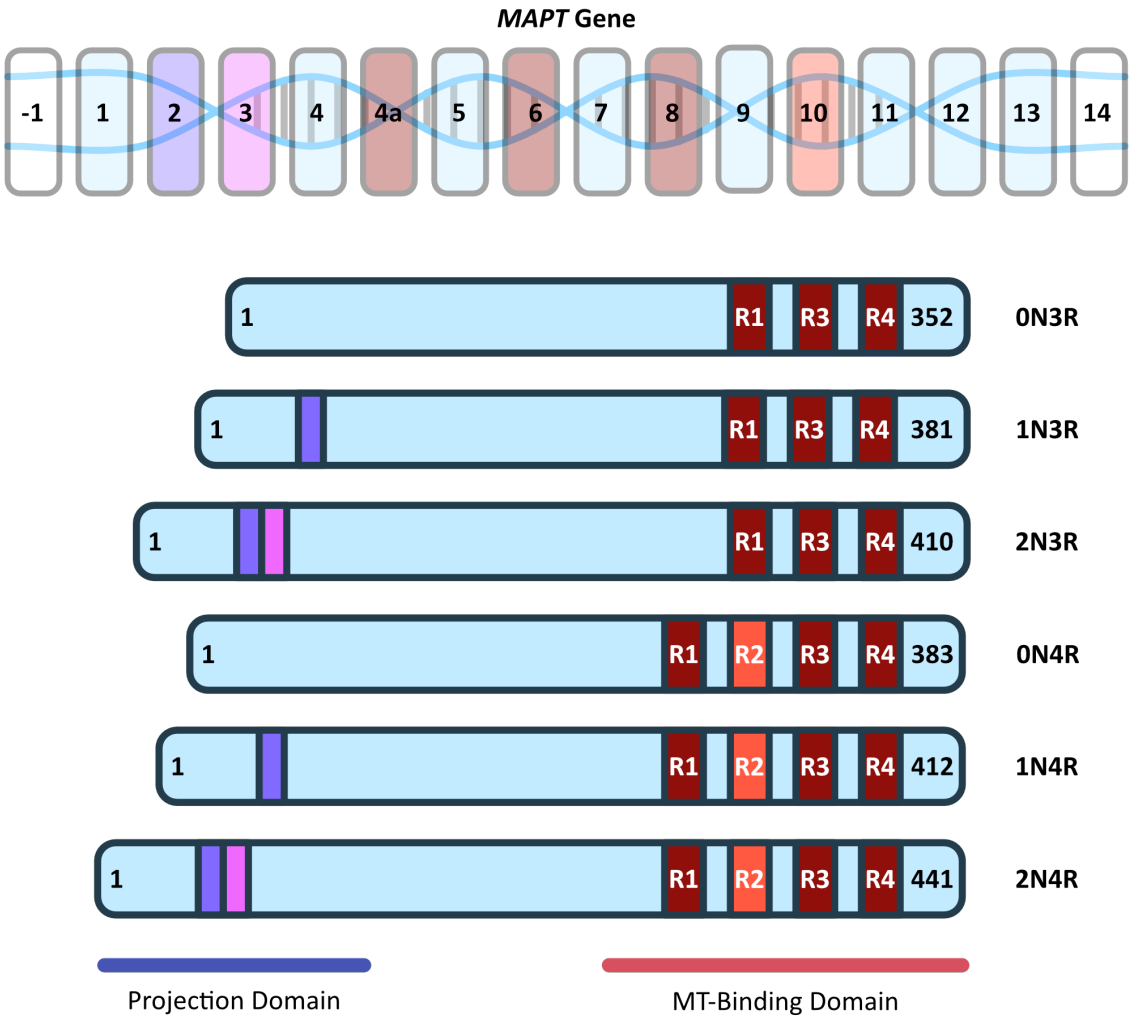

Figure 1.1: Different isoforms of tau. Human *MAPT* gene on chromosome 17, (exon numbers shown) can be alternatively spliced to give 6 different isoforms of tau. Each contain at least three repeat regions, with three isoforms containing an extra repeat region from exon 10 and are known as 3R and 4R respectively. In addition, there are two N-terminus regions that can be included from exons 2 and 3 to give either 0N, 1N, or 2N tau.

MAPT dysfunction is heavily implicated in other forms of neurodegenerative diseases known as tauopathies (Roy et al., 2005), including FTDP-17, an autosomal dominant frontotemporal dementia and parkinsonism linked to a mutation in tau on chromosome 17 (Wszolek et al., 2006) that is used in mouse models to simulate tau pathology (Berger et al., 2007). Although the exact mechanism of MAPT-induced neurodegeneration is unknown, a loss of MT-stabilisation is highly

likely to play a role. A reduction in MT function would be detrimental to neuronal structure, and axonal transport. Perturbations in axonal transport have been linked to many other neurodegenerative diseases, including certain forms of motor neuron disease and Huntington's disease (Roy et al., 2005), highlighting the importance of this process for proper neuronal health. In addition, certain MT-stabilising drugs such as paclitaxel, which targets tubulin, appear to have some ability in being able to reduce neurodegeneration in transgenic mouse models of certain tauopathies, corroborating the involvement of impaired axonal transport in tau-related neurodegeneration (Trojanowski et al., 2005).

AD could also be classed as a secondary tauopathy; MAPT hyperphosphorylation and tangle formation correlates well with synapse loss and extent of cognitive decline in AD patients (Augustinack et al., 2002; Braak and Braak, 1995) and MAPT binds to a variety of other structures and enzymes, including presenilin 1 (PS1), mutations in which are closely linked with familial, early onset AD (Takashima et al., 1998). The histopathological presence of Aβ plaques, however, differentiates AD as a disease from pure tauopathies. Although there is certainly a link between abnormal MAPT handling and neurodegeneration, the extent of the involvement of dysfunctional tau in driving AD pathology has been a subject of much debate. There are a number of studies that suggest that neurofibrillary tangles are not the original initiator of AD pathology; Santacruz *et al.* found that mice expressing a repressible human tau developed neurofibrillary tangles together with neuronal loss, however, when tau was suppressed, this neuronal loss was stabilised, but the neurofibrillary tangles still remained (Santacruz et al., 2005). In addition to this, strikingly, the tauopathies caused by mutations in *MAPT* are characteristically frontotemporal dementia with Parkinsonism, with no accompanying Aβ deposits as seen with AD (Hardy et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Furthermore, transgenic mice with an over expression of mutant Aβ (Tg2576) and mutant human tau (JNPL3) present a marked increase in the formation of tau tangles compared to mice expressing human tau alone, but no increase in

plaque deposition (Lewis et al., 2001). This evidence, therefore, suggests that, although in other dementia aberrant phosphorylation and accumulation of tau is an early event that drives further disease progression, in AD it is a downstream pathological process that is recruited by some other pathology.

1.1.1.2 Amyloid β Processing and Pathology

The physiological functions of endogenous Aβ remain poorly understood, but there is growing evidence that it is important at the synapse. Aβ results from the altered cleavage of amyloid precursor protein (APP), normally handled by α-, β-, and γ-secretases (Figure 1.2). The cloning of APP revealed that it is localised on chromosome 21 (Goldgaber et al., 1987), possibly accounting for the overwhelming prevalence of early onset AD in trisomy 21 (Down's syndrome) patients (Tanzi et al., 1987).

Alongside Aβ, APP is a precursor to a variety of different proteins, secreted APP β (sAPPβ), secreted APP α (sAPPα), CTFα, CTFβ, APP intracellular domain (AICD), and P3 (Figure 1.2). These non-amyloidogenic cleavage products also have synaptic properties, and some show varying degrees of neurotoxicity, including CTFβ, AICD, and P3, but show less propensity for aggregation (Nhan et al., 2015).

Aβ is produced upon sequential cleavage of APP by β-secretase, identified as BACE1, and γ-secretase (Figure 1.2), and both are required for Aβ production (Cai et al., 2001; De Strooper et al., 1998). This cleavage produces a peptide that is predominantly 40 amino acid residues in length ($A\beta_{1–40}$), but has been found to exist in lengths between 38 and 42 residues (Chow et al., 2010). The 42 amino acids long variant ($A\beta_{1–42}$) is particularly prone to oligomerisation in comparison to the other variants, and it appears that it is increased production of this variant that leads to most

Aβ oligomerisation; computational analysis showed that this increased propensity for oligomerisation is due to an increased β-turn and β-hairpin-like structure in $A\beta_{1–42}$ compared to $A\beta_{1–40}$ caused by a Valine 36-Glycine 37 turn (Roychaudhuri et al., 2013).

Insight into the importance of Aβ and APP processing in AD has been shown by studies of the early onset forms of AD known collectively as familial AD (fAD). fAD is linked to a variety of mutations, all which are in genes involved in either the cleavage or handling of APP (Hatami et al., 2017). Two proteins that lead to fAD are presenilin 1 (PS1) (Clark et al., 1995; Sherrington, 1995) and presenilin 2 (PS2) (Levy-Lahad et al., 1995; Rogaev et al., 1995). Although this could be due to a loss of their ability to act as endoplasmic reticulum Ca^{2+} leak channels (Tu et al., 2006), studies *in vitro* and *in vivo*, in humans and using mouse models, indicate that these PS1 and PS2 mutations seem to cause accentuated AD pathology (Holcomb et al., 1998), in particular elevated extracellular levels of aggregation prone $A\beta_{1–42}$ compared to the more common $A\beta_{1–40}$ (Borchelt et al., 1996; Duff et al., 1996; Esler and Wolfe, 2001; Lemere et al., 1996; Scheuner et al., 1996), suggesting that Aβ aggregation is important for AD pathology. Work performed in neuronal cultures of PS1 deficient mice found that γ-secretase cleavage of APP was prevented (De Strooper et al., 1998), and further mutagenesis of PS1 also found a reduction in γ-secretase activity, accompanied by an increase in the accumulation of APP C-terminal α- and β-secretase products (Wolfe et al., 1999). This evidence led to the proposition that PS1 and PS2 could, in fact, be γ-secretase itself, with Wolfe *et al.* suggesting that both presenilins autocatalytically activate themselves and then cleave APP to give Aβ (Wolfe et al., 1999). In addition to cleaving APP, presenilins have been identified as having a key role in notch signalling (De Strooper et al., 1999), a versatile, but ubiquitous cellular process that makes blockade of presenilins an intractable target for altering Aβ levels.

Other variants of fAD involve the APP gene, particularly those favouring β- and γ-secretase cleavage (Cai et al., 1993; Citron et al., 1992). Once again, these mutations appear to promote the formation of $A\beta_{1-42}$, increasing the propensity for oligomerisation and plaque formation (Suzuki et al., 1994). As noted previously, Down's syndrome patients, who possess three copies of chromosome 21 upon which the APP gene is found, have a profound likelihood of developing fAD (Tanzi et al., 1987). Other mutations directly to APP such as the Swedish mutation (APP-swe) lead directly to fAD (Mullan et al., 1992). Current mouse models for AD take advantage of these and PS1 mutations to simulate AD-like histopathology together with learning and memory deficits and synapse loss (Hall and Roberson, 2012; Lewis et al., 2001) and these are now widely used to further inform understanding of the disease. Although useful, these models do have many limitations. A more detailed discussion of these will occur later in the introduction.

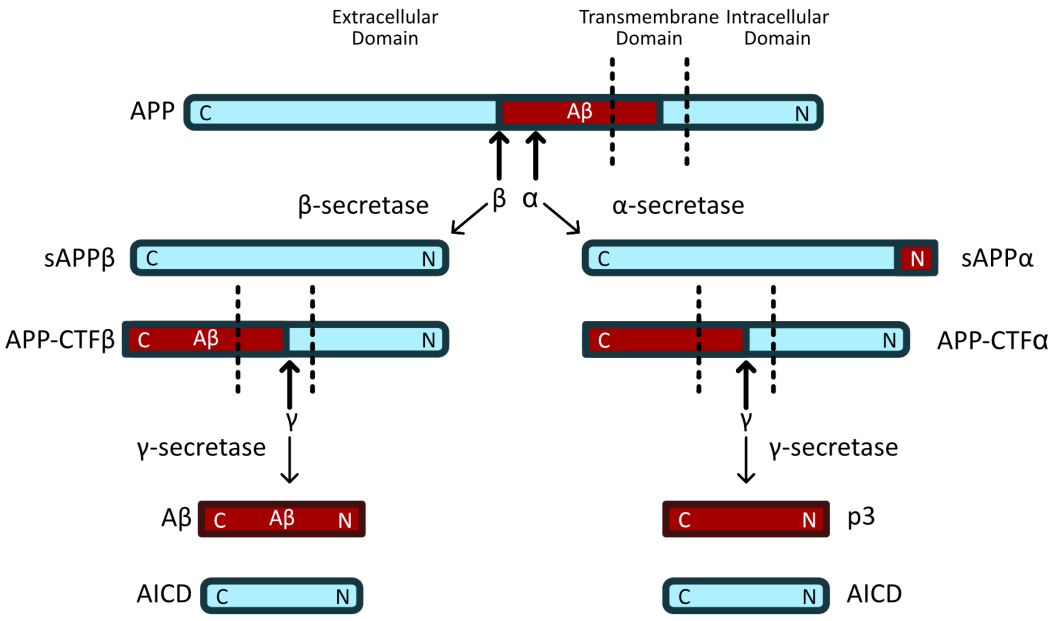

Figure 1.2: Schematic of APP processing by α-, β-, and γ-secretases. Aβ motif is shown in maroon; C and N denote C- and N-termini. Dotted lines indicate where proteins sit in the membrane.

Despite the compelling evidence that Aβ, and particularly the aggregation prone $A\beta_{1-42}$, are important for AD pathogenesis, there is only a loose correlation between areas of plaque formation and synapse loss or extent of cognitive decline in AD patients (Arriagada et al., 1992; Hardy and Selkoe, 2002). This suggests that it is not Aβ plaques that are the neurotoxic species that drives AD.

Insight into a possible candidate was provided by early work on the kinetics of plaque formation. The β sheet rich structure of the Aβ protein increases its propensity for protein-protein interactions via larger area hydrogen bonding, resulting in aggregation. It was found that plaque formation occurs via a nucleation dependant polymerization (Xue et al., 2008). By this mechanism, soluble monomers aggregate together in thermodynamically unfavourable and kinetically slow processes to give a "nucleus". Once the nucleus is formed, the more favourable enthalpic interactions upon monomer addition to the nucleus of Aβ proteins overcomes the unfavourable entropic contributions, resulting in a swift and thermodynamically favourable aggregation to give amyloid fibrils. The rate limiting step in this model, therefore, is the nucleation process, where monomers aggregate together to form a high energy intermediate oligomer. Once this has happened, fibril growth occurs rapidly (Figure 1.3). $A\beta_{1-42}$ has been found to undergo the nucleation process more quickly than $A\beta_{1-40}$, thereafter able to provide a nucleus for aggregation of both $A\beta_{1-42}$ and $A\beta_{1-40}$ monomers (Figure 1.3) (Jarrett and Lansbury, 1993). As well as this Primary (1°) nucleation independent of previously formed fibrils, work done on islet amyloid protein (IAPP), which has similar aggregating properties as Aβ, has revealed that fibril formation also occurs via a secondary (2°) nucleation whereby monomer conversion to fibrils is catalysed by the presence of existing fibrils (Ruschak and Miranker, 2007). Indeed, it seems that this 2° nucleation is responsible for the majority of oligomer formation, resulting in a positive feedback loop, whereby generation of Aβ fibrils leads to an increase in rate of monomer oligomerisation

(Cohen et al., 2013). All of these processes are reversible, meaning that monomers exist in dynamic equilibrium with oligomers and that oligomers are in equilibrium with plaques as well (Figure 1.3).

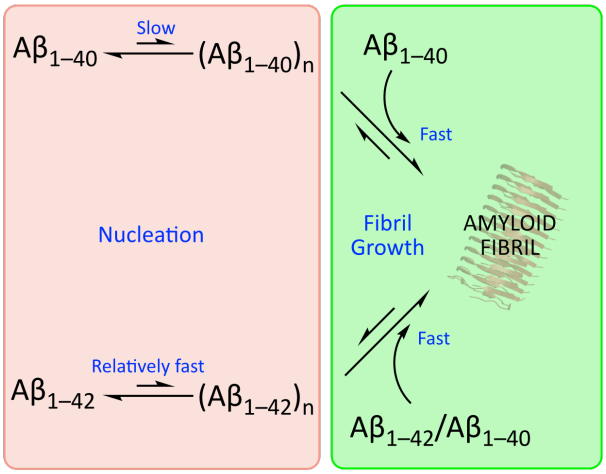

Figure 1.3: Proposal for amyloid fibril formation based on kinetic studies, with nucleation (slow), followed by fibril growth (fast). Nucleation of the amyloidogenic $A\beta_{1–42}$ occurs at a greater rate. Image PDB ID: 2mxu (Xiao et al., 2015).

Although the Aβ plaques had been extensively studied, little was known about the oligomers that lie as intermediates between the Aβ monomer and development of the insoluble plaque. With advancements in detection techniques, such as enzyme-linked immunosorbent assays (ELISAs) (Lue et al., 1999) and Europium Immunoassays (EuIAs) (Näslund et al., 2000; Wang et al., 1999) it became possible to detect the kinetically soluble oligomers of a variety of lengths that are formed during the nucleation period, or lag phase, of Aβ aggregation prior to formation of insoluble Aβ plaques. Studies performed using a variety of different assays able to detect these soluble Aβ oligomers (Aβo) showed that disease progression correlates well with the concentration of these soluble Aβo (Lue et al., 1999; McLean et al., 1999; Näslund et al., 2000; Wang et al., 1999; Welikovitch et al., 2018) and have helped to identify that Aβo accumulation can trigger an early inflammatory response in the brain that precedes other AD pathology including plaque formation (Welikovitch et al., 2020). Early work in rodents showed that addition of soluble Aβo were able

to cause neurotoxicity *in vitro* (Lambert et al., 1998) and that they could cause learning and memory impairments *in vivo* (Walsh et al., 2002).

Further confirmation that these Aβo were the primary pathogenic species in AD remained elusive for some time, as there were a variety of different sizes of oligomers in solution that were hard to separate. In addition, it was hard to confirm the toxicity of these oligomers using synthetic Aβo, since there was large irregularity in composition. More physiological and consistent oligomers have been obtained by using human Aβo secreted into cultures of Chinese hamster ovary, expressing mutated APP. Work performed *in vivo* injecting oligomers produced in this way showed that these soluble Aβ oligomers disrupt cognitive function in rats (Cleary et al., 2005). Studies carried out *in vitro* also showed that smaller, globular oligomers are neurotoxic, with denaturing electrophoresis suggesting that the identity of the toxic species was in the range of dimers to 24mers (Chromy et al., 2003). With new improvements in Aβo production, both synthetic and human Aβo, particularly the longer aggregation prone isoform ($A\beta_{1-42}$) (Viola and Klein, 2015), have been shown to cause spine loss and reduced synaptic plasticity (attenuation of long-term potentiation (LTP), with facilitation of long-term depression (LTD)) both *in vitro* and *in vivo*, resulting in eventual synaptic death (Lambert et al., 1998; Li et al., 2009; Shankar et al., 2007; Shankar et al., 2008; Sheng et al., 2012; Walsh et al., 2002).

1.1.2 Possible Mechanisms of Aβo-induced Pathogenesis

Despite identification of Aβo as key drivers of AD pathology, the mechanism by which this occurs remains unknown. Aβo have been shown to have a high binding affinity with a number of different proteins, and each has been proposed to be a receptor through which Aβo act (Table 1.1) (Jarosz-Griffiths et al., 2016; Mroczko et al., 2018; Viola and Klein, 2015). Of these, there have been several promising candidates, including Prion protein (PrP) (Lauren et al., 2009), the insulin

receptor (IR) (Townsend et al., 2007), α7-containing nicotinic acetyl choline receptors (α7nAChR) (Wang et al., 2000a; Wang et al., 2000b) human leukocyte immunoglobulin-like receptor B2 (LilrB2) / the murine equivalent paired immunoglobulin-like receptor B (PirB) (Fernandez-Vizarra et al., 2012; Kam et al., 2013; Kim et al., 2013), triggering receptor expressed on myeloid cells 2 (TREM2) (Lessard et al., 2018), and tyrosine kinase ephrin type-B receptor 2 (EphB2) (Shi et al., 2016).

Receptor	Abbreviation
N-Methyl-D-aspartate receptor	NMDAR
α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor	AMPAR
Metabotropic glutamate receptor mGluR	mGluR
Cellular prion protein	PrP^{C}
β2 -Adrenergic receptor	β2AR
α7 nicotinic acetylcholine receptor	α7nAChR
Insulin receptor	IR
p75 neutrotrophin receptor	p75NTR
Human leukocyte immunoglobulin-like receptor B2	LilrB2
Paired immunoglobulin-like receptor B	PirB
Fragment crystallizable gamma receptor II b	FcγRIIb
Triggering receptor expressed on myeloid cells 2	TREM2
Tyrosine kinase ephrin type-A receptor 4	Eph4A
Tyrosine kinase ephrin type-B receptor 2	EphB2
Receptor for advanced glycation end products	RAGE
Megalin (glycoprotein 330, low density lipoprotein-related protein 2)	gp330, LRP2
Formyl Peptide Receptor 2	FPR2
Frizzled	
Neuroligin	NLGN

Table 1.1: List of suggested Aβo receptors.

1.1.2.1 Prion Protein

Of the receptors linked with mediating Aβo toxicity, PrP is a propitious contender for mediating Aβo toxicity. This protein is also linked with neurodegeneration in its own right, involving the conversion of normal cellular PrP (PrP^{C}) to an infectious form (PrP^{Sc}) (Aguzzi et al., 2008), with pathologies that are shared with AD (Hainfellner et al., 1998). The first evidence of some possible

connection between PrP and AD was found using PrP^C immunoreactivity studies which show increased expression of PrP^C in several areas of the brain in AD patients (Voigtländer et al., 2001). Furthermore, genetic studies have shown that the gene encoding PrP^C (*PRNP*) is an AD susceptibility gene (Bertram et al., 2007), and that a Met/Val 129 polymorphism in *PRNP* is a risk factor for AD (Del Bo et al., 2006; Dermaut et al., 2003; Riemenschneider et al., 2004).

Further work using transgenic mice expressing mutant APP and hamster PrP^C revealed that prion protein may, in fact, promote plaque formation: animals expressing mutant APP and PrP^C together had increased Aβ plaque formation compared to those expressing mutant APP alone. Furthermore, $Aβ_{1-40}/Aβ_{1–42}$ ratios were the same with or without mutant PrP^C expression, and double-labelling immunostaining showed co-localisation of PrP^C and Aβ plaques suggesting that PrP promotes aggregation without altering the $Aβ_{1–40}/Aβ_{1–42}$ ratio (Schwarze-Eicker et al., 2005).

In addition, PrP^C has been shown to interact with and potentially mediate the neurotoxic effects of Aβo. PrP^C binds to Aβo with nanomolar affinity (Lauren et al., 2009) and Aβ-dependent plasticity deficits were not observed in slices of mice lacking PrP, or in the presence of PrP^C antibodies that prevent Aβ binding to PrP^C (Barry et al., 2011; Gimbel et al., 2010; Lauren et al., 2009). It is possible, therefore, that PrP^C mediates the neurotoxicity of Aβo; recent work has discovered that a PrP^C-Aβ complex causes an activation of Fyn, a kinase that phosphorylates the NR2B subunit of the NMDAR, a key protein in many forms of plasticity (Grant, 1996). Moreover, work using neuronal cultures derived from $Prn^{-/-}$ mice, $Fyn^{-/-}$, and APP/PS1 AD model mice shows that PrP^C-Aβ activation of Fyn leads to an initial increase, followed by a loss of surface NMDARs (Um et al., 2012). These findings would be consistent with other observations that Aβ oligomers lead to an impairment of synaptic plasticity. Further work in cell cultures has shown

that metabotropic glutamate receptor 5 (mGluR5) complexes with both PrP^C and Fyn, and that it is necessary for Aβo-PrP^C-induced synaptic dysfunction (Um et al., 2013).

The role of PrP^C in AD is still not clear. Although the normal cellular function of PrP^C is unknown, it has been linked with protection against oxidative stress (Linden et al., 2008) and with a neuroprotective action in rat retinal explants (Chiarini et al., 2002). Furthermore, Studies have indicated that PrP^C interacts with β-secretase, reducing Aβ production; overexpression of PrP^C reduces Aβ formation, with a depletion of PrP^C leading to an increase in Aβ production in cell cultures (Parkin et al., 2007). In addition to this, the amyloid intracellular domain (AICD), fragments of APP produced upon γ-secretase cleavage of APP (Figure 1.2), regulates the expression of PrP^C. These two pieces of evidence suggest that PrP^C could be involved in a feedback controlled regulation of Aβ production (Vincent et al., 2009): at high levels of Aβ production, there will be higher levels of AICD produced, resulting in an increased expression of PrP^C, triggering suppression of β-secretase activity, leading to a reduction in Aβ synthesis (Kellett and Hooper, 2009).

Furthermore, some studies indicate that PrP^C is, in fact, not necessary for Aβo induced reduction in LTP and neurotoxicity. Infection of organotypic slices with Sindbis virus driving expression of APPct100, an Aβ formation prone variant of APP, showed that there was no change in plasticity deficits between wild type and *Prnp*$^{-/-}$ slices. In addition, the *Prnp*$^{-/-}$ mice showed no alleviation in the loss of dendritic spines in the APPct100 infected slices, or in slices where $A\beta_{1–42}$ was added. These findings were corroborated *in vivo*, via injections of $A\beta_{1–42}$ into mice (Kessels et al., 2010). A further study showed that, although a nanomolar affinity was found for PrP^C by Aβo, no difference in the Aβo-induced reduction of learning and memory was found between mice expressing PrP^C and the *Prnp*$^{-/-}$ mice (Balducci et al., 2010).

These findings appear to be contradictory. Many agree that there is a high affinity between Aβo and PrP^C (Balducci et al., 2010; Kessels et al., 2010; Lauren et al., 2009), but there seem to be varying effects in different AD models with and without PrP^C knockouts. For instance APP/PS1 mice (coexpressing mutant APP and mutant PS1) show no significant differences in the Aβ-dependent reduction in learning and memory when over-expressing or lacking of PrP^C (Calella et al., 2010). Some studies have found that there is a mild attenuation in the plasticity deficits of APP/PS1 mice crossed with Glycosylphosphatidylinositol (GPI)-anchorless PrP^C, suggesting that secreted PrP^C might interfere with Aβ neurotoxic pathways in a neuroprotective way (Benilova and De Strooper, 2010; Calella et al., 2010).

As a result of these two conflicting strands of evidence, the extent of the involvement of PrP^C in Aβo-induced neuronal dysfunction is still a subject of much debate. One explanation for some of the findings from certain mouse models could be that the *Prnp* gene is located near to a quantitative trait locus (QTL) for Aβ levels (Ryman et al., 2008). QTLs are sections of DNA that correlate with changes in phenotype expression (Miles and Wayne, 2008). It has been demonstrated that QTLs can heavily affect the levels of Aβ (Calella et al., 2010), and so, it is possible that the PrP^C knockout mice may have some interference with these QTLs, resulting in an impact on Aβ production. The debate surrounding the involvement of PrP^C in AD pathology remains active, therefore, with many still believing that PrP^C could play some role in mediating an element of Aβo neuronal toxicity.

1.1.2.2 Insulin Signalling

A study looking at the connection between diabetes and dementia has thrown up another potential pathway involved in Aβ induced neurotoxicity; this study found that there was a greatly increased prevalence of diabetes in those who had dementia, particularly those with vascular dementia or

AD (Ott et al., 1996; Ott et al., 1999). This led to observations that AD cases have reduced brain glucose metabolism and impaired insulin signalling (De Felice et al., 2014; Steen et al., 2005). Insulin signalling is important for neuronal survival (Apostolatos et al., 2012), brain function, and even memory formation (Chiu et al., 2008; Zhao et al., 2011), suggesting that a disruption in the signalling cascade could play a part in the mechanism of Aβo-induced neurodegeneration and cognitive impairment.

Studies examining the effect of soluble oligomers of $A\beta_{1-42}$ on various proteins involved in insulin signalling have corroborated that Aβo do have some interaction with this important neuroprotective pathway. Application of varying concentrations of $A\beta_{1-42}$ oligomers increased expression of glycogen synthase kinase 3 (GSK-3β) and reduced that of the insulin receptor (IR), resulting in increased activity of GSK-3β in neuroblastoma cells (Bartl et al., 2013). Given that the overactivity of GSK-3 is linked with memory impairment, tau hyperphosphorylation, increased $A\beta_{1-42}$ production, and inflammatory responses, aberrant activity of this kinase is of extreme interest (Hooper et al., 2008). In addition, soluble $A\beta_{1-42}$ oligomers also appear to affect the activity of other important metabolic proteins, including inhibiting monoamine oxidase B (MAO-B), a protein thought to have neuroprotective effects (Bartl et al., 2013), and decreasing phosphorylation of Akt (protein kinase B (PKB)), a measure of insulin signalling activity (Pearson-Leary and McNay, 2012).

The impairment of insulin signalling by $A\beta_{1-42}$ oligomers could, in part, be due to direct interactions between the oligomers and IRs; application of soluble Aβo causes neuronal surface IR removal in hippocampal neuronal cultures (De Felice et al., 2009; Zhao et al., 2008), and the oligomers have been found to bind to IRs directly, preventing their autophosphorylation (Townsend et al., 2007). In addition, an antagonist of the IR produces a similar pattern of kinase

inhibition to that induced by Aβo (Townsend et al., 2007). It could be that Aβo directly cause neuronal insulin resistance, at least in part, via interacting with the surface IRs, and this is supported by evidence showing that Aβo are a direct competitive inhibitor of insulin binding to IRs (Xie et al., 2002).

As well as the fact that Aβo have a detrimental effect on neuronal insulin signalling, insulin also appears to have protective effects against accumulation of Aβo: genetically unmodified rabbits, which can form human-sequence Aβ, showed a large accumulation of Aβo in the brain and retina after diabetes was induced via the use of alloxan (Bitel et al., 2012), while application of insulin to mature neuronal slices completely prevents Aβo-induced spine loss, oxidative stress, and IR removal (De Felice et al., 2009; Zhao et al., 2009). In addition, the effects submaximal doses of insulin can be boosted by insulin-sensitizing drugs (De Felice et al., 2009). Mechanistically, this insulin-induced protection is dependent on IR tyrosine kinase activity, suggesting that this protective effect is as a result of IR signalling to give clearance of Aβo, or downstream removal of Aβ binding sites (De Felice et al., 2009). Therefore, it appears that there is a vicious cycle whereby impaired insulin signalling leads to an accumulation of Aβo, which, in turn, leads to damage of synapses and further impairment of neuronal insulin signalling, and so on. This cycle is an important discovery and mirrors the accelerating nature of the disease progression.

Many similarities have been drawn between type 2 diabetes mellitus (T2DM) and AD, leading to the suggested definition of AD as type 3 diabetes mellitus (T3DM) (Suzanne, 2014; Suzanne and Wands, 2008). T2DM and AD cases show metabolic stress together with proinflammatory signalling that leads to a reduction in insulin signalling and a decrease in the cells ability to respond to insulin (De Felice et al., 2014). This insulin resistance has large impacts on the cell's ability to metabolise (De Felice et al., 2014). The mechanism for the development of insulin resistance could

be the same in T2DM and AD. Both diseases show altered gene expression and activity of many proteins all involved in insulin signalling, with application of Aβo able to recreate this altered expression (Lourenco et al., 2013; Pearson-Leary and McNay, 2012; Steen et al., 2005). Furthermore, the anti-diabetes drug exendin-4 (exenatide) was able to prevent many of the effects of Aβo induced pathogenies, including impaired axonal transport, in mice (Bomfim et al., 2012). Although both T2DM and AD show this very similar pathology and gene expression, there are distinct differences between the two. T2DM is a common comorbidity with AD, however, it does not necessarily lead to AD. In addition, insulin signalling deficiencies present in AD are found predominantly in the brain, unlike T2DM, implying that the two diseases, although linked, are not one and the same, resulting in a possible classification of AD as a separate type of diabetes (Suzanne, 2014; Suzanne and Wands, 2008).

Despite this evidence that insulin signalling is impaired in AD and in AD models, it is unclear how directly it drives other aspects of the disease such as impaired plasticity and cognitive decline. It is clear, however, that damaging insulin signalling resistance increases risks of AD, and so, strengthens the case for a healthy diet and exercise in reducing the risks of cognitive decline and AD (Ngandu et al., 2015; Scarmeas et al., 2009).

1.1.2.3 α7nAChR

Since there is widespread loss of cholinergic neurons, particularly of the basal forebrain (Mobley and Chen, 2019), and early observations of AD pathology have shown loss of presynaptic nAChRs (Whitehouse et al., 1986), it seemed possible that nAChRs could play a role in Aβo-induced neurodegeneration. Of the many subtypes of nAChRs, α7nAChRs have been highlighted as the most involved, since they are upregulated in the hippocampi of AD patients (Hellström-Lindahl et al., 1999) and neurons expressing them having greater susceptibility to Aβo neurodegeneration

(Oddo et al., 2005). Indeed, immunoprecipitation and ligand-binding studies have revealed that $A\beta_{1-42}$ oligomers bind to α7nAChRs with a picomolar affinity (Wang et al., 2000a; Wang et al., 2000b) and more recent studies have shown that Aβo bind to α7nAChRs and can act as an antagonist (Liu et al., 2001; Sadigh-Eteghad et al., 2014).

There appears to be some controversy as to the effects of α7nAChRs on Aβo-induced pathology. Several studies show that α7nAChR is important for AD pathology, with α7nAChR KO mice exhibiting protection against Aβo-induced synaptic dysfunction and learning and memory deficits (Dziewczapolski et al., 2009; Wang et al., 2003), however, other studies demonstrate that α7nAChRs have a neuroprotective action against Aβo; AD transgenic (Tg2576) α7nAChR KO mice exhibit enhanced learning and memory deficits together with elevated accumulation of Aβ compared to the pure AD transgenic (Hernandez et al., 2010). Several studies *in vitro*, *in vivo*, and in humans have hinted that the mechanism of this protective effect could be via direct α7nAChR-dependent internalisation of extracellular Aβo (Baker-Nigh et al., 2015; Nagele et al., 2002; Oddo et al., 2005; Wang et al., 2002). Another study has revealed that a partial α7nAChR agonist, SSR180711, could restore Aβo-induced plasticity deficits (Kroker et al., 2013). This ability of α7nAChR to rescue LTP changes could be a contributing mechanism as to how AChIs are able to mask AD symptoms.

1.1.2.4 LilrB2/PirB and FcγRIIb

Recently, it has been shown that two immune receptors LilrB2 (and its murine homologue PirB) and FcγRIIb bind Aβo (Fernandez-Vizarra et al., 2012; Kam et al., 2013; Kim et al., 2013). In addition, both proteins appear to be necessary for the plasticity deficits seen in AD mouse models: FcγRIIb deficient AD model mice (hAPP J20) exhibited a reduction in the LTP attenuation

observed in mice expressing FcγRIIb (Kam et al., 2013), and LilrB2/PirB are required for Aβo induced memory, learning, and plasticity deficits observed in AD mouse models (APPSwe/PS1ΔE9) (Kim et al., 2013). Interestingly, PirB is involved in the attenuation of LTP in mice that occurs with aging (Bochner et al., 2014), with PirB deficient mice showing strengthened synaptic plasticity (Djurisic et al., 2013). It could be that Aβo interact in some way with these proteins to enhance their suppression of synaptic plasticity.

1.1.2.5 TREM2 and ApoE ε4

As well as looking at the mutations that lead to fAD, genetic studies of late-onset (sporadic) AD cases can shed light on the mechanism of action of AD. Genome wide association studies have shown numerous 'risk factors' for late-onset AD, but of these, two proteins linked with the immune system have a striking potency. These are the ε4 variant of the apolipoprotein E (ApoE ε4) (Corder et al., 1993; Harold et al., 2009; Saunders et al., 1993; Strittmatter et al., 1993a), and triggering receptor expressed on myeloid cells 2 (TREM2) (Guerreiro et al., 2012; Jonsson et al., 2012).

TREM2 is a microglia specific protein (Hickman and El Khoury, 2014) that is upregulated in plaque associated tissue in AD transgenic mice (APP23) and colocalises with microglia (Frank et al., 2008), while ApoE is a glycoprotein that mediates transport of lipids and cholesterol (Kanekiyo et al., 2014). Interestingly, other alleles of ApoE, ε2 and ε3, give a lowered risk of AD (Castellano et al., 2011), with ApoE ε2 conferring protection against late-onset AD (Corder et al., 1994). The three ApoE variants stimulate neuronal signal transduction cascades, including APP synthesis and synapse generation, in neuronal stem cell models in an ApoE ε4>ApoE ε3>ApoE ε2 manner (Huang et al., 2019). Both ApoE (LaDu et al., 1994; Strittmatter et al., 1993a; Strittmatter et al.,

1993b; Wisniewski et al., 1993) and TREM2 bind Aβ with high affinity (particularly $A\beta_{1-42}$ (Lessard et al., 2018)), and AD-related mutations in TREM2 reduce this binding affinity (Zhao et al., 2018).

It appears that ApoE and TREM2 interact together and are important in microglial phagocytosis of Aβ: ApoE is a ligand of TREM2, and Aβ bound ApoE is taken up by microglia in a TREM2 dependent manner, with uptake and ApoE binding being impaired in AD- linked mutated TREM2 cell culture models (Atagi et al., 2015; Yeh et al., 2016). Further evidence points to the importance of TREM2 in microglial removal of Aβ; in AD mouse models, TREM2 KO mice show fewer microglia surrounding plaques and greater neuritic damage (Wang et al., 2015; Wang et al., 2016b). This has also been observed in TREM2 heterozygous knockouts, together with a reduction in the expression of immune signalling molecules (Ulrich et al., 2014).

Clearance of, and impairment of Aβ clearance is increasingly becoming recognised as an important part of AD pathogenesis. This TREM2-ApoE interaction appears to be vital to our ability to remove Aβ, which becomes impaired in old age (Kress et al., 2014). Treatments such as Aβ immunotherapy are diminished in TREM2 deficient mice (Xiang et al., 2016), further highlighting the importance of functioning microglia in resisting and possibly treating AD. Although targeting TREM2 could provide interesting therapeutic interventions to AD, studies have shown that its ability to ameliorate amyloid pathology change as the disease progresses. Over-expression of TREM2 reduces amyloid-related pathology early on (Jiang et al., 2014), but has no effect in aged animal models (APPSwe/PS1ΔE9) (Jiang et al., 2017). Other studies have also suggested that, although TREM2 is protective against Aβ pathology in the early stages of AD, it in fact augments it later on in the disease progression in AD transgenic mice (APPSwe/PS1 L166P) (Jay et al., 2017; Jay et al., 2015). Despite this, it is clear that preventing accumulation of Aβo is important

for normal physiological function, and this offers a wealth of potential therapeutic areas of intervention.

1.1.2.6 EphB2

Given that Aβo induce changes in synapse morphology, density, and plasticity (Lambert et al., 1998; Li et al., 2009; Selkoe, 2008; Shankar et al., 2007; Shankar et al., 2008; Sheng et al., 2012; Walsh et al., 2002), there has been interest in the Tyrosine kinase EphB2. EphB2 and other EphB receptors interact with NMDARs upon binding Ephrin (Dalva et al., 2000) leading to phosphorylation of NMDARs and activation of NMDAR-dependent gene expression (Takasu et al., 2002). Furthermore, mice lacking EphB2 have normal hippocampal morphology, but show plasticity deficits (Grunwald et al., 2001), including reduced hippocampal LTP at CA1 and dentate gyrus (DG) synapses, coupled with attenuated synaptic NMDAR currents (Henderson et al., 2001). EphB receptors are involved in synapse formation and morphogenesis (Henkemeyer et al., 2003), with, presynaptic EphB2 regulating synaptic density and formation via interactions with postsynaptic Ephrin-B3 (McClelland et al., 2010).

There is evidence, for changes in EphB2 signalling in AD models. APP transgenic mice (hAPP J20 and Tg2576) show a reduction in EphB2 receptor expression and downstream EphB2 signalling, with this occurring prior to impairment of object recognition and spatial memory (Simon et al., 2009). Furthermore, it appears that EphB2 signalling changes are involved in Aβo mediated AD pathogenesis, including learning and memory alterations; EphB2 depletion in hAPP transgenic mice enhances cognitive impairment and plasticity deficits that can be reversed by increasing EphB2 levels (Cisse et al., 2011). Overexpression of EphB2 improves memory deficits in APPSwe/PS1ΔE9 mice together with a reduction in Aβo-induced depletion of EphB2 and NR2B NMDARs (Hu et al., 2017) in a manner involving interaction between the EphB2 PDZ-

binding domain and AMPAR GluA2 subunit (Miyamoto et al., 2016) and activation of EphB2 receptor induces tau dephosphorylation and inhibition of the tau kinase GSK-3β (Jiang et al., 2015). Interestingly, there is recent evidence that EphB2 binds Aβo directly, and that blocking this not only rescues Aβo-induced depletion of EphB2, but also improves memory deficits in transgenic mice and phosphorylation and surface expression of NR2B NMDARs (Shi et al., 2016). This evidence clearly suggests an important role for EphB2 in AD pathogenesis and suggests EphB2 signalling as a potential druggable target.

1.1.2.7 Properties of APP in Physiology and Disease

As well as looking at the interactions between Aβo and other proteins, there may be some clues to AD pathogenesis in the endogenous properties of both Aβo and APP. Although the physiological functions of both proteins remain poorly understood, they appear to play a role in a variety of processes including neurogenesis, synaptic formation, and strengthening (Conboy et al., 2005; Doyle et al., 1990; Huber et al., 1993; Mileusnic et al., 2000; Soba et al., 2005; Southam et al., 2019; Wang et al., 2014; Wang et al., 2009).

Although it can be hard to differentiate between the functions of APP itself and those of the APP cleavage products, some endogenous functions have been suggested; APP is upregulated during neuronal development (Loffler and Huber, 1992), and has been linked, both then and post development, with neurogenesis (Southam et al., 2019; Wang et al., 2014; Wang et al., 2009), memory formation (Conboy et al., 2005; Mileusnic et al., 2000), and memory retention (Doyle et al., 1990; Huber et al., 1993). In addition, APP KO mice show cognitive and LTP deficits (Dawson et al., 1999) with lower neuronal survival rates in the hippocampus (Wang et al., 2016a).

Interestingly, APP can also form dimers (Dahms et al., 2010) and it seems that these dimers are important for cell adhesion (Soba et al., 2005; Wang et al., 2009), controlling Aβo levels (Eggert et al., 2009), and enhancing presynaptic glutamate release (Fogel et al., 2014). Indeed, APP appears to be involved in regulating presynaptic function in various ways. APP and other members of the APP family (APP like protein 1 (APLP1) and APP like protein 2 (APLP2)) are part of the presynaptic active zone around docked vesicles (Lassek et al., 2013), and APP deletion reduces the expression of the synaptic vesicle proteins synaptophysin, synaptotagmin-1, and synaptic vesicle protein 2A (SV2A) (Lassek et al., 2014). In addition, APP is a regulator of the presynaptic active zone in mouse hippocampal neurons (Lassek et al., 2016), with the APP intracellular zone being found to facilitate glutamate release at hippocampal boutons (Fanutza et al., 2015).

APP also appears to interact with a variety of proteins (Table 1.2), including F-Spondin (Ho and Sudhof, 2004), Neurogenin 2 (Bolos et al., 2014), Slit (Wang et al., 2017a), Notch receptors (Chen et al., 2006; Oh et al., 2005), Nogo-66 (Park et al., 2006), Netrin-1 (Lourenco et al., 2009), and Aβ (Lorenzo et al., 2000). It seems that APP is required for Aβo-induced changes in neuronal network excitability (specifically the excitatory/inhibitory (E/I) balance (Wang et al., 2017b) and for Aβo toxicity (Lorenzo et al., 2000). Interestingly, APP also to plays a role in regulating Aβo levels in a neuronal activity-dependent manner (Kamenetz et al., 2003), and APP interactions with Nogo-66 (Park et al., 2006) and Netrin-1 (Lourenco et al., 2009) appear to regulate Aβo levels, suggesting that APP is involved in some feedback mechanism for Aβo production.

Taken together, this evidence shows that APP performs a variety of tasks, particularly in neural development, and promoting neurotransmitter release. It is of note that APP is required for Aβo-induced pathology, and that it plays a role in regulating Aβo levels. It could be that changes in physiological APP functions upon interacting with Aβo are behind some of the pathology of AD.

Receptor	Interaction	Reference
F-Spondin	Inhibit β-secretase cleavage of APP	(Ho and Sudhof, 2004)
Neurogenin 2	Regulate neurogenesis	(Bolos et al., 2014)
Slit	Mediate axonal guidance	(Wang et al., 2017a)
Nogo-66	Reduce Aβ plaque formation	(Park et al., 2006)
Netrin-1	Regulate Aβ formation	(Lourenco et al., 2009)
Notch Receptors	Unknown	(Chen et al., 2006; Oh et al., 2005)
Aβ	Required for Aβ pathology	(Lorenzo et al., 2000)

Table 1.2: Summary of APP interactions.

1.1.2.8 Properties of sAPPα in Physiology and Disease

As well as having physiological functions as a full-length peptide, there is much evidence that the derivatives of APP (Figure 1.2) play a physiological role. Of these, the α-secretase cleavage product of APP, sAPPα, has been extensively studied.

The functions of APP and sAPPα overlap. This could be because a lot of the properties observed in APP models are actually mediated by sAPPα. Among these overlapping functions, sAPPα has neurotrophic actions: sAPPα promotes neuronal differentiation in U251 glioma cells (Jiang et al., 2013) and is required for nerve growth factor (NGF)-mediated enhancement of neuronal viability in a manner involving insulin signalling (Akar and Wallace, 1998; Luo et al., 2001; Wallace et al., 1997a; Wallace et al., 1997b). In addition, APP KO mice show spine density deficits that can be rescued by knock in of sAPPα (Hick et al., 2015; Ring et al., 2007; Weyer et al., 2014). As well as rescuing, or enhancing spine density, sAPPα addition or overexpression can enhance LTP (Hick et al., 2015; Livingstone et al., 2019; Ring et al., 2007; Taylor et al., 2008) and learning and memory (Meziane et al., 1998), in addition to NMDAR currents (Taylor et al., 2008), with LTP facilitation involving activation of Arc synthesis (Livingstone et al., 2019). There appears to be some

controversy, however, as to the synaptic strengthening properties of sAPPα, as a recent study has shown that it has an inhibitory effect on presynaptic function by interacting with GABA receptors in mice (Rice et al., 2019). This suggests that sAPPα has a more complex role in modulating synaptic transmission than simply enhancing or inhibiting it.

sAPPα also appears to have a regulatory function on Aβ production via an interaction with β-secretase (also known as BACE-1) (Obregon et al., 2012; Peters-Libeu et al., 2015), with blockade of sAPPα increasing Aβ build-up, and overexpression leading to a reduction in Aβ levels (Obregon et al., 2012). This interaction seems to affect tau phosphorylation as well; β-secretase also appears to activate GSK-3 (a crucial kinase of tau) in induced pluripotent stem cells (Israel et al., 2012) and sAPPα overexpression inhibits GSK-3β phosphorylation of tau in mice (Deng et al., 2015), possibly via its regulation of β-secretase activity.

This evidence shows that sAPPα has a partially neuroprotective function against Aβ, suggesting that increasing sAPPα production might be a good area of therapeutic intervention. Given that there is evidence for reduced activity and expression of α-secretase in AD patients (Colciaghi et al., 2002; Marcinkiewicz and Seidah, 2000; Nistor et al., 2007; Tang et al., 2006), part of AD pathology might result from a change in the balance between sAPPα production and Aβ production.

1.1.2.9 Endogenous Properties of Aβ

Although much research is directed at pathophysiological functions of Aβ, it is possible that some clues as to the mechanism of Aβo toxicity can be found in any physiological function that Aβ might have. There has been some doubt as to whether Aβ is just a pathological by-product of APP

processing, or whether it serves a physiological role itself. There is evidence, however, in healthy brains, of constant, low levels of Aβ (picomolar range in rodents (Puzzo et al., 2008; Schmidt et al., 2005) and for humans 1,500 pM for $A\beta_{1-40}$, 200 pM for $A\beta_{1-42}$ in CSF; 60 pM for $A\beta_{1-40}$, 20 pM for $A\beta_{1-42}$ in plasma (Giedraitis et al., 2007)), suggesting that it may have a physiological function at these low concentrations (Cirrito et al., 2003). Aβ levels are influenced by synaptic activity (Cirrito et al., 2008; Cirrito et al., 2005) and this could involve APP (Kamenetz et al., 2003), further suggesting that Aβ plays some role beyond being an unwanted cleavage product of APP. More recent studies, able to detect low levels of Aβ and manipulate them by small amounts have shed more light on any physiological functions.

Similarly to APP and sAPPα, Aβ also shows properties that promote neuronal growth and differentiation; Aβ appears to have neurotrophic action (Yankner, 1996), encourage neuronal growth and survival (Bishop and Robinson, 2004; Giuffrida et al., 2010), protect against excitotoxic cell death (Giuffrida et al., 2009; Luo et al., 1996), and induce neurogenesis in a dose-dependent manner (Lopez-Toledano and Shelanski, 2004). This protective function of Aβ goes further; there is evidence that Aβ has antioxidant effects (Baruch-Suchodolsky and Fischer, 2009; Kontush et al., 2001; Nadal et al., 2008), the ability to bind and remove harmful substances (Robinson and Bishop, 2002), and some antimicrobial activity against common microorganisms (Soscia et al., 2010), with patients and animals showing increased vulnerability to infection upon treatment to lower Aβ levels (Dominguez et al., 2005; Green et al., 2009).

Strikingly, Aβ also has effects on synaptic plasticity, learning, and memory (Abramov et al., 2009; Garcia-Osta and Alberini, 2009; Morley et al., 2010; Mura et al., 2012; Puzzo et al., 2011; Zucker and Regehr, 2002). Minor, acute elevations of endogenous Aβ by inhibition of the Aβ clearing

peptidase, meprilysin, using the drug thiorphan, show elevated LTP together with activity dependent elevated neurotransmitter release probability in rodent hippocampal cultures and slices (Abramov et al., 2009) and further work in rat brain has revealed that this enhanced release is dose-dependent (Mura et al., 2012). Interestingly, Aβo-enhanced LTP requires α7nAChRs (Dineley et al., 2002), while low levels of Aβ enhance hippocampal ACh production in mice *in vivo* (Morley et al., 2010). Low doses of Aβ show an ability to improve cognitive function in rodents *in vivo*, including improved reference and contextual memory (Puzzo et al., 2012), memory retention (Morley et al., 2010), memory consolidation (Garcia-Osta and Alberini, 2009), and memory induction (Puzzo et al., 2011), in a dose dependent manner with higher, more pathological concentrations reducing memory and learning (Puzzo et al., 2012).

Therefore, it appears that low, more physiological levels of Aβ enhance presynaptic function, and so augment plasticity, learning, and memory. It could be that at higher concentrations, there is either a loss of this function, or even, an increase in presynaptic function, leading to the synaptic deficits that occur early on in AD.

1.1.3 Amyloid β oligomers and plasticity

Much of the evidence of studies looking at the endogenous effects of APP, sAPPα, and Aβ, and the interactions of Aβ with other proteins suggest a role for Aβ in synaptic transmission and plasticity. Synaptic plasticity is dysfunctional in AD, and it is becoming increasingly clear that these plasticity deficits are early pathological events and could even drive further AD pathology such as tau phosphorylation, and synapse loss (Mucke and Selkoe, 2012; Selkoe, 2008).

Various preparations of low molecular weight Aβo (Yang et al., 2017) (dimers (Hu et al., 2008; Muller-Schiffmann et al., 2016; Shankar et al., 2008; Welzel et al., 2014) and trimers (Townsend et

al., 2006)), and a variety of Aβ isoforms, including $A\beta_{1–42}$ (Chen et al., 2000; Hu et al., 2009a; Kimura et al., 2012; Ma et al., 2011; Nomura et al., 2005; Nomura et al., 2012; Rammes et al., 2018; Ripoli et al., 2014; Zhao et al., 2004), $A\beta_{1–40}$ (Chen et al., 2000; Hu et al., 2008; Nomura et al., 2012; Rammes et al., 2018; Schmid et al., 2008; Wu et al., 2008), and $A\beta_{25–35}$ (Chen et al., 2000; Holscher et al., 2007; Wu et al., 2008; Zhang et al., 2015), induce deficits in LTP (Lambert et al., 1998). $A\beta_{1–42}$ shows much higher potency for LTP inhibition compared to $A\beta_{1–40}$ (Nomura et al., 2012), while it seems that the active part of the Aβo is the N-terminus of the $A\beta_{35–45}$ isomer (Chen et al., 2000).

This inhibition of LTP in rodent hippocampus also appears to be accompanied by an associated facilitation of LTD, whereby LTD can be achieved with subthreshold stimuli such as 300 stimuli at 1 Hz (Huang et al., 2018; Li et al., 2009; Ondrejcak et al., 2019; Salgado-Puga et al., 2017; Shankar et al., 2008), 180 stimuli at 1 Hz (Chen et al., 2013) or 900 stimuli at 3 Hz. Some studies have even shown that LTD can be induced using more high frequency, LTP-like induction protocols in the presence of Aβo (Kervern et al., 2012; Moreno-Castilla et al., 2016; Sanchez-Rodriguez et al., 2019), suggesting that pathological concentrations of Aβo induce a metaplastic change in synaptic tuning.

This Aβo-induced change in plasticity has been extensively studied using varied Aβo preparations, in a variety of models (particularly at glutamatergic synapses), and is thought to be among the very early pathological changes that occur in AD. Indeed, it could be that these changes in plasticity drive the rest of the disease progression; there is much evidence that normalising Aβo-induced plasticity deficits also rescue memory and learning deficits and also spine loss in transgenic mice (5xfAD – B6SJL-Tg) and upon oligomer addition (Hwang et al., 2017; Li et al., 2013). Indeed, synaptic weakening has been linked with spine shrinkage (Zhou et al., 2004) and even spine elimination (Wiegert and Oertner, 2013; Wiegert et al., 2018).

The exact mechanism of these Aβo-induced plasticity deficits still remain a mystery. The majority of the work has focussed on the post synaptic terminus and the Aβo-induced changes there. One of the proteins that appears to be affected in the presence of Aβo is the AMPAR. Since movement of AMPARs to the synapse is necessary for LTP to occur (Penn et al., 2017; Roth et al., 2019), it could be that Aβo affect synaptic expression of these in some way. Indeed, Aβo induce a reduction in synaptic surface AMPAR expression in animals (Hsieh et al., 2006; Minano-Molina et al., 2011; Roselli et al., 2005; Zhao et al., 2010). This AMPAR internalisation appears to be dependent on GluA3 containing AMPARs (Reinders et al., 2016), postsynaptic Ca^{2+}-influx (Minano-Molina et al., 2011) and subsequent calcineurin activation (Minano-Molina et al., 2011; Zhao et al., 2010). Interestingly, Aβo also seem to induce a change in AMPAR subtype-expression from GluA2 to the more Ca^{2+}-permeable GluA1 (Gilbert et al., 2016; Whitcomb et al., 2015) which could have impacts on homeostatic plasticity as well as other cellular signalling.

Alongside AMPARs, NMDARs are heavily implicated in mediating the pathological effects on synaptic transmission and learning and memory. This is, perhaps not surprising given the importance of NMDARs in synaptic plasticity (Citri and Malenka, 2008). Not only do Aβo induce changes in postsynaptic NMDAR function (Cerpa et al., 2010; Deng et al., 2014; Dewachter et al., 2009; Dinamarca et al., 2008; Ferreira et al., 2012; Goto et al., 2006; He et al., 2011; Kessels et al., 2013; Kurup et al., 2010; Lacor et al., 2007; Li et al., 2009; Li et al., 2011; Niidome et al., 2009; Rammes et al., 2018; Shankar et al., 2007; Sinnen et al., 2016; Snyder et al., 2005) but also, there is much evidence suggesting that NMDAR activation is required for Aβo-induced synaptic plasticity deficits (Hu et al., 2009a; Kessels et al., 2013; Klyubin et al., 2011; Lacor et al., 2007; Li et al., 2011; Opazo et al., 2018; Rammes et al., 2017; Rammes et al., 2018; Roselli et al., 2005; Shankar et al., 2007; Shankar et al., 2008; Tamburri et al., 2013; Varga et al., 2014, 2015; Wei et al., 2010) possibly

via a metabotropic action (Tamburri et al., 2013). In particular, NR2B containing NMDARs are implicated in Aβo pathogenesis; it appears that NR2B NMDAR activation is required for Aβo-induced plasticity deficits (Hanson et al., 2015; Hu et al., 2009a; Li et al., 2011; Opazo et al., 2018; Rammes et al., 2017; Rammes et al., 2018), with some studies in rodents implicating extrasynaptic NR2B-containing NMDARs (Hanson et al., 2015; Li et al., 2011).

There is evidence for altered postsynaptic NMDAR expression and function in the presence of Aβo, with some work showing a reduction in the expression of synaptic NMDARs/NMDAR-mediated excitatory postsynaptic currents (EPSCs) (Dewachter et al., 2009; Dinamarca et al., 2008; Goto et al., 2006; Kurup et al., 2010; Lacor et al., 2007; Rammes et al., 2018; Snyder et al., 2005), possibly due to NMDAR endocytosis (Dewachter et al., 2009; Dinamarca et al., 2008; Goto et al., 2006; Kurup et al., 2010; Lacor et al., 2007), and some a change from NR2B-containing NMDARs to NR2A, that depends on the metabotropic function of NR2B NMDARs (Kessels et al., 2013). There is also a suggestion that Aβo interact with NMDARs to reduce NMDAR-mediated excitotoxicity; pre-treatment with Aβo reduces NMDAR-mediated excitotoxicity via inhibition of NMDAR Ca^{2+} influx (Dewachter et al., 2009; Ferreira et al., 2012; Goto et al., 2006; He et al., 2011; Niidome et al., 2009) however, one study shows that Aβo interfere with neuroprotective mechanisms against NMDAR-mediated excitotoxicity, with Aβo (0.1–1 μM) preventing GSK-3-dependent removal of synaptic NR1 containing NMDARs (Deng et al., 2014).

There appears to be an increase in the extent of extrasynaptic NR2B NMDAR activation (Hanson et al., 2015; Li et al., 2009; Li et al., 2011) that is not due to additional expression of extrasynaptic NR2B NMDARs in the presence of Aβo (Li et al., 2011). This increase in the activation of extrasynaptic NMDARs has been suggested to be a result of elevated extracellular glutamate levels (Li et al., 2009; Li et al., 2011; O'Shea et al., 2008). Indeed, elevation of extracellular glutamate via

blockade of excitatory amino acid transporters (EAATs) has been shown to cause spillover of glutamate from the synapse and activation of extrasynaptic NMDARs (Harney et al., 2008; Li et al., 2011), enhanced LTD (Dutar and Potier, 2019; Valtcheva and Venance, 2019) and reduced LTP (Kervern et al., 2012; Li et al., 2011). The implication of extrasynaptic NMDARs is particularly relevant as they have been shown to be important for LTD induction (Papouin et al., 2012).

A wealth of other proteins have been implicated in Aβo-induced changes in plasticity, including tumour necrosis factor α (TNFα) (Samidurai et al., 2018; Wang et al., 2005), nAChRs (Lazarevic et al., 2017), Caspase-3 (Jo et al., 2011; Yi et al., 2018), GSK-3 (Deng et al., 2014; Jo et al., 2011; Shipton et al., 2011; Yi et al., 2018), Akt (Jo et al., 2011; Yi et al., 2018), CDK5 (Lazarevic et al., 2017), and calcineurin (Alzoubi et al., 2011; Lazarevic et al., 2017). Many of these proteins are heavily involved in plasticity (Bradley et al., 2012), and so, it may come as no surprise that they are necessary for the manifestation of Aβo-induced plasticity deficits.

1.1.3.1 Presynaptic release

The evidence for Aβo-induced postsynaptic changes is all very much in agreement, showing altered Glu receptor function and expression (Dewachter et al., 2009; Dinamarca et al., 2008; Goto et al., 2006; Kurup et al., 2010; Lacor et al., 2007; Minano-Molina et al., 2011; Rammes et al., 2018; Roselli et al., 2005; Snyder et al., 2005; Zhang et al., 2018; Zhao et al., 2010), impaired LTP expression (Li et al., 2011; Shankar et al., 2008), and facilitated LTD (Li et al., 2009). The effects of Aβo on the presynaptic terminus, however, are less clear. As has been discussed, more endogenous, physiological concentrations Aβ monomers and oligomers (pM) show facilitation of presynaptic release (Mura et al., 2012) and enhanced learning and memory (Garcia-Osta and Alberini, 2009; Morley et al., 2010; Puzzo et al., 2011; Puzzo et al., 2012), but this enhanced

presynaptic release appears to be dose dependent, with low concentrations augmenting release and high concentrations inhibiting it (Mura et al., 2012; Parodi et al., 2010; Puzzo et al., 2012). In addition, glutamate release appears enhanced early on in APPSwe/PS1 M146V transgenic mice, but this effect is lost later on in development (Cummings et al., 2015). Several other studies have shown that pathological levels of Aβo have no effect on presynaptic release probability (Cerpa et al., 2010; Li et al., 2009; Shankar et al., 2008; Talantova et al., 2013; Ting et al., 2007), but others have shown an Aβo-induced enhancement of release (Brito-Moreira et al., 2011; Dolev et al., 2013; Kabogo et al., 2010; Merlo et al., 2016; Russell et al., 2012; Wang et al., 2017b).

Experimental evidence is, therefore, confused. One reason for this is the difficulty of observing presynaptic release directly. Traditional methods of investigating presynaptic function rely on indirectly extrapolating information from postsynaptic responses, in the form of excitatory postsynaptic potentials (EPSPs), or large-scale, multisynaptic postsynaptic responses, field excitatory postsynaptic potentials (fEPSPs); delivering two action potentials in quick succession (up to 50 ms) induces a form of presynaptic plasticity known as paired pulse facilitation (PPF). The postsynaptic response to the second pulse is, generally, potentiated as a result of residual presynaptic Ca^{2+}, with the extent of the potentiation dependent on the release probability at the bouton; a high release probability causes a depletion of the readily releasable pool (RRP) of synaptic vesicles, leading to a reduced facilitation of the second pulse. An alternative method, pioneered by the Svoboda lab and Emptage *et al.*, involves the detection of all-or-nothing excitatory synaptically evoked postsynaptic Ca^{2+} transients (EPSCaTs) in response to single action potential (AP) stimuli to provide information on release probability (Emptage et al., 1999; Oertner et al., 2002; Padamsey et al., 2019). Although both of these systems provide insight into presynaptic function under basal conditions, they are confounded by changes to the postsynaptic terminus (Chang et al., 2006; Li et al., 2009; Shankar et al., 2007; Shankar et al., 2008; Whitcomb et al., 2015;

Zhang et al., 2018), as occurs in AD, where Aβo cause postsynaptic weakening, possibly due to AMPAR desensitisation (Li et al., 2009).

Recent advances in optical techniques have allowed for more direct measurements of the presynaptic terminus, without any of the confounds of postsynaptic changes. These involve the fluorescent dye, FM 1-43 (Ryan et al., 1997; Tokuoka and Goda, 2008), and two genetically encoded probes SynaptopHluorin (SypH), a reporter of vesicle fusion expressed in the lumen of synaptic vesicles that fluoresces at neutral pH and is quenched at the low pHs observed within the acidified synaptic vesicles (Figure 1.4) (Zhu et al., 2009), and iGluSnFR, a synaptically expressed fluorescing Glu sensor (Marvin et al., 2013; Marvin et al., 2018). All three techniques provide valuable information about the presynaptic terminus, however, whereas FM 1-43 and SypH allow for direct imaging of release, iGluSnFR is sensitive only to Glu, and so, is less able to inform about separate involvement of Glu reuptake compared to Glu release.

Both FM 1-43 dye (Brito-Moreira et al., 2011; Parodi et al., 2010), and SypH (Lazarevic et al., 2017) have been used to probe the effects of Aβo on presynaptic release to show that Aβo enhance release probability. Given the involvement of endogenous APP, APLP1 and APLP2 in release mechanisms (Lassek et al., 2013), and the evidence for physiological Aβo in enhancing release (Abramov et al., 2009; Mura et al., 2012), there is a strong suggestion for a presynaptic role for Aβo.

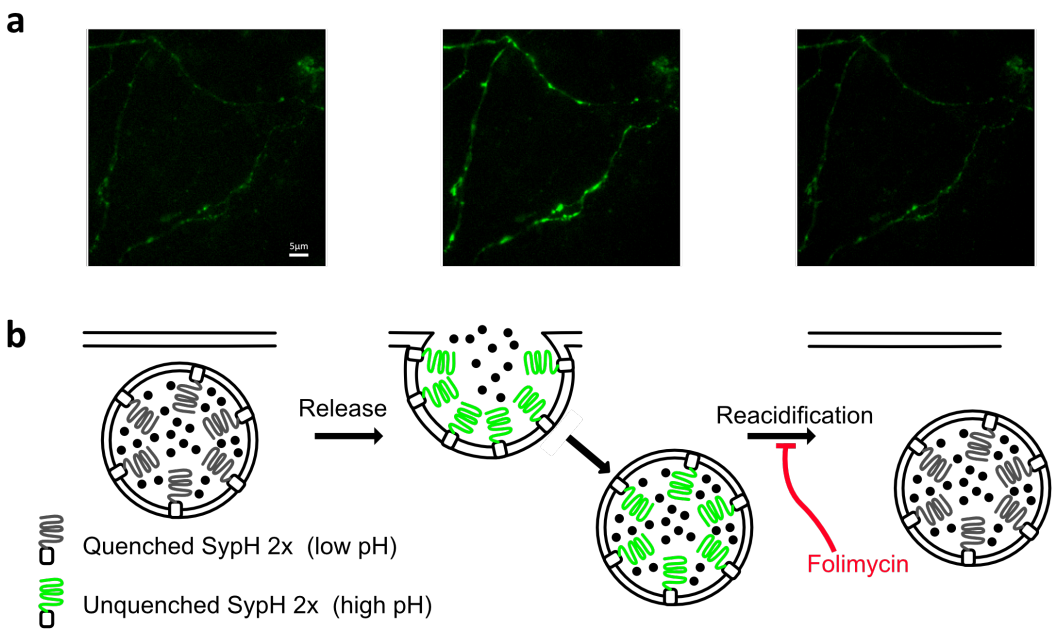

Figure 1.4: Representation of SypH 2x live image acquisition. **a)** Representative fields of dissociated hippocampal neurons transfected with SypH 2x, left to right – before release, after release (post-delivery of 100 AP at 10 Hz), after endocytosis and reacidification of vesicles (100 s later). **b)** mechanism of SypH 2x fluorescence, left to right – before release, after release and unquenching of SypH 2x, after endocytosis and reacidification of vesicles

1.1.4 Homeostatic plasticity and network dysfunction in AD

Homeostatic plasticity refers to the negative feedback mechanisms that keep neuronal excitability and firing anchored around a 'set-point' (Pozo and Goda, 2010). Traditional Hebbian plasticity, LTP and LTD, act as positive feedback systems, whereby undergoing synaptic strengthening increases the excitability of that synapse, and so, its ability to strengthen further, and vice versa if synaptic weakening occurs. This would result in excessive synaptic strengthening or weakening, which in turn impacts neuronal excitability. Therefore, without mechanisms that anchor neuronal excitability and synaptic strength, there would be a loss of synaptic input specificity and encoding of relative synaptic weights, leading to a loss of information encoding (Pozo and Goda, 2010; Turrigiano, 2012). In addition, given that neurons exist as parts of local and wider networks, homeostatic plasticity is vital for maintaining their stability and coordination, critical for normal brain function and memory encoding. A breakdown in homeostatic mechanisms would, therefore,

result in network-wide changes, including hyperactivity and seizures (Styr and Slutsky, 2018) that would significantly impair brain function and are implicated in several diseases, including epilepsy (Turrigiano, 2011), schizophrenia (Dickman and Davis, 2009), autism (Nelson and Valakh, 2015), and migraine (Welch, 2003).

As well as altering Hebbian plasticity, there is evidence that Aβo affect homeostatic plasticity (Styr and Slutsky, 2018) and this appears to have a profound knock-on effect on neural circuit function (Mucke and Selkoe, 2012; Palop and Mucke, 2016). These changes are apparent in AD patients decades before symptoms of cognitive impairment evolve (Bateman et al., 2012; Filippini et al., 2009; Mondadori et al., 2006; Reiman et al., 2012; Sperling et al., 2009) and appear to be important for much of AD pathology, especially learning and memory impairments (Palop and Mucke, 2016).

There are multiple proposed mechanisms by which neurons and networks homeostatically maintain stability by compensating to any changes (Turrigiano, 2012). These include activity-dependent regulation of neuronal firing (Marder and Prinz, 2003; Zhang and Linden, 2003), synaptic scaling (Davis, 2006; Turrigiano and Nelson, 2004), balancing network E/I ratio (Gonzalez-Islas and Wenner, 2006; Maffei et al., 2004), changes in synapse number (Kirov et al., 1999; Wierenga et al., 2006), changes in metaplasticity (Bienenstock et al., 1982), and regulation of intrinsic neuronal excitability (Marder and Goaillard, 2006; Turrigiano, 2011). A vast number of pathways are involved in implementing these feedback mechanisms, however, the identity of many of the proteins involved remain unknown. Of those that are known, a number are affected in AD or AD models (Table 1.3) (Styr and Slutsky, 2018). The dysregulation of these molecular 'players' would have an impact on a variety of homeostatic mechanisms; for example, down regulation of repressor element-1 silencing transcription factor (REST), as occurs in AD (Lu et al., 2014), results in an impairment of the presynaptic and intrinsic ability of neurons to adapt to hyperactivity in networks (Pecoraro-Bisogni et al., 2018; Pozzi et al., 2013) and dysregulation of the mammalian

target of rapamycin (mTOR) pathway, as has been implicated in AD (Lipton and Sahin, 2014), increases E/I ratio, leading to hyperexcitability (Bateup et al., 2013), possibly indicating an impairment to set-point regulation (Styr and Slutsky, 2018).

Protein	Involvement in Homeostatic plasticity	Involvement in AD
PS1	PS1 mutation disrupts synaptic scaling	Mutations leads to FAD; Involved in cleaving APP to give Aβ
BACE1 (β-secretase)	BACE1 KO mice show lack of synaptic scaling	Involved in cleaving APP to give Aβ
REST	Important for tuning presynaptic strength and intrinsic excitability to hyperactivity	Downregulated in AD
TNF α	Postsynaptic upscaling to inactivity	Increases Aβ production and inhibits secretion of sAPP; increases BACE1 expression and suppresses Aβ degradation
BDNF	Postsynaptic scaling and E/I balance; presynaptic adaptation	BDNF treatment improves neuronal loss in early AD model; BDNF interacts with APOE polymorphism to affect memory decline in AD
CDK5	Synaptic scaling; presynaptic adaptation	Hyperactivation promotes neurodegeneration
Arc	Synaptic scaling	Regulates activity-dependent Aβ production; reduced Arc mRNA in AD models
NPTX2	Regulate synaptic scaling of excitatory synapses onto PV interneurons	Downregulated in AD
mTOR	Regulates E/I balance and firing rate; regulates presynaptic homeostatic adaptations	Reduction in mTOR signalling improves AD-pathology in models
CaMKK2	Inhibition occludes synaptic scaling	Inhibition rescues Aβ-induced spine loss
CaMKII	Presynaptic and postsynaptic adaptations	Reduced in synapses in AD
CaN	Inhibition leads to homeostatic synaptic plasticity	Hyperactivated in AD
VGCCs	Mediate presynaptic adaptation and postsynaptic scaling	APP regulates L-type VGCCs in interneurons
RyR	Synaptic scaling	Increased RyR-mediated Ca^{2+} release in AD models
STIM2-SOC-CaMKII	Spine stability	Downregulation causes spine loss in PS1 M146V knock in mice
Retinoic Acid	Synaptic scaling	Rescues AD-like pathology in AD mice (APPSwe/PS1ΔE9)
$GABA_BR$	Presynaptic and postsynaptic adaptations; firing rate homeostasis	APP is a core component of presynaptic $GABA_BR$ complex; regulates Aβ40/42 ratio
Adenosine Receptors	Sleep homeostasis; Increased adenosine is antiepileptic	Overexpressed in hippocampus of AD patients and mice; regulate Aβ40/42 ratio during spike bursts

Table 1.3: Summary of the proteins that are implicated in both AD and homeostatic plasticity mechanisms. Adapted from Styr and Slutsky (2018). BDNF, brain derived neurotrophic factor; Arc, activity-regulated cytoskeleton-associated protein; NPTX2, Neuronal pentraxin-2; CaMKK2, Ca^{2+}/calmodulin-dependent protein kinase 2; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CaN, calcineurin; VGCC, voltage gated Ca^{2+} channel; RyR, ryanodine receptor; STIM2, stromal interaction molecule 2; SOC, store operated Ca^{2+}; $GABA_BR$, gamma aminobutyric acid receptor B;

Although research into how Aβo impair a neuron's ability to adjust to perturbations is in its infancy, the downstream impacts on a network level, and the implications to behaviour, learning, and memory are better characterised. A variety of AD animal models show neuronal hyperactivity (Busche et al., 2012; Busche et al., 2008; Busche et al., 2015; Maier et al., 2014; Rudinskiy et al., 2012), while AD patients (Bateman et al., 2012; Filippini et al., 2009; Mondadori et al., 2006; Reiman et al., 2012; Sperling et al., 2009) show similar alterations in network activity that can be observed prior to the manifestation of clinical symptoms. Such changes can also be seen early in other neurological disorders such as schizophrenia (Seidman et al., 2014) and epilepsy (Fahoum et al., 2013; Oser et al., 2014). In AD patients, one of the earliest observations is lowered deactivation of regions known collectively as the default mode network (DMN), comprising the medial prefrontal cortex, posterior cingulate cortex, and angular gyrus (Sperling et al., 2009), that show reduced activity during active brain states such as memory formation (Boyatzis et al., 2014; Raichle et al., 2001), together with hyperactivity of areas of the brain specifically involved in memory encoding such as the hippocampus (Bookheimer et al., 2000; Filippini et al., 2009; Quiroz et al., 2010; Sepulveda-Falla et al., 2012; Sperling et al., 2009; Trivedi et al., 2008). In late-stage AD, hippocampal hypoactivation is normally observed (O'Brien et al., 2010), although, reduced deactivation of the DMN remains (Celone et al., 2006; Persson et al., 2008; Reiman et al., 2012; Sperling et al., 2009). Interestingly, deactivation of the DMN is a better correlate for memory performance than excitation of regions that are actively involved (Sperling et al., 2010) and its

excessive activation could account for the prevalent appearance of amyloid plaques in these regions in AD transgenic mice (APP695) and upon Aβ addition (Bero et al., 2011; Palop and Mucke, 2016; Yamamoto et al., 2015). In addition to activity changes, network hypersynchrony has been observed early on in AD, resulting in epileptiform and seizure activity (Minkeviciene et al., 2009; Palop et al., 2007; Palop and Mucke, 2016). Indeed, AD is a risk factor for epileptic seizures (Hauser et al., 1986; Romanelli et al., 1990), particularly autosomal dominant, early-onset fAD (Palop and Mucke, 2016). Reducing this network hyperactivity has been shown to improve cognitive performance in MCI patients (Bakker et al., 2015; Bakker et al., 2012) and in AD model mice (Nygaard et al., 2015a; Sanchez et al., 2012) and remains a promising area of therapeutic intervention, with antiepileptic drugs such as leviteracetam showing some cognitive benefits in animal models (Sanchez et al., 2012) and AD patients (Bakker et al., 2015; Bakker et al., 2012).

These network-wide alterations may arise from impairment of more local circuit function. Specific areas of the brain have coordinated oscillatory activity at varying frequencies that are altered during different brain states. High frequency, gamma oscillations, are increased in amplitude in areas involved during memory encoding, whereas low frequency oscillations (alpha, beta, and theta) that are present in other cortical areas are depressed (Bentley et al., 2016; Lachaux et al., 2005; Matsumoto et al., 2013; Tallon-Baudry et al., 1996). Gamma oscillations are cross-frequency coupled to theta oscillations (Buzsaki et al., 2012; Buzsaki and Draguhn, 2004; Buzsaki and Wang, 2012), and gamma oscillatory activity correlates well with successful memory formation (Jensen et al., 2007; Matsumoto et al., 2013; Sederberg et al., 2007a; Sederberg et al., 2007b; Uhlhaas et al., 2009; Yamamoto et al., 2014). Gamma oscillations are decreased in AD and AD models (Cramer et al., 2012; Gurevicius et al., 2013; Herrmann and Demiralp, 2005; Ittner et al., 2014; Rubio et al., 2012; Verret et al., 2012) and there is a loss of coupling between low and high frequency oscillations (Jeong, 2004). In addition, there is evidence for increased epileptic activity when gamma oscillations are reduced in mice (Maheshwari et al., 2016), suggesting a causal link with the neuronal

hyperactivity observed in AD (Palop and Mucke, 2016). At a cellular level, oscillatory activity is controlled by inhibitory interneurons (Buzsaki and Draguhn, 2004), with gamma oscillations particularly relying on the coordinated activity of parvalbumin-positive (PV^+) interneurons (Cardin et al., 2009; Kemere et al., 2013; Lapray et al., 2012; Sohal et al., 2009). These interneurons are functionally impaired in AD models (APP23/PS45 and hAPP J20) or upon $A\beta_{1-42}$ addition (Busche et al., 2008; Chung et al., 2020; Kurudenkandy et al., 2014; Verret et al., 2012) possibly due to reduced expression of the voltage gated Na^+ channel $Na_V1.1$ (Corbett et al., 2013; Kim et al., 2007; Kim et al., 2014), and there is evidence that improving interneuron function improves memory deficits (Hunt et al., 2013; Southwell et al., 2014; Tong et al., 2014; Verret et al., 2012). There are increasing indications that reduced inhibitory tone does indeed drive much of the network hyperactivity in AD models (Busche and Konnerth, 2016); elevated neuronal activation occurs near to plaques (Busche et al., 2008), or upon Aβo addition (Busche et al., 2012), that is synaptic in origin, arising from reduced $GABA_A$ergic inhibition of the hyperexcited neurons (Busche et al., 2008), and enhancing GABAergic inhibition can rescue behavioural deficits and memory impairments in AD (Andrews-Zwilling et al., 2010; Sun et al., 2012) as well as reducing network hyperactivity and hypersynchrony (Busche et al., 2008; Busche et al., 2015).

There is also a suggestion that extracellular Glu plays a role in the aberrant increase in the E/I balance that occurs in AD and upon Aβo addition (Busche and Konnerth, 2016). As discussed, there is evidence that Aβo can increase extracellular Glu (Fogel et al., 2014; Li et al., 2009) and a recent study using *in vivo* imaging techniques has shown that addition of Aβo can induce neuronal hyperactivity as a result of increased extracellular Glu (Zott et al., 2019). Interestingly, this neuronal excitability and epileptic activity can be mimicked by increasing extracellular Glu with DL-threo-β-Benzyloxyaspartate (DL-TBOA) (Campbell et al., 2014). Therefore, it appears that these wide-ranging Aβo-induced network effects arise from a change in the E/I balance as a result of reduced

inhibitory tone together with increased excitatory neuronal activity. It is more than likely that the effects of Aβo on Hebbian and homeostatic synaptic plasticity also play an important role in maintaining these changes and therefore the resultant deficits in cognitive performance (Styr and Slutsky, 2018). Being able to rescue the E/I changes, therefore, is an important area of therapeutic intervention, that has already shown promising results from treatments such as the anti-epileptic drug levetiracetam (Bakker et al., 2015; Bakker et al., 2012) or the use of designer receptors exclusively activated by designer drugs (DREADDs) (Yuan and Grutzendler, 2016).

1.2 Tau phosphorylation

Although Aβo are the upstream trigger of AD pathogenesis (Mucke and Selkoe, 2012), the generation of pathologically phosphorylated and aggregated tau is also critical for the pathophysiological progression of AD (Ittner et al., 2010; Rapoport et al., 2002; Roberson et al., 2011; Roberson et al., 2007), and neurons from tau knockout mice are resistant to Aβo-induced deficits (Rapoport et al., 2002). Given the perceived importance of tau in proper neuronal function, however, and the relatively normal physiology of these tau knockout mice there is possibly some compensatory protein activity occurring that could confound evidence from these models (see section on models for more discussion). Aberrantly phosphorylated tau has a reduced affinity for MT binding, and is also prone to oligomerisation, like Aβo, and the formation of distinctive, soluble paired helical filaments (PHF), which then lead to intracellular insoluble NFTs (Ittner and Ittner, 2018; Spires-Jones et al., 2009). It is still not entirely clear which of these assemblies mediate the majority of tau neurotoxicity, although most evidence points to soluble tau oligomers and PHFs rather than NFTs (Busche, 2019). However, it is evident that tau hyperphosphorylation can cause neurodegeneration in its own right (Hanger et al., 2009), and also contributes to AD pathology.

Tau is enabled to perform a variety of its physiological functions through post-translational modifications. These modifications take the form of phosphorylation, acetylation, methylation, and ubiquitination (Mandelkow and Mandelkow, 2012; Morris et al., 2015; Noble et al., 2013) and are important in altering both the binding properties of tau, and its localisation within the neuron (Ittner and Ittner, 2018; Mandell and Banker, 1996). As well as playing an important role in stabilising MTs and in axonal transport (Ballatore et al., 2007), recent evidence points to a role for tau at the synapse (Ahmed et al., 2014; Ittner and Ittner, 2018; Ittner et al., 2010; Kimura et al., 2014; Mondragon-Rodriguez et al., 2012; Regan et al., 2015; Regan et al., 2017), including a possible regulatory role of NMDARs involving Fyn and PSD 95 (Ittner et al., 2010; Lopes et al., 2016; Mondragon-Rodriguez et al., 2012) and ERK (Bi et al., 2017), as well as a part in controlling synaptic expression of AMPARs, involving an interaction with kidney and brain (KIBRA) protein (Tracy et al., 2016), and protein C kinase 1 (PICK1) alongside the GluA2 AMPAR subunit (Regan et al., 2015).

KIBRA is a known regulator of AMPAR trafficking (Makuch et al., 2011), while the PICK1-mediated endocytosis of AMPARs is an important step in the mechanism of LTD expression (Citri et al., 2010; Hanley and Henley, 2005; Penn et al., 2017; Roth et al., 2019), suggesting that tau may play a direct role in plasticity. Most studies show that tau knockout mice exhibit normal LTP (Kimura et al., 2014; Shipton et al., 2011), however, it appears that tau is required for physiological LTD to occur (Kimura et al., 2014; Regan et al., 2015) in a process that involves GSK-3 phosphorylation of tau at serine 396 and serine 404, in addition to an interaction with PICK1 and the GluA2 subunit of the AMPAR, resulting in AMPAR internalisation (Regan et al., 2015) (Figure 1.5). Given that Aβo facilitate LTD (Li et al., 2011; Shankar et al., 2008), this could be a key link between the two main proteins of AD.

1.2.1 Tau kinases and sites involved

Of the post-translational modifications that tau undergoes, phosphorylation is by far the most prevalent, with 85 possible sites having been highlighted (Table 1.4) (Martin et al., 2013). These sites are phosphorylated by a number of different kinases, implicated in a variety of physiological and pathological processes and can be affected by a number of factors, including diet, with high levels of homocysteine exacerbating pathological tau phosphorylation in an APPS/PS1/tau transgenic mouse model (Li et al., 2014). Many of these kinases have been implicated in AD pathology, including GSK-3β, CDK5, ERK, JNK, CK, P38, CaMKII, PKA, PKB (Akt), and PKC (Martin et al., 2013).

Of these, GSK-3β is of note, having the most tau phosphorylation sites, many of which are only seen in AD brains (Table 1.4) (Martin et al., 2013). In addition to this, GSK-3β is heavily implicated in both physiological plasticity (Hooper et al., 2007; Peineau et al., 2007) and Aβo-induced plasticity deficits (Deng et al., 2014; Jo et al., 2011; Shipton et al., 2011; Yi et al., 2018), including facilitated LTD (Li et al., 2009). Previous studies have shown that GSK-3β phosphorylates tau in a manner dependent on NMDARs in the presence of Aβo (Tackenberg and Brandt, 2009; Tackenberg et al., 2013). Recent findings that GSK-3β-mediated phosphorylation of tau at serine 396 and serine 404 is vital for LTD to occur (Regan et al., 2015), raise the possibility that Aβo-induced overstimulation of NMDARs could lead to enhanced activation of GSK-3β and subsequent phosphorylation of tau. GSK-3β activity changes upon LTP and LTD induction and is regulated, in part, by phosphorylation at serine 9, whereby phosphorylation by Akt, as happens following LTP induction, inhibits GSK-3β activity (Hong and Lee, 1997), and dephosphorylation by protein phosphatase 1 (PP1) activates it (Bennecib et al., 2000). PP1 is activated upon LTD induction, via a pathway involving NMDAR activation, calcineurin induced dephosphorylation of inhibitor-1 (I-1) and subsequent activation of PP1 (Mulkey et al., 1994). Taken together, this

suggests a mechanism by which tau is normally phosphorylated in response to LTD induction, leading to AMPAR endocytosis (Figure 1.5) (Bradley et al., 2012). However, many of these proteins are also implicated in AD pathogenesis, and it is possible that this could be a mechanism by which Aβo recruit pathological tau, and, indeed, phosphorylation of tau at serine 396 has been shown to occur early on in AD (Mondragon-Rodriguez et al., 2014).

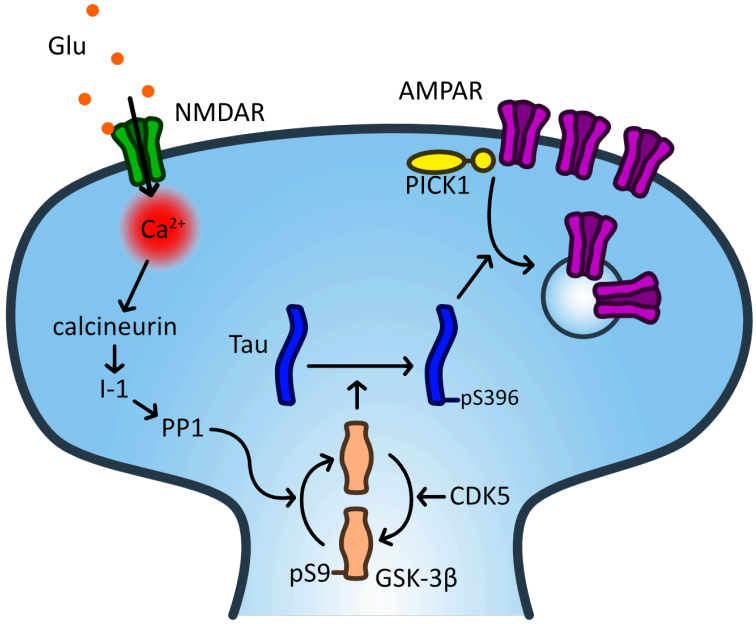

Figure 1.5: Possible mechanism for LTD-induced AMPAR endocytosis involving activation of GSK-3β and tau phosphorylation. Activation of NMDARs activates calcineurin. This dephosphorylates and activates I-1, which in turn activates PP1. PP1 dephosphorylates GSK-3β at serine 9, resulting in increased activity and resultant phosphorylation of tau at serine 396. Phosphorylated tau then binds to the PICK1 and causes AMPAR endocytosis.

Another NMDAR-dependent process that could involve GSK-3β phosphorylation is the tau mediated regulation of NMDAR excitotoxicity. As discussed, Aβo induce phosphorylation of tau at Threonine 231 (Mondragon-Rodriguez et al., 2012), leading to formation of a complex of tau, Fyn, and PSD 95 which interacts with NR2B-containing NMDARs to increase NMDAR excitotoxicity (Ittner et al., 2010; Lopes et al., 2016; Mondragon-Rodriguez et al., 2012). This,

regulatory activity towards NMDARs could be a physiological function that becomes toxic in the presence of Aβo and might make up a feedback process, whereby activity of NMDARs can then further tune NMDAR activity.

Some phosphorylation of tau, however, appears to have a protective role against Aβo toxicity; recent evidence shows that tau phosphorylation of tau by the p38-mitogen-activated protein kinase (MAPK) p38γ confers protection against Aβo-mediated toxicity by phosphorylating tau at threonine 205 and reducing PSD 95-tau-Fyn complexes in APP23 transgenic mice (Ittner et al., 2016). Interestingly, phosphorylation at threonine 205, a site also linked with Aβo treatment, in response to LTP-like synaptic activity localises tau to the dendrite (Frandemiche et al., 2014) and so could be an upstream event required for more pathological phosphorylation, since dendritic tau is responsible for mediating Aβo toxicity (Ittner et al., 2010).

Tau Phosphorylation Sites			
Only AD	AD and Physiological	Physiological	Putative
Y18	S46	T17	T30
S68	T181	Y29	S61
T69	S198	T39	T63
T71	S190	T50	S64
S113	S202	T52	T76
T123	T205	S56	S129
T153	T212	T95	S137
T175	T217	T101	Y310
S191	T231	T102	T319
Y197	S235	T111	T377
S208	S396	T131	
S210	S400	T135	
S214	S404	T149	
S237	S412	T169	
S238	S413	S195	
S258	S416	T220	
S262		S241	
S289		T245	
S356		T263	
Y394		S285	
T463		S293	
S409		S305	
S422		S316	
T427		S320	
S433		S324	
S435		S341	
		S352	
		T361	
		T373	
		T388	

Table 1.4: List of known tau phosphorylation sites in AD patients, and healthy individuals. All sites are for the longest, 441 residue, isoform of tau. Residues shown in green are phosphorylated by GSK-3β. S: Serine, T: Threonine, Y: Tyrosine. Table adapted from Martin *et al.* (2013).

1.2.2 Pathological tau and network function

At the synapse, tau has effects on excitability via its interactions with NMDARs and AMPARs that help to mediate Aβo toxicity. How these synaptic effects might impact on a network level are still poorly understood. Tau knockout mice show normal EEG spectral waveforms under basal conditions (Bi et al., 2017; Ittner et al., 2016), but in the presence of Aβ, tau expression is required for network dysfunction in the cortex and hippocampus (Ittner et al., 2016; Roberson et al., 2011), in a manner involving postsynaptic tau-Fyn interactions (Roberson et al., 2011) and modulation of postsynaptic tau complexes (Ittner et al., 2016).

Recent evidence suggests a contrasting effect of pathological tau on network function to that of APP or PS1 overexpression in mice, which results in network hyperactivity and hypersynchrony (Busche and Konnerth, 2016; Mucke and Selkoe, 2012; Palop and Mucke, 2016), since tau transgenic mice show reduced neuronal excitability (Busche et al., 2019; Hatch et al., 2017; Menkes-Caspi et al., 2015). Interestingly, mice coexpressing APPSwe/PS1ΔE9 with mutant tau (rTg4510) show suppression of cortical neuronal activity and enhanced silencing of neurons that dominates the usual Aβo-dependent neural excitability, an effect that depends on soluble tau rather than NFTs (Busche et al., 2019). It appears, therefore, that tau performs a dual role in AD network pathology, both mediating enhanced Aβo-induced hyperexcitability, and causing later neuronal silencing. This may help to explain the contrasting network effects that occur in AD, where the cortical and hippocampal hyperactivity of preclinical AD gives way to suppressed activity as the disease progresses, possibly reflecting the progression of tau pathology interacting with Aβo pathology.

1.2.3 Tau as a possible therapeutic target

Tau performs many roles in the axon, including MT stabilisation (Aronov et al., 2001; Hirokawa et al., 1996; Kanai and Hirokawa, 1995) and axonal transport (Ballatore et al., 2007), and at the synapse, where it appears to play a key role in LTD (Kimura et al., 2014; Regan et al., 2015) and possibly in regulating NMDAR activity (Ittner et al., 2010; Lopes et al., 2016; Mondragon-Rodriguez et al., 2012). Aberrant tau phosphorylation is associated with neurodegeneration in its own right as evidenced by numerous tauopathies, but potential interactions between tau and Aβo make AD even more complex. Without doubt, tau is a key player in AD, as evidenced by its importance in transducing Aβo pathology AD (Ittner et al., 2010; Rapoport et al., 2002; Roberson et al., 2011; Roberson et al., 2007). It is, therefore, vital that the full pathological effects of tau are better understood, as well as the mechanisms by which Aβo recruit pathological tau.

There have been some efforts to target tau. Reducing tau levels with anti-sense oligonucleotides can lower human tau levels in transgenic mice (P310S) and non-human primates to alleviate neuronal loss (DeVos et al., 2017), and passive immunisation with human tau antibodies could reduce behavioural deficits in human mouse tau transgenics (P301L) (Nisbet et al., 2017). It is unclear, however, whether lowering levels of tau will be a viable strategy; the early protective function of tau against Aβo toxicity may be impaired (Ittner et al., 2016), along with the many important physiological processes that tau is involved with, such as axonal transport (Ballatore et al., 2007) and LTD (Kimura et al., 2014; Regan et al., 2017). Some efforts have also been made to affect some of the proteins that tau interacts with; saracatinib, a broad Src kinase inhibitor, is in placebo-controlled phase 2 trials (Ittner and Ittner, 2018; Nygaard et al., 2015b) to test whether reducing the interaction between tau and Fyn might ameliorate AD pathology.

Although some of these treatments show some promise, there is still a long way to go in targeting tau-related pathways to treat AD. A better understanding of the physiological functions of tau may help to shed more light on possible areas of intervention, particularly at the synapse where Aβo act. Indeed the involvement of tau in LTD (Kimura et al., 2014; Regan et al., 2017) is an important discovery, helping us to understand the basis of tau recruitment by Aβo, and, therefore open up more therapeutic targets for AD.

1.3 Models and systems for studying AD

1.3.1 Models used and their limitations

The majority of studies performed investigating AD are done in rodents. Rodents do not develop AD naturally, and so, AD pathophysiology can be partially simulated by addition of pathological variants of Aβo, or by the introduction of one or more mutations that have been observed in fAD patients. As has been discussed these mutations all involve proteins that produce, or process Aβ and APP, including PS1, PS2, BACE1, and APP itself. Although these models are able to capture elements of the disease, none of them reliably display full AD clinical symptoms, including generation of tau tangles (Drummond and Wisniewski, 2017; LaFerla and Green, 2012). In addition, the effects of these mutations vary based on the genetic background of the individual animals. Because of this, there is a relatively wide and conflicting range of evidence produced from these models and this could help to account for the high failure rate of clinical trials that have shown promise in rodent models (Banik et al., 2015; Cummings et al., 2014; Schneider et al., 2014). In order to capture the tau pathology of AD, some studies use transgenic animals also expressing a mutated form of tau from tauopathy models. Although these mice do generate tangles (Drummond and Wisniewski, 2017; Gotz and Ittner, 2008), they bring their own confounds, as AD is a disease involving unmodified tau, not mutated tau.

A number of AD animal models involve the use of transgenic animals that express an extra, mutated copy of human APP. Although these animals do display many of the symptoms of AD, including cognitive impairment, they do also display some phenotypes that could be due to the overexpression of APP. These animals carry double the number of copies for APP, and so, it is important to recognise that this could be the cause of some of these presentations. More recently, studies have begun using APP knock-in mice that do show less severe AD symptoms, but are also lacking many of the phenotypes that arise due to APP overexpression (Saito et al., 2014).

As well as transgenic and knock-in animal models, studies can also use knock out models to inform about the importance of a proteins in physiology and disease, such as the work done using tau knockouts described above (Rapoport et al., 2002). Although these models can be useful in informing us about the importance of these proteins in pathology, it is also important to recognise that there may be some compensatory activity of other proteins occurring in the models. This can arise as other proteins take over some of the roles that the ablated protein plays normally.

Therefore, although we can gain a lot of information from studies performed in these rodent models, it is crucial to recognise that none of them is an exact model of AD, and that the findings can be confounded by other processes that are occurring such as compensatory activity or overexpression.

1.3.2 The Hippocampus

As well as studying overall behavioural and physical changes *in vivo*, we can learn a lot about more cellular based disease changes from various *in vitro* preparations. Although these bring with them the confounds of being a partial system, lacking factors such as connections between wider brain regions, they do facilitate study of changes on a cellular level with fewer variables involved. One of the most widely used brain regions studied in AD is the hippocampus, being intimately involved

in memory formation, particularly episodic, declarative, and spatial memory (Bird and Burgess, 2008; Burgess and O'Keefe, 1996) and is an area severely affected in AD (Halliday, 2017; Scheff and Price, 2003; Scheff et al., 2006; Townsend et al., 2006). It has a tightly ordered structure that is categorised into three distinct subregions: the dentate gyrus (DG), the main part of the hippocampus containing CA3, CA2, and CA1, and the subiculum. The key area of interest in this thesis is the main body of the hippocampus containing CA3, CA2, and CA1, particularly the Schaffer collateral-CA1 synapse (Figure 1.6). The hippocampus contains a wide range of different cell classes, including GABAergic, glutamatergic, and glial cells all of which interact with each other. Within these different cell classes there is also a large amount of diversity in cell types such as GABAergic PV^+ basket cells and a variety of different glutamatergic pyramidal cells. Although the main cell-types investigated in this thesis are glutamatergic pyramidal neurons, it is important to consider that these are also surrounded by a wide range of other cells.

The standard model of the hippocampus is of a tri-synaptic feedforward pathway. All subregions of the hippocampus receive input from the entorhinal cortex, although this is particularly pronounced for the DG. The DG then projects forwards to the proximal apical dendrites of the CA3 pyramidal neurons. The CA3 forms numerous recurrent connections, leading to it being regarded as an autoassociative network, and also projects via Schaffer collateral axons to the stratum radiatum and forms excitatory glutamatergic synapses with CA1 dendrites (Megias et al., 2001). These synapses have been particularly extensively studied, partially on account of the highly ordered arrangement of axonal fibre bundles projecting along the stratum radiatum of CA1 which makes this area more amenable to electrophysiological investigation. In addition, the connections of the hippocampus, particularly those of the tri-synaptic pathway described above are mainly confined to a two-dimensional plain, which, therefore, allows them to be studied in *ex vivo* brain preparations, such as acute and organotypic slices. In this thesis, I shall take advantage of this

morphology and our extensive knowledge of the excitatory, glutamatergic Schaffer collateral-CA1 synapse to probe some of the synaptic pathology of AD.

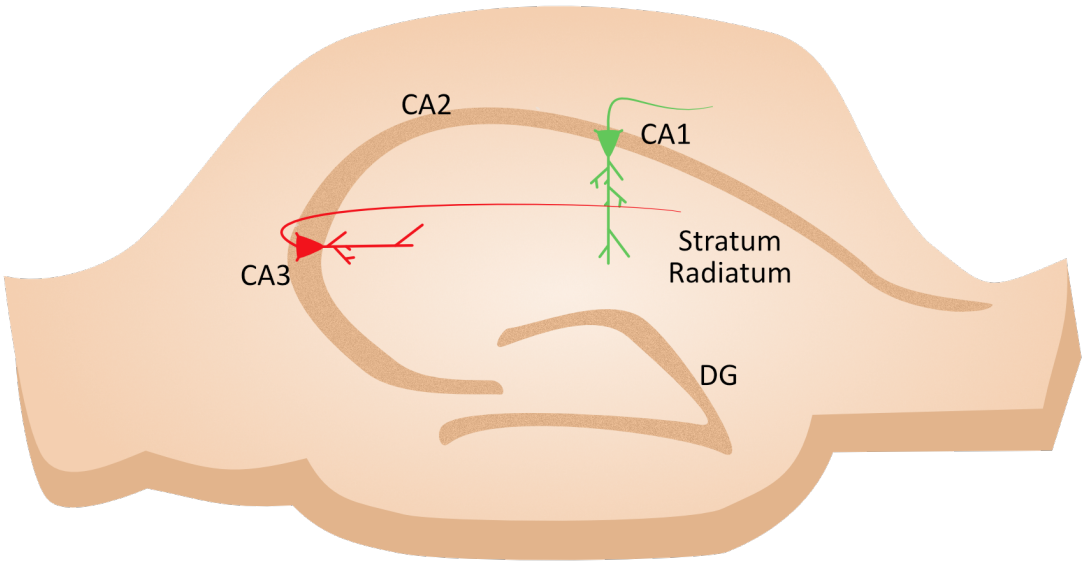

Figure 1.6: The anatomy of the hippocampus. Illustration of the anatomy of the hippocampus with Schaffer collateral axons from CA3 neurons projecting to dendrites of the CA1 in the stratum radiatum. Cell body layer shown in darker brown. Diagram kindly provided by Rudi Tong.

1.3.2.1 Acute hippocampal slices

Acute hippocampal slices are obtained from adult rodent brains for immediate use on the day of preparation. They possess advantages over other hippocampal preparations, in that they maintain the majority of their cytoarchitecture and synaptic circuits (Lein et al., 2011). This conservation of hippocampal morphology makes acute slices an ideal model for use in examining synaptic plasticity.

1.3.2.2 Organotypic hippocampal slices

Unlike the acute slice, organotypic slices can be maintained for much longer periods of time, as much as several weeks, allowing for more chronic studies (Bahr, 1995). There are, however, some disadvantages to organotypic slices; they must be prepared from young animals (around P7) from

developing hippocampi, and so, cannot be used as an aged model in the way that acute slices can. Development occurs over the first few weeks after culturing, and this can lead to some of the circuits and function of the hippocampus being changed. Although the general cytoarchitecture and connectivity are maintained, there are reorganisations of the connections, enhanced dendritic branching, and increased spontaneous and recurrent activity due to increased connectivity as a result of lack of external connections and experience-driven activity, and confinement in a two-dimensional structure (Collin et al., 1997; De Simoni et al., 2003; Debanne et al., 1995; Gahwiler, 1981; Gahwiler et al., 1997; Muller et al., 1993). Despite this, these changes are small, making them a suitable system to model changes in general hippocampal biology, as well as being easily accessible for optical and genetic intervention.

1.3.2.3 Dissociated Primary Hippocampal Neurons

In comparison to acute and organotypic slices, primary dissociated neuronal cultures do not possess any of the organised cytoarchitecture or pathways of the hippocampus. In addition to this, they appear to have some differences in ion channel subunit expression pattern, composition (Marder and Goaillard, 2006), and subcellular distribution (Lai and Jan, 2006), as well as some functional differences such as increased mEPSC frequency (Cingolani and Goda, 2008), despite showing a comparable, or reduced synapse density compared to the brain (Boyer et al., 1998). They are made from very young animals (P1), and are ideal for interrogating individual synapses away from network level interference, particularly when studying homeostatic plasticity (Pozo and Goda, 2010). In addition to this, they are extremely accessible to pharmacological perturbations, since they are a grown as a single layer of cells on a flat surface.

1.4 Thesis Aims

AD is one of the most prevalent diseases in the world, with a huge range of pathological processes implicated. As yet, however, our understanding of the early mechanisms of pathogenesis are poor,

and there is no effective treatment. Aberrant accumulation and aggregation of Aβ appears to lie at the heart of the disease, and there are several proposed mechanisms, including altered processing, increased production, and reduced clearance (Selkoe, 2001). Beyond this, how Aβo act to give the widely seen pathology of AD has not been fully demonstrated. Elucidating the early Aβo-induced pathological events will be key to our understanding of the disease, and to effective treatments, that will require both early diagnosis and appropriate therapeutic targets.

One process that is impaired early on in AD is synaptic plasticity, specifically the Aβo-induced impairment of LTP and facilitation of LTD (Lambert et al., 1998; Li et al., 2009; Shankar et al., 2007; Shankar et al., 2008; Sheng et al., 2012; Walsh et al., 2002), however the mechanism of how this happens remains unknown. One possible driver for the plasticity changes could be an alteration in neurotransmitter release at the presynaptic terminus; as discussed, there is evidence that the physiological function of both APP (and its derivatives) and Aβ is to enhance presynaptic release (Abramov et al., 2009; Fogel et al., 2014; Mura et al., 2012) and that higher, pathological concentrations of Aβo also enhance presynaptic function (Brito-Moreira et al., 2011; Dolev et al., 2013; Kabogo et al., 2010; Russell et al., 2012). Recent evidence from the Emptage laboratory, collected using direct optical measurement of presynaptic release, together with electrophysiological recordings, in a variety of experimental model AD systems has shown that pathological concentrations of Aβo induce enhanced release via increased activity of presynaptic $Ca_V2.1$ VGCCs (Jeans et al., Unpublished). In this thesis, I intend to build upon these observations, exploring further the role of functional presynaptic changes in AD pathogenesis. More specifically, I intend to test the hypothesis that changes in transmitter release properties drive aberrant synaptic plasticity and the recruitment of pathological tau.

I shall first explore whether facilitated presynaptic release drives the plasticity deficits that are an early hallmark of AD. Plasticity can be induced by different patterns of pre- and postsynaptic activation, often in response to varying patterns of neurotransmitter release. Therefore, it is possible that alterations in presynaptic release probability (Pr) can recruit different changes in plasticity. Given that there is a large body of evidence that Aβo-affect Pr, it is possible that Aβo-induced presynaptic changes could drive postsynaptic ones. I therefore ask whether reducing presynaptic release returns Aβo-induced plasticity changes to normal?

Secondly, I shall explore whether chronic Aβo-induced synaptic activity changes drive recruitment of pathological tau phosphorylation. As discussed, Aβo facilitate LTD, and tau phosphorylation is key for LTD induction. These observations suggest that Aβo-induced enhancement of presynaptic release might result in elevated extracellular Glu that enhances LTD induction. In addition, chronic, repeated LTD induction might recruit further, pathological phosphorylation of tau, presenting a mechanism by which hyperphosphorylated tau is recruited by Aβo. Therefore, in light of this evidence, I ask several questions: Can Aβo-induced pathological tau phosphorylation be rescued by suppression of presynaptic function? Can Aβo-induced pathological tau phosphorylation be mimicked by chronic changes in extracellular Glu? Can Aβo-induced pathological tau phosphorylation be mimicked by chronic induction of LTD?

Thirdly, I explore whether Aβo induce changes in non-evoked release. In addition to evoked release, occurring in response to APs arriving at the presynaptic terminus, neurons release vesicles in a spontaneous, non-evoked manner. These non-evoked release events are increasingly being recognised as playing a role in processes that tune synaptic strength such as homeostatic plasticity (Gonzalez-Islas et al., 2018; Kavalali, 2015; Pozo and Goda, 2010; Turrigiano, 2012). I sought to develop a novel, quantitative optical technique for measuring non-evoked release on a synaptic

level, and to see whether this revealed any Aβo-induced changes to non-evoked release. Previous evidence has shown that spontaneous miniature release (minis), recorded in the form of miniature excitatory postsynaptic currents (mEPSCs), frequency is reduced in the presence of Aβo (Kamenetz et al., 2003; Nimmrich et al., 2008; Shankar et al., 2007; Talantova et al., 2013). As with many studies of presynaptic release, however, these measurements of non-evoked release are made at the soma of the postsynaptic cell, and, as such, can be confounded by postsynaptic weakening. Given that evoked and non-evoked release are normally well-correlated, with mini frequency often used as an indicator of presynaptic strength (Prange and Murphy, 1999), these result seem to contradict the evidence that Aβo enhance evoked presynaptic release function (Brito-Moreira et al., 2011; Dolev et al., 2013; Jeans et al., Unpublished; Kabogo et al., 2010; Russell et al., 2012). This could possibly be a result of confounds of the experimental set-up. Therefore, I set out to explore non-evoked release with a direct optical technique in order to resolve these ambiguities.

Finally, I shall investigate whether Aβo-enhanced release can be affected by pharmacological manipulation of processes integral for release. Our laboratory has already shown that blockade of $Ca_V2.1$ with ω-agatoxin IVA can reduce Aβo-enhanced presynaptic release (Jeans et al., Unpublished), however, global alteration of $Ca_V2.1$ could have unwanted side effects. Sphingolipids are a class of membrane lipids that are abundant in neurons and have been heavily implicated in regulation of the synaptic vesicle cycle underlying neurotransmitter release (Rohrbough and Broadie, 2005), and this class of lipids has also been established as viable and promising drug targets, with their levels being readily modulated by small molecule inhibitors of enzymes in the various sphingolipid synthetic pathways (Wymann and Schneiter, 2008). In addition, sphingolipids are dysregulated in the brains of AD patients (Haughey et al., 2010). Manipulation of sphingolipid levels could, as a result, represent a valuable therapeutic approach to alleviating Aβo induce effects on the presynaptic release. Therefore, I explored whether the

pharmacological manipulation of sphingolipid metabolism, and so relative sphingolipid levels, could alter release, and whether this could counteract the effects of Aβo, possibly introducing sphingolipids as a novel area of therapeutic intervention for AD.

2. Materials and Methods

2.1 Preparation of Hippocampal Slices and Cultures

2.1.1 Acute hippocampal slices

Acute hippocampal slices were prepared from 7/8-week-old C57 Black 6 mice (n = 87 mice). Mice were sacrificed by cervical dislocation and decapitation. The brain was then extracted and placed in ice-cold dissection media (65 mM sucrose, 85 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 7 mM $MgCl_2 \cdot 6H_2O$, and 0.5 mM $CaCl_2 \cdot 6H_2O$) saturated with 95% O_2 and 5% CO_2. The cerebellum was removed manually by cutting coronally with a scalpel to create a flat surface with which to secure the brain. The brain as then glued onto a designated platform for the vibratome (Microm HM 650V, Thermo Scientific) together with a block of 2% agar by the dorsal side of the brain to support the brain when slicing. 350 μm coronal slices were then cut, and the hemispheres of the brain were separated manually using a scalpel. Slices were then transferred into a custom-made recovery chamber containing oxygenated artificial cerebral spinal fluid (ACSF) (120 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 11 mM D-glucose, 1 mM $MgCl_2 \cdot 6H_2O$, and 2 mM $CaCl_2 \cdot 6H_2O$) which was bubbled with 95% O_2 and 5% CO_2. Slices were allowed to recover for 8 min at 37 °C, followed by 60 min at room temperature. Slices were maintained in the bubbled ACSF at room temperature for up to 4–5 h.

2.1.2 Organotypic hippocampal slices.

Organotypic hippocampal slices were prepared from postnatal day 6–7 (P6–7) male Wistar rat pups (Harlan UK, Nihon SLC) (n = 26 rats). Rats were sacrificed by cervical dislocation and decapitation. The brain was then extracted and placed into ice-cold Earle's Balanced Salt solution (EBSS)-based dissection buffer with added 21 mM Hepes and 27.8 mM D-glucose (pH adjusted to 7.2–7.4 with NaOH) and the hippocampus of both hemispheres were isolated. These were transferred to a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd. And Cavey

Laboratory Engineering Co. Ltd.) and cut into slices of 350 μm thickness. Slices were manually vetted, discarding any with incomplete hippocampal anatomy or tissue damage. Slices were then placed onto Millicell CM culture plate inserts (polytetrafluoroethylene filter, pore size 0.4 μm, diameter 12 mm) in a six-well Millicell culture plate (both supplied by Merck Millipore) with 1 mL culture medium (78.8% minimum essential medium, GlutaMAX (Gibco), 20% heat-inactivated horse serum, 1% B27 plus (Gibco), with added 1 mM $CaCl_2$, 30 mM Hepes, 26 mM D-glucose, 5.8 mM $NaHCO_3$, and 2 mM $MgSO_4$) and stored at 34.5 °C. Culture media were renewed every 3–4 days and slices were maintained at 37 °C and 5% CO_2 (Foster et al., 2018).

2.1.3 Dissociated Primary Hippocampal Neurons

Dissociated hippocampal cultures were prepared from neurons of P1 Wistar rats (n = 32 rats). Rats were sacrificed by cervical dislocation and decapitation . The brain was then extracted and placed into ice-cold Hanks' Balanced Salt Solution (HBSS) and the hippocampus of both hemispheres were isolated. Once the meninges had been removed, dissected hippocampi were transferred to a trypsin solution at 37 °C for 15 min. They were then washed with a solution of HBSS containing 2% Foetal Bovine Serum (FBS), followed by HBSS. The hippocampi were then transferred to a solution of Neurobasal medium A (NBA) and were triturated. Once separated, the cells were counted and diluted with more NBA. They were then seeded onto poly-D-lysine coated coverslips and placed into wells containing NBA supplemented with 2% FBS, 2% B27 Plus, 1% GlutaMAX, and 1% penicillin/streptomycin. The day following plating, half the medium was exchanged for NBA supplemented with 2% B27 Plus and 1% GlutaMAX only, which was used for all subsequent feeds (every 3 days).

2.2 Transfection

2.2.1 Transfection of organotypic slices with Channelrhodopsin 2

Channelrhodopsins (ChRs) are light gated ion channels that were first isolated from the green algae Chlamydomonas (Nagel et al., 2002; Nagel et al., 2003). Two forms were originally isolated, Channelrhodopsin 1 (ChR1) and Channelrhodopsin 2 (ChR2), and the latter of the two was shown to be able to facilitate light-induced depolarisation of mammalian cells (Nagel et al., 2003). Previous investigations had already used different methods to explore the control of neuronal firing using light (Banghart et al., 2004; Zemelman et al., 2002), however studies expressing ChR2 in retina, hippocampal neurons, spinal chicken embryonic cells, mouse brain slices, and transgenic worms (Bi et al., 2006; Boyden et al., 2005; Ishizuka et al., 2006; Li et al., 2005; Nagel et al., 2005) demonstrated that ChR2 allowed for easy and precise control of neuronal firing in a variety of systems, making it a key protein for the field of optogenetics. The expression of ChR2 in mammalian neurons to control neuronal firing is now used in a wealth of applications (Kim et al., 2017), and has been shown to work in mammals *in vivo* (Cardin et al., 2010).

At 7 days *in vitro* (DIV), organotypic slices were removed from the incubator and a gradated glass pipette was used to inject a solution containing recombinant AAV carrying fluorescently tagged ChR2, ChR2-eYFP (Addgene plasmid 20298, pAAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA (AAV1); titre 3×10^{12} GC/mL; together with Addgene plasmid 105553, pENN.AAV.hSyn.Cre.WPRE.hGH (AAV1); titre 1×10^{13}), and phenol red into CA3. Slices were then returned to the incubator for 5 more days before ascertaining whether transfection had occurred successfully (at DIV 12). pAAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA (AAV1) was a gift from Karl Deisseroth (Addgene viral prep # 20298-AAV1; http://n2t.net/addgene:20298; RRID: Addgene_20298) and pENN.AAV.hSyn.Cre.WPRE.hGH was a gift from James M. Wilson (Addgene viral prep # 105553-AAV1 ; http://n2t.net/addgene:105553; RRID:Addgene_105553).

2.2.2 Transfection of dissociated cultures with SypH 2x

pHluorins were first developed to investigate vesicular release dynamics, taking advantage of the pH dependence of green fluorescent protein (GFP) (Miesenbock et al., 1998; Sankaranarayanan et al., 2000). The pHluorin moiety can be targeted to the interior of synaptic vesicles by fusion with other synaptic vesicle proteins such as synaptobrevin (to give synaptopHluorin) (Miesenbock et al., 1998), synaptotagmin (Diril et al., 2006), VGLUT1 (Voglmaier et al., 2006), or synaptophysin (to give SypH) (Granseth et al., 2006) (Figure 1.4). SypH has since been optimised with the inclusion of two pHluorin proteins to give SypH 2x which has an enhanced signal-to-noise ratio over the other pHluorin variants (Zhu et al., 2009). This has been used in a variety of studies, including to look at release in response to single action potential (AP) stimuli (Jeans et al., 2017; Zhang et al., 2009).

The SypH 2x plasmid was originally a gift of Dr Y. Zhu and was stored as a glycerol stock at -80 °C. The plasmid was amplified and purified using a QIAGEN® Plasmid Maxi Kit (QIAGEN). In short, the glycerol stock was grown on an agar plate containing 0.1% ampicillin overnight. Single colonies were then transferred to 2 mL Luia-Bertani (LB) broth containing 0.1% ampicillin and incubated at 37 °C for 8 h, before being transferred to 250 mL LB broth containing 0.1% ampicillin and incubated at 37 °C for a further 12 h. Bacterial cells were then harvested by centrifugation and the medium was removed. The cells were then resuspended in resuspension buffer (50 mM Tris·HCl, pH 8.0, 10 mM EDTA) containing RNase A (26 mg/mL in 10 mM sodium acetate, pH 5.2). Lysis buffer (0.2 M NaOH, 1% sodium dodecyl sulphate (SDS)) was then added and gently mixed, before allowing the resultant solution to incubate at room temperature for 5 min. After lysis, chilled neutralisation buffer (3.0 M potassium acetate, pH 5.5) was mixed in and the resultant solution was incubated on ice for 15 min. This mixture was then filtered using a QIAfilter Cartridge, and the resultant solution was transferred a QIAGEN-tip that had been

equilibrated with equilibration buffer (50 mM MOPS, 0.75 M NaCl, 15% isopropanol, 0.15% Triton®X-100). The QIAGEN tip was then washed twice with wash buffer (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol) before the DNA was eluted using elution buffer (1.6 M NaCl, 50 mM Tris·Cl, pH 8.5, 15% isopropanol). The eluted DNA was then precipitated with isopropanol and extracted using a PureLink™ HiPure Precipitator Module (Thermo Fischer Scientific). The precipitation was loaded onto a PureLink™ HiPure Precipitator and washed with 70% ethanol. Once dried, the DNA was extracted from the module with 60 °C TE buffer (10 mM Tris·Cl, pH 8.0, 1 mM EDTA) and the concentration and purity were measured using a NanoDrop™ Spectrometer (Thermo Fischer Scientific).

At DIV 8, dissociated cultures were transfected with SypH 2x (gift of Dr Y. Zhu). DNA (3 μg/coverslip) was mixed with Lipofectamine 2000 (Invitrogen) (3 μL/coverslip). Conditioned media was collected from each well, and the wells were topped up with NBA supplemented with 2% B27 Plus and 1% GlutaMAX. The DNA/Lipofectamine 2000 solution was added to each well and incubated for 1 h. Following this, all media was removed and subsequently replaced with conditioned media.

2.3 Electrophysiology

2.3.1 Field recordings in acute slices

Slices were transferred to a custom-built interface chamber perfused with ACSF (120 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 11 mM D-glucose, 1 mM $MgCl_2 \cdot 6H_2O$, and 2 mM $CaCl_2 \cdot 6H_2O$) heated to near-physiological temperature (33–35 °C) with a custom-made heater and bubbled with 95% O_2 and 5% CO_2. For drug treatments, slices were incubated for >2 h in ω-agatoxin IVA (50 nM), Aβo (50 nM), or both, and recordings were performed in ACSF containing drug. A bipolar stimulating electrode was placed in the Schaffer collaterals of CA3

neurons to deliver stimuli. A borosilicate glass recording electrode filled with ACSF was placed in the stratum radiatum of CA1 neurons. Data was acquired using Digitimer NeuroLog amplifier and WinWCP software (Strathclyde Electrophysiology Software). An appropriate stimulating intensity was identified (one that could produce fEPSPs of around 0.5 mV without inducing population spikes) and neurons were then stimulated every 25 s, with a paired pulse stimulation (50 ms after the first stimulus) delivered every four stimuli. Once a stable response was observed, a baseline recording was made for 10–15 min.

2.3.1.1 ω-agatoxin IVA titration

In order to ascertain the appropriate concentration of ω-agatoxin IVA that could partially reduce synaptic function by a similar amount that Aβo enhance it (Jeans et al., Unpublished), ω-agatoxin IVA (Alomone Labs) (50 nM) was added to the bath after a stable baseline had been recorded for 10 mins, and the effects on fEPSP and PPR were monitored.

2.3.1.2 LTP

Once a stable baseline had been recorded, LTP was induced with a 20x theta burst stimulus (TBS), 4 bursts at 100 Hz repeated 20 times over 20 s (Capocchi et al., 1992; Larson et al., 1986). The recording was normalised to the average of the baseline fEPSP slope, and LTP was measured as the average fEPSP slope 55–60 min after induction compared to the average baseline fEPSP slope.

2.3.1.3 LTD

Once a stable baseline had been recorded, LTD was induced with a 900 x 1 Hz low frequency stimulus (LFS). The recording was normalised to the average of the baseline fEPSP slope, and LTD was measured as the average fEPSP slope 55–60 min after the induction protocol compared to the average baseline fEPSP slope.

2.3.1.4 Analysis

Electrophysiological traces were analysed in Clampfit 9 Version 10.6.2.2, Molecular Devices). The initial slope of the fEPSP was used as a measure of the response in order to remove any confounds of population spiking. PPR was analysed for every fourth trace as:

$$PPR = \frac{fEPSP_2}{fEPSP_1}$$

Where $fEPSP_1$ and $fEPSP_2$ are the slopes of the responses to the first and second paired pulse stimuli respectively (interstimulus interval of 50 ms). Kruskal-Wallis test followed by Dunn's multiple comparison test were used to determine statistical significance.

2.3.2 Electrophysiological recordings in organotypic slices

Organotypic slices were transferred to the recording chamber by cutting the membrane of the culture insert around the slice and fixing to the bottom of the chamber using a thin layer of grease (glisseal® HV, Borer). The recording chamber was constantly perfused (1–2 mL/min) with ACSF (145 mM NaCl, 2.5 mM KCl, 1.2 mM KH_2PO_4, 16.0 mM $NaHCO_3$, 11.0 mM D-glucose, 3.0 mM $CaCl_2$, and 2.0 mM $MgCl_2$), heated to near-physiological temperature (33–35 °C) with a custom-made heater, and bubbled with 95% O_2 and 5% CO_2.

Data was acquired using an Axoclamp 2B amplifier and WinWCP software (Strathclyde Electrophysiology Software).Whole-cell patch clamp recordings were performed on pyramidal neurons using low (4–8 MΩ) resistance patch electrodes filled with standard internal solution (135 mM KGluconate, 10 mM HEPES, 2 mM $MgCl_2$, 2 mM Na_2ATP, and 0.4 mM Na_3GTP, pH 7.2–7.4).

2.3.2.1 NMDA LTD

For chemical LTD induced with NMDA, a glass electrode (filled with ACSF) was placed in stratum radiatum, nearer to CA3 than the recording electrode. Stimulation intensity was adjusted to evoke a 5–10 mV excitatory postsynaptic potential (EPSP). Once a stable response was found, a 5 min baseline was recorded before perfusion of ACSF containing NMDA (Sigma Aldrich) (20 μM) for 3 min. EPSPs were then recorded for a further 32 min. EPSP slope was then measured, comparing average slope post-NMDA addition (35–40 min after the recording started) with that of control (without any NMDA addition) at the same timepoint, normalised to the average baseline EPSP slope.

2.3.2.2 Optogenetically induced LTD

For optogenetic low frequency stimulation (LFS) LTD, slices transfected in CA3 with ChR2 were used from DIV 12–19. The CA3 and CA1 areas were imaged using confocal fluorescence microscopy, and z-stacks (0.5 μm) were taken to visualise transfection levels (Figure 2.1a). Confocal imaging was conducted on an Olympus BX50WI microscope equipped with a 60x water immersion objective (NA=0.9 or 1.1) and a BioRad Radiance 2000 confocal scanhead (BioRad/Zeiss) using a 488 nm argon laser. Images were acquired using Lasersharp software as z-stacks. A blue LED (450–485 nm) (Cree®) was placed by the bath (within 1 cm) and EPSPs were recorded from transfected CA3 neurons. Stimulus intensity and duration of stimulation were varied in order to find an appropriate pulse that could reliably elicit one AP (Figure 2.1b, c). CA1 cells were then recorded from and EPSPs were elicited via optogenetic stimulation. After a 5 min baseline, LTD was induced, either with 500 x 1 Hz pulses, or 900 x 1 Hz pulses and EPSPs were then recorded for a further 30 mins post induction. This was repeated with the mGluR blocker LY351495 (100 μM) or the NMDAR blocker APV (50 μM) for the 500 x 1 Hz protocol or 900 x 1 Hz protocol respectively. EPSP slopes were then normalised to baseline EPSP slopes, and

average EPSP slope 35–40 min post induction was compared to the same timepoints for respective drug treatments.

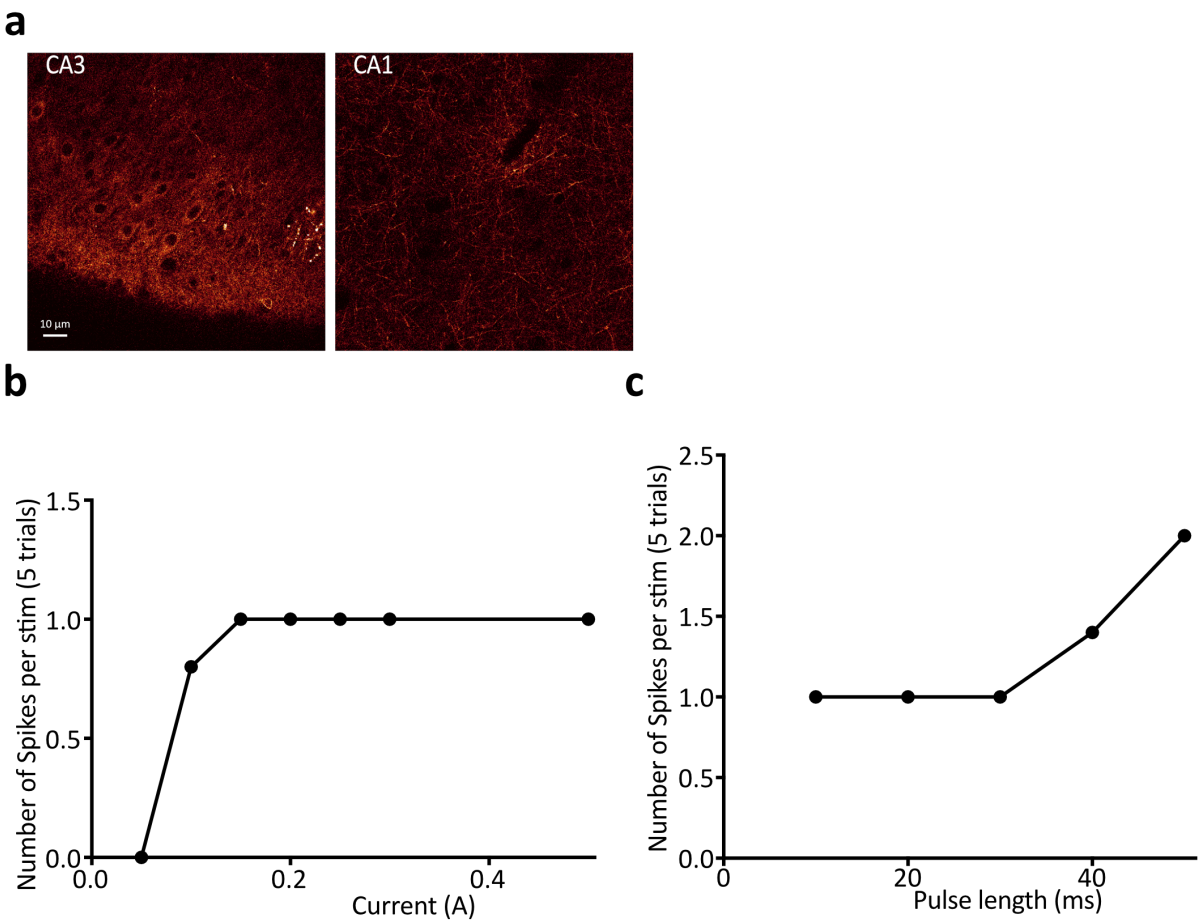

Figure 2.1: Confirmation of transfection with ChR2 and optimisation of stimuli with input-output curves of CA3 neurons transfected with ChR2 responses to different intensities of blue LED light and different pulse widths. **a)** Images of hippocampal slices transfected with ChR2-eYFP in CA3 at CA3 cell body layer (left) and at CA1 stratum radiatum (right). **b)** Number of spikes elicited per stim (averaged over 5 trials) at different intensities of light stimulation (as measured by the current delivered to the LED) with 50 ms pulse length (n = 1 slice). **c)** Number of spikes elicited per stim (averaged over 5 trials) at different pulse durations with 0.3 A delivered to the LED (n = 1 slice).

2.3.2.3 Analysis

Electrophysiological traces were analysed in Clampfit 9 (Version 10.6.2.2, Molecular Devices), measuring EPSP responses as the slope of initial rise.

2.3.3 Electrophysiological recordings in dissociated cultures

mEPSCs were recorded from dissociated hippocampal cultures at DIV 14–18. Coverslips with dissociated cultures at DIV 14–18 were transferred to the recording chamber in Tyrode's buffer (120 mM NaCl, 2.5 mM KCl, 20 mM HEPES, 30 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) containing tetrodotoxin (TTX) (1 μM). Recordings were carried out in whole-cell voltage-clamp, holding cells to -70 mV, using low resistance patch electrodes (4–8 MΩ) filled with Cs internal solution (135 mM CsCl, 2 mM $MgCl_2$, 2 mM Na_2-ATP, 0.2 mM Na-GTP, 10 mM HEPES). The internal solution was allowed to diffuse for 2 min before mEPSCs were recorded using an Axopatch 2A amplifier (Axon Instruments) and WinWCP software (Strathclyde Electrophysiology Software) for 5 min. For experiments where cells had been incubated in Aβo, coverslips were incubated in Aβo (200 nM) for over an hour and Aβo was also present during the experiment.

2.3.3.1 Analysis

Recordings were analysed using Mini Analysis (Synaptosoft), and mEPSCs were detected manually.

2.4 Live cell imaging

pHluorin experiments were carried out on dissociated hippocampal cultures at DIV 14–18 as per Jeans *et al.* (unpublished). Coverslips with transfected dissociated cultures were mounted in a Chamlide EC-B18 stimulation chamber on the stage of an Olympus IX-71 inverted microscope fitted with a 100X, NA 1.40 UPlanSApo objective and an Andor iXon EM CCD camera. Fluorescence illumination was supplied by a 100 W mercury lamp used with appropriate neutral density filters and shuttered (Uniblitz CS25, Vincent Associates) during all non-data acquisition

periods. Stimulation, image acquisition and shuttering were all under the co-ordinated control of WinWCP software (Strathclyde Electrophysiology Software) and all pHluorin imaging was performed out at 1 Hz. Experiments were carried out at room temperature in a standard Tyrode's buffer (120 mM NaCl, 2.5 mM KCl, 20 mM HEPES, 30 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) containing 10 μM NBQX and 50μM APV to block recurrent activity (DeLorenzo et al., 1998; Sombati and Delorenzo, 1995). The total SypH 2x signal was obtained by syphoning off the original Tyrode's solution from the chamber and addition of a Tyrode's solution high in NH_4Cl where 50mM NH_4Cl was added in substitution of 50mM of NaCl. Suitable fields of boutons were selected using the readily visualised resting fluorescence signal exhibited by SypH 2x, the additional criterion being that boutons show normal, healthy morphology, indicated by puncta that were not overly bright or large and which responded well to the NH_4Cl unquenching. Where cells had been incubated with Aβo or drug, these were premixed in culture media and the coverslips were incubated for over an hour before imaging and were present throughout the experiment. Drugs were used at the following concentrations: Aβo, 200 nM; D-e-MAPP, 5μM; K6PC-5, 10 μM; cambinol, 10 μM.

2.4.1 100 AP experiments

After recording a 10 s baseline signal, APs were evoked by passing 40 V, 1 ms current pulses from a custom-made stimulation box via platinum electrodes at 10 Hz over 10 s. Imaging was then continued for a further 100 s, before unquenching by alkalisation using a buffer containing NH_4Cl over a further 20 s to reveal the maximal SypH 2x response at each terminal.

2.4.2 1 AP experiments

SypH 2x-expressing boutons were serially imaged over 10 s, while giving a single stimulus to evoke a single AP by passing 20 V, 1 ms current pulses from a custom-made stimulation box via platinum

electrodes. This was repeated 23 times with 30 s rest between each one before unquenching by alkalisation using a buffer containing NH_4Cl over the last 10 s to reveal the maximal SypH 2x response at each terminal.

2.4.3 Miniature release experiments

SypH 2x-expressing boutons were imaged in the presence of TTX (μM). A baseline fluorescence was recorded over 10 s, before addition of the cell-impermeable vesicular ATPase inhibitor folimycin (10 nM) which blocks reacidification of synaptic vesicles (Ertunc et al., 2007). After 10 min, images were acquired over 30 s, and maximal SypH 2x response at each terminal was revealed by unquenching by alkalisation using a buffer containing NH_4Cl over the last 10 s.

2.4.4 SypH 2x analysis

Images were analysed in ImageJ (http://rsb.info.nih.gov/ij) using the Time Series Analyzer plugin (http://rsb.info.nih.gov/ij/plugins/time-series.html). Visible varicosities were selected for analysis with a 2 μm diameter ROI if they showed a response to application of NH_4Cl. All responses were background adjusted and normalised to the maximal response in order to control for inter-terminal differences in pHluorin expression level (Jeans et al., 2017).

2.4.4.1 100 AP analysis

Analysis was performed in MATLAB, using a custom-written script. Following background adjusting and normalisation, responses were measured as the average response over 2 s following stimulation compared to baseline response.

2.4.4.2 Miniature release analysis

Boutons were selected using a 2 μm diameter ROI. pHluorin imaging experiments were analysed using a custom-written MATLAB script. For analysis of minis, background fluorescence was subtracted from data points, and normalized to the individual NH_4Cl signal to normalise for changes in expression of SypH 2x between boutons. The difference in fluorescence between baseline and 10 min after Folimycin incubation was then calculated. The fluorescence changes (the responses) of every bouton were classified into bins of fixed-width, and this binned data was used to calculate a frequency distribution. A Gaussian mixture model was then fitted to the (unbinned) data, with the optimal number of Gaussians determined by the model resulting in the lowest Akaike Information Criterion (AIC). The Gaussian distributions were ranked according to their expected values, with the rank corresponding to the number of release events (q). Having identified the intersections between Gaussian distributions, I assigned each bouton within those intersections as having the q corresponding to that Gaussian distribution. In order to compare between the different conditions containing different numbers of boutons, the proportion of boutons in each Gaussian distribution was used to detect changes in mini frequency across conditions.

2.4.4.3 1 AP analysis

Analysis was performed using a custom-written MATLAB script. Data points were background adjusted as before, and for each stimulation a response was measured as: response (average two points after stimulation) - baseline (average of first five points). These were then normalized to NH_4Cl signal. A frequency distribution was then generated in the same range as before, and two Gaussians fitted. Having found the intersection of the Gaussians, observations below the intersection were designated as not releasing, while those above were assigned as a release event. Pr was then calculated for each bouton, discarding any bouton that did not release at all or had fewer than 15 usable measurements.

2.5 Chronic treatment of organotypic slices and measurement of tau phosphorylation

2.5.1 Chronic incubation experiments

Drugs were added to the culture medium of organotypic slices from 7 DIV for 7 days. Upon feeding (twice a week), drugs were also replaced. Drugs were mixed in the culture medium prior to feeding. Conditions used were: control (culture medium), Aβo (200 nM), D-APV (50 μM) (Abcam), Aβ oligomers (200 nM) with D-APV (50 μM), ω-agatoxin IVA (50 nM) (Alomone labs), Aβo (200 nM) with ω-agatoxin IVA (50 nM), DL-TBOA (20 μM) (Tocris), DL-TBOA (20 μM) with D-APV (50 μM). Slices were then lysed in RIPPA buffer (Abcam) on DIV 14 using a microtube pestle and stored at -80 °C until required.

2.5.2 Chronic LTD

For chemical LTD, from DIV 7 onwards, NMDA (20 μM) (Sigma-Aldrich) was added for 3 min to the top of the slice in culture medium before being syphoned off, once a day for 7 days. Slices were then lysed in RIPPA buffer on DIV 14 using a microtube pestle and stored at -80 °C until required.

For optogenetic LTD, transfected slices were subjected to LTD protocols (500 x 1 Hz, 900 x 1 Hz, 900 x 1 Hz every 2 h) from DIV 12 for 7 days. Optogenetic stimulation was controlled by a custom-built stimulation box and was delivered by a custom-built array of blue LEDs in the incubator. Slices were then lysed in RIPPA buffer on DIV 19 using a microtube pestle and stored at -80 °C until required.

2.5.3 Western blotting

Lysed samples were dissolved in 2X Laemmli sample buffer with 5% β–mercaptoethanol, heated to 60°C for 3 min and run on a precast 4–20% gradient SDS-PAGE gel (Thermo Scientific). The separated samples were transferred to a nitrocellulose membrane (Bio-Rad) before blocking with 5% milk and 1% horse serum in TBS with 0.05% Tween-20 (TBST) and subsequent probing with a mixture of the following antibodies: anti-phosphorylated tau (AT8, 1:400; ThermoFischer); anti-total tau (E178, 1:1000; Abcam) for 12 h at 5 °C. After three TBST washes, bound antibodies were detected with IRDye 680LT donkey anti rabbit IgG (Li-Cor, 1:20,000) and IRDye 800CW goat antimouse IgG (Li-Cor, 1:15,000) fluorescent secondary antibodies, washed three times in TBST and imaged on a Li-Cor Odyssey system.

2.5.3.1 Analysis

Images were analysed quantitatively using Image Studio Lite software (Li-Cor Biosciences). The ratio of AT8:E178 was measured and then, for each blot, values were normalised to the average of the corresponding control group to allow for discrepancies between runs. In order to check for outliers, the ROUT method was used (Q = 1%), and any outliers identified were excluded. This method is better at detecting more than one outlier than the Grubb's test, and so, I decided to use this for the Western blot data, as the n values are relatively high and allow for the possibility of multiple outliers.

2.6 Aβo synthesis

Experiments were conducted using a single batch of $A\beta_{1–42}$ peptide (Abcam) and oligomers were synthesised according to a validated protocol (Klein, 2002). This widely-used and highly reproducible preparation has previously been characterised in detail and found to contain predominantly the low molecular weight species most associated with synaptotoxicity (Lambert et

al., 2007; Velasco et al., 2012) (see Figure 2.2 from Jeans *et al.* unpublished data). Briefly, solid $A\beta_{1-42}$ was dissolved in cold hexafluoro-2-propanol (HFIP) (Sigma Aldrich). The peptide was incubated at room temperature for at least 1 hour to establish monomerization and randomisation of the structure. The HFIP was aliquoted and allowed to evaporate overnight, followed by 10 min in a Savant Speed Vac. The resulting peptide was stored as a film at -80 °C. When required, the film was dissolved in anhydrous dimethylsulfoxide (DMSO) (Sigma Aldrich) to 5 mM, diluted to approximately 100 μM with Ham's F12 (without phenol red, with glutamine) (Caisson Laboratories, Logan, UT) and briefly vortexed. The solution was incubated at 4 °C for 22–24 hours, and soluble oligomers obtained by centrifugation at 14000 g for 10 min at 4 °C.

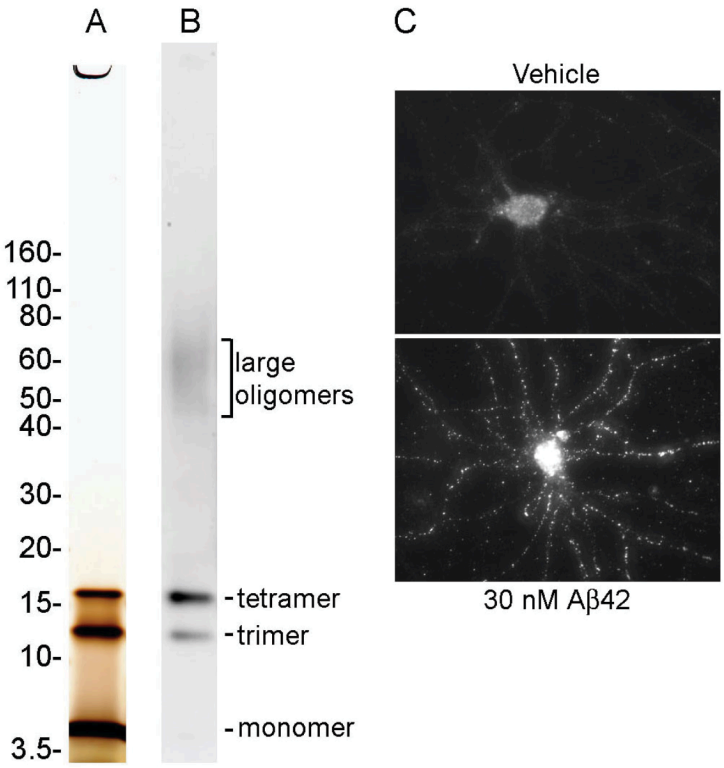

Figure 2.2: Analysis of Aβ oligomers and lack of effects on action potentials and neuronal excitability. (A) Silver-stained SDS-PAGE gel of Aβ oligomers (left) shows a predominant monomer band in addition to trimers and tetramers. The smaller species are largely the result of the SDS-sensitivity of larger oligomers (Velasco et al., 2012). Numbers shown are in kDa. (B) Western blot immunostaining (middle) with oligomer-specific monoclonal antibody NU2 detected the presence of large SDS-stable oligomers in addition to trimers and tetramers. (C) Mature (26 DIV) hippocampal cell cultures (right) incubated for 30 min at 37°C with oligomers formed at 30 nM $A\beta_{1-42}$ peptide shows specific synaptic binding detected by immunolabelling with oligomer-specific monoclonal antibody NU4. Figure taken from Jeans *et al.* (unpublished data).

2.7 Statistics

All statistical tests were performed using Graphpad Prism and are stated where used. Error bars represent ± standard error of the mean (SEM) unless otherwise stated. Significance is denoted as

follows: ns: not significant, *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. In all tests, alpha = 0.05. Outliers were calculated using the Robust regression and Outlier removal (ROUT) method (GraphPad Prism version 7, using the coefficient, Q = 1%, and any outliers were removed. This test allows for the identification of multiple outliers. Data from acute slices were treated as n = 1 per animal. Data collected from the same animal was averaged and treated as n = 1. Data from organotypic slices were treated as n = 1 per slice on account of the differentiation that occurs to slices as they develop. Data from primary dissociated hippocampal neurons were treated as n = 1 per coverslip.

3. Reducing presynaptic function normalises Aβo-induced plasticity deficits

3.1 Introduction

Understanding the early pathological events that occur in AD is vital for helping us to find an effective treatment. A key hallmark of Aβo-induced pathology is dysfunctional synaptic plasticity, which appears to occur soon after introducing pathological levels of Aβo (Mucke and Selkoe, 2012; Selkoe, 2008; Viola and Klein, 2015). Synaptic plasticity is considered to be a key mechanism in altering the strength of synaptic transmission and the formation of memories (Citri and Malenka, 2008), and so, it is probable that any dysfunction of synaptic plasticity would drive the symptoms that are observed in AD, including aberrant network dynamics (Busche and Konnerth, 2016; Palop and Mucke, 2016), neurodegeneration, and the memory and behavioural deficits that present clinically (Mucke and Selkoe, 2012).

Although some effects of Aβo on synaptic plasticity are well understood, including attenuation of LTP and facilitation of LTD (Hsieh et al., 2006; Li et al., 2009; Li et al., 2011; Shankar et al., 2008; Walsh et al., 2002), the mechanism by which they occur is unclear. A number of postsynaptic targets have been identified as possible mediators of Aβo-induced toxicity (Viola and Klein, 2015), but none appear to lie at the heart of Aβo-driven synaptotoxicity. Studies have shown that Aβo-induced plasticity and network deficits depend on enhanced extracellular glutamate, which has been proposed to result from impaired glutamate clearance (Li et al., 2009; Li et al., 2011; Zott et al., 2019), leading to spillover of glutamate and activation of extrasynaptic NMDARs (Hanson et al., 2015; Li et al., 2009; Li et al., 2011). Indeed, elevation of extracellular glutamate levels via blockade of EAATs using DL-TBOA also results in activation of extrasynaptic NMDARs (Harney et al., 2008; Li et al., 2011), enhanced LTD (Dutar and Potier, 2019; Valtcheva and Venance, 2019) and reduced LTP (Kervern et al., 2012; Li et al., 2011) in rodents. Other evidence, however,

suggests that pathological levels of Aβo can enhance synaptic glutamate release (Brito-Moreira et al., 2011; Dolev et al., 2013; Kabogo et al., 2010; Russell et al., 2012) and this would also account for the elevated extracellular glutamate levels. Recent, unpublished work from our laboratory has confirmed that Aβo enhance glutamate release and has identified elevated activity of the presynaptic VGCC $Ca_V2.1$ as a potential cause (Jeans et al., Unpublished).

It is still unclear whether Aβo-induced attenuation of LTP and facilitation of LTD are mechanistically or causally linked. The two processes have differences in the mechanism of expression and signal transduction, and are induced under different conditions (Citri and Malenka, 2008). The direction of synaptic plasticity at excitatory synapses depend upon different patterns of pre- and postsynaptic activation, often in response to varying patterns of neurotransmitter release. The most commonly studied form of LTP, NMDAR-dependent LTP, tends to be induced by a large Ca^{2+} influx as a result high frequency bursts of presynaptic activation, leading to swift release of glutamate and activation of NMDARs (Malenka, 1991; Malenka and Nicoll, 1993). In contrast, LTD seems to depend on sustained, low levels of Ca^{2+} influx (Cummings et al., 1996), and can be induced by long lasting, low frequency presynaptic activation that results in steady, low levels of glutamate release (Dudek and Bear, 1992; Mulkey and Malenka, 1992). In this context synapses with a high probability of release, as occurs in AD, will function more as low-pass filters, allowing for reliable transmission of low frequency activity (Abbott and Regehr, 2004). Therefore, I hypothesised that this would facilitate the conditions required for LTD (and so attenuate LTP), namely tonic low levels of synaptic glutamate. I set out to build upon the previous work from Jeans *et al.* (described in the introduction) to investigate whether reducing presynaptic function by partial blockade of $Ca_V2.1$ might mitigate Aβo-induced plasticity changes. Using electrophysiological measurements of plasticity in adult (age 7/8 weeks) mice, I show that partially reducing presynaptic function normalises both aberrant LTP and LTD in the presence of Aβo.

This finding supports the hypothesis that the well-documented plasticity deficits observed in AD are a result of Aβo-induced changes in presynaptic function, suggesting that these changes in presynaptic function drive downstream AD pathology.

3.2 A low dose of ω-agatoxin IVA partially reduces presynaptic function

Ca^{2+} influx at hippocampal excitatory boutons depends upon activation of a combination of $Ca_V2.2$ and $Ca_V2.1$ VGCCs (Takahashi and Momiyama, 1993). Since neurotransmitter release is critically dependent on this Ca^{2+} influx, with which it has a non-linear cooperative relationship (Sinha et al., 1997) full blockade of $Ca_V2.1$ would drastically reduce synaptic transmission and prevent the induction of plasticity completely. Therefore, I set out to determine a concentration of ω-agatoxin IVA, a specific $Ca_V2.1$ blocker, that could offset the Aβo-induced increase in synaptic transmission without completely blocking it.

fEPSPs were recorded from Schaffer collateral-CA1 synapses in acute hippocampal slices from 7–8 week-old mice (Figure 3.1a) and ω-agatoxin IVA (50 nM) was bath applied. 50 nM was the first concentration attempted, and fortunately, after addition of 50 nM ω-agatoxin IVA fEPSP was reduced by 18% (Figure 3.1b), enough to offset the effects of Aβo-mediated release enhancement (20% as measured by Jeans *et al.*), accompanied by an increase in PPR (Figure 3.1c), confirming that this decrease in synaptic transmission was presynaptic in origin.

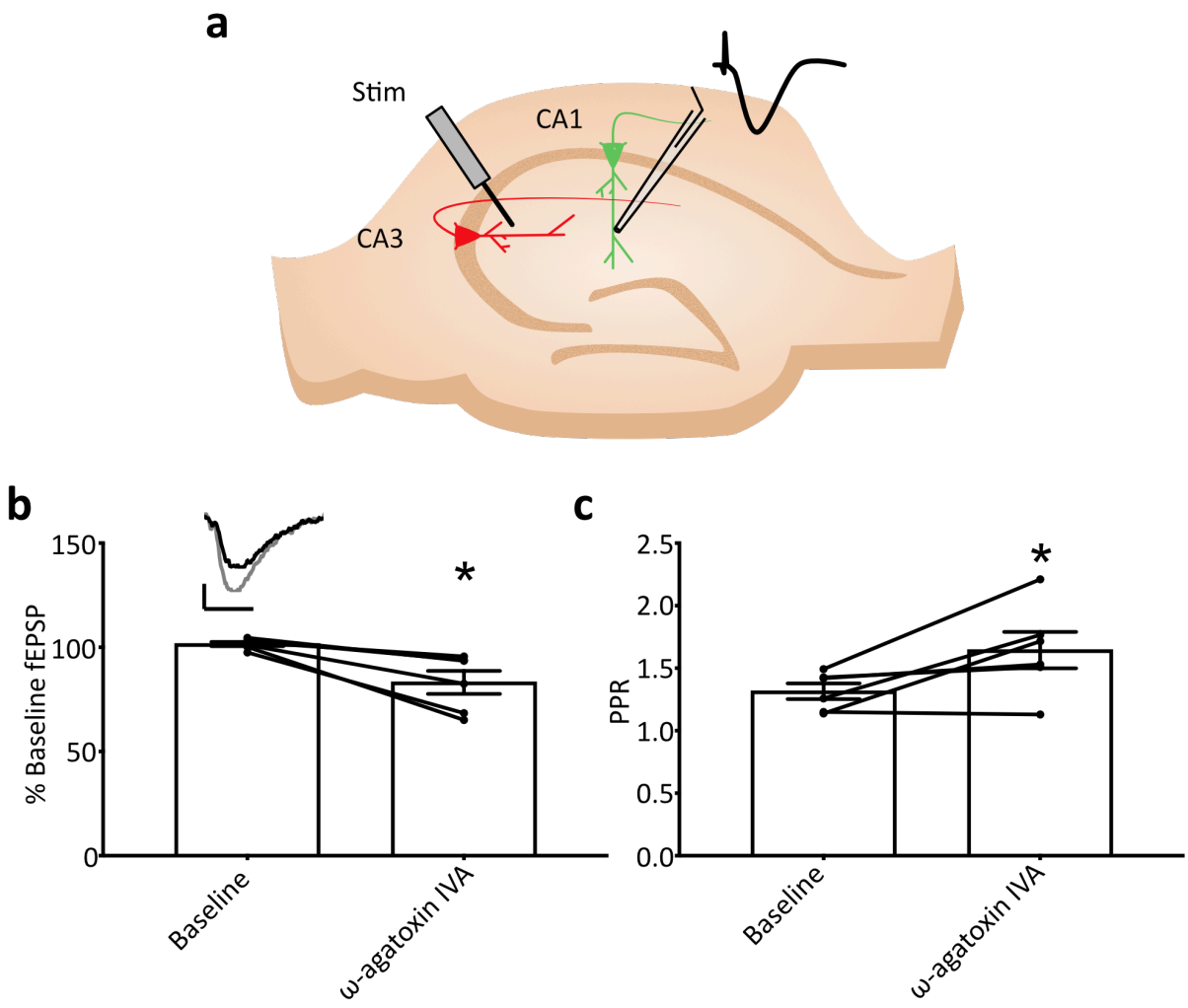

Figure 3.1: 50 nM ω-agatoxin IVA partially reduces synaptic transmission. **a)** Outline of the experimental setup, with a bipolar stimulation electrode placed in CA3 Schaffer collaterals, and a recording electrode in stratum radiatum in CA1, measuring fEPSPs elicited. Hippocampus adapted from an illustration by Rudi Tong. **b)** 50 nM ω-agatoxin IVA reduces fEPSP by 18% (before ω-agatoxin IVA addition: 101.6 ± 1.04% baseline fEPSP, n=6 animals; after ω-agatoxin IVA addition 83.17 ± 5.53% baseline fEPSP, n=6), inset traces represent fEPSPs before (grey) and after (black) ω-agatoxin IVA addition, vertical line represents 0.2 mV, horizontal line represents 5 ms. Paired t test **c)** 50 nM ω-agatoxin IVA increases PPR (before ω-agatoxin IVA addition: 1.32 ± 0.06, n=6 animals; after ω-agatoxin IVA addition 1.65 ± 0.15, n=6) paired t test.

3.3 A low dose of ω-agatoxin IVA rescues Aβo-induced LTP attenuation

Having ascertained that a partial block of $Ca_V2.1$ could be achieved with a concentration of 50 nM agatoxin, I asked whether this reduction in presynaptic function could normalise Aβo induced LTP deficits. fEPSPs were recorded from acute hippocampal slices and LTP was induced using a 20x theta burst stimulus (TBS). This protocol was chosen as it better resembles physiological stimuli compared to standard HFS (Otto et al., 1991; Perez et al., 1999) and was able to elicit robust LTP in control and ω-agatoxin IVA (50 nM) incubated conditions (Figure 3.2a, b).

In contrast, slices incubated in Aβo (50 nM) showed only mild potentiation that was significantly different to control ($p= 0.049$) (Figure 3.2a, b), in line with previous studies (Shankar et al., 2008). Slices incubated in Aβo with ω-agatoxin IVA, however, showed a strong potentiation, similar to that of control and ω-agatoxin IVA only conditions (Figure 3.2a, b). Although every effort was taken to achieve a stable baseline, these results show a degree of runup in the baseline. As such, it is important to acknowledge that the degree of LTP obtained here could be over reported. Despite this, the level of runup is consistent between the groups, and so, the conclusions here comparing the different conditions are likely to be valid.

An analysis of the PPR showed that, upon LTP induction, there was no change (Figure 3.2c, d), suggesting that for all conditions the potentiation was postsynaptically expressed, with no presynaptic component.

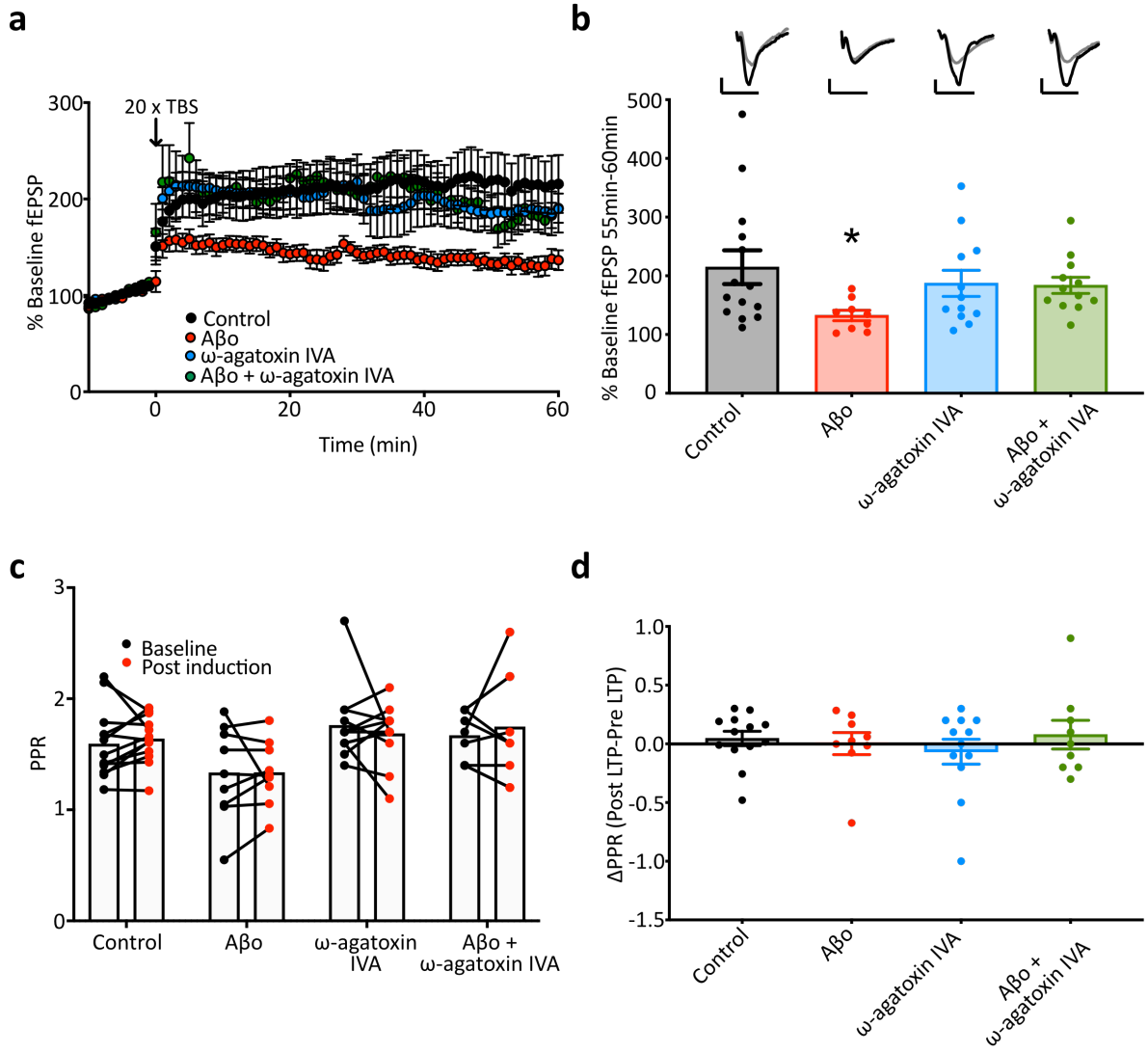

Figure 3.2: Partial block of Cav2.1 mitigates the effects of Aβo on hippocampal LTP. **a)** Summary traces showing LTP following 20x TBS. **b)** Mean fEPSP slope 55–60 min post LTP induction (Control: n = 14 animals, 214.7 ± 28.7 % baseline fEPSP; Aβo: n = 9 animals, 132.4 ± 8.9 % baseline fEPSP; ω-agatoxin IVA n = 14 animals, 187.2 ± 22.6% baseline fEPSP; ω-agatoxin IVA + Aβo n = 14 animals, 183.7 ± 13.76 % baseline fEPSP). Inset traces represent single trace fEPSPs before (grey) and after (black) LTP induction, vertical line represents 0.5 mV, horizontal line represents 5 ms. Kruskal-Wallis followed by Dunn's multiple comparison test. **c)** PPR changes before (black) and after (red) LTP induction post incubation in Aβo, ω-agatoxin IVA, Aβ + ω-agatoxin IVA. **e)** Change in PPR (post LTP induction – pre LTP induction). post incubation in Aβo, ω-agatoxin IVA, Aβo + ω-agatoxin IVA (Control: n = 13, 0.05 ± 0.06; Aβ0: n = 9, 0.00 ± 0.09; ω-agatoxin IVA: n = 12, -0.07 ± 0.11; Aβ + ω-agatoxin IVA: n = 9, 0.08 ± 0.12) Kruskal-Wallis test followed by Dunn's multiple comparisons test.

3.4 ω-agatoxin IVA can mitigate Aβo-induced LTD facilitation

I have hypothesised that Aβo-induced elevation of presynaptic function drives both attenuation of LTP and facilitation of LTD. Therefore, I asked whether Aβo-enhanced LTD could be rescued by partial reduction in presynaptic function, aimed at offsetting the presynaptic enhancement elicited by Aβo. As above, for this, I used a 50 nM concentration of ω-agatoxin IVA and induced LTD with a low-frequency stimulation (LFS) protocol of 900 stimuli at 1 Hz. Recording fEPSPs of Schaeffer collateral synapses in CA1 of acute hippocampal slices, I found that, under control conditions, LFS depressed fEPSPs of control slices to 77.26 ± 4.79% of baseline (n = 7 animals), while incubation with Aβo (50 nM) robustly increased the magnitude of LTD ($p = 0.033$), depressing fEPSP to 41.46 ± 9.08% of baseline (n = 8 animals) (Figure 3.3a, b).

Adding 50 nM ω-agatoxin alone had no effect on LTD compared to control (80.26 ± 8.99 % of baseline; n = 5 animals), however, ω-agatoxin fully rescued the effect of Aβo on LTD, which was restored to control levels (76.08 ± 4.64 % of baseline; n = 6 animals) (Figure 3.3a, b), suggesting that enhanced presynaptic function is required for the Aβo-mediated enhancement of LTD.

An examination of the PPR showed significance, but a multiple comparisons test showed no significant change between any group, with the Aβo treated condition compared to control having $p = 0.12$ (Figure 3.3c, d).

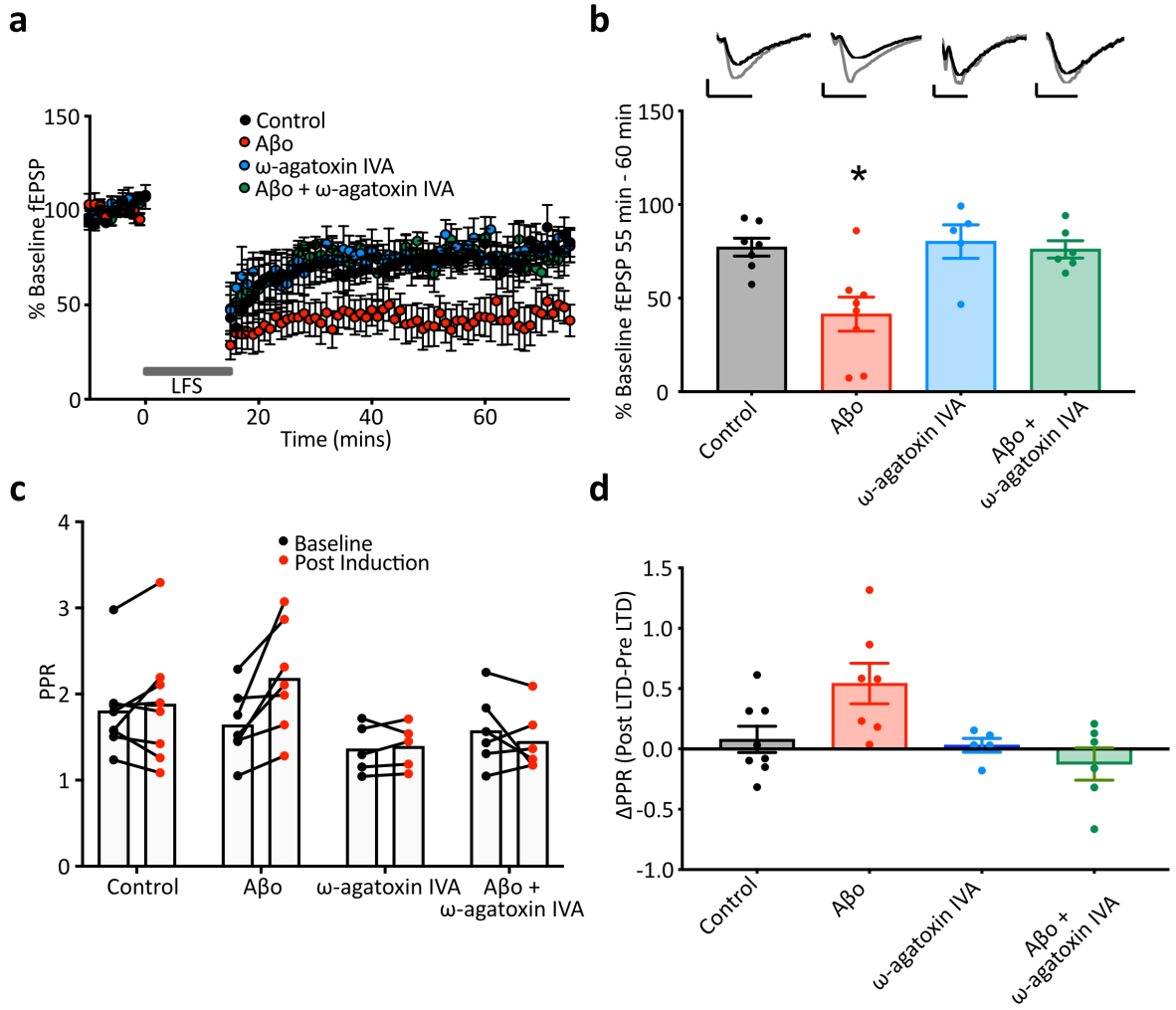

Figure 3.3: Aβo enhance LFS LTD and this can be rescued by ω-agatoxin IVA **a)** Summary traces showing LTD following LFS (900 x 1 Hz stimulation). **b)** Mean fEPSP slope 55–60 min post LTD induction (Control: n = 7 animals, 77.26 ± 4.79 % baseline fEPSP; Aβo: n = 8 animals, 41.46 ± 9.08 % baseline fEPSP; ω-agatoxin IVA n = 5 animals, 80.26 ± 8.99% baseline fEPSP; ω-agatoxin IVA + Aβo n = 6 animals, 76.08 ± 4.64 % baseline fEPSP). Inset traces represent fEPSPs before (grey) and after (black) LTD induction, vertical line represents 0.5 mV, horizontal line represents 5 ms. Kruskal-Wallis followed by Dunn's multiple comparison test. **c)** PPR changes before (black) and after (red) LTD induction post incubation in Aβo, ω-agatoxin IVA, Aβo + ω-agatoxin IVA. **d)** Change in PPR (post LTD induction – pre LTD induction). post incubation in Aβo, ω-agatoxin IVA, Aβo + ω-agatoxin IVA (Control: n = 8 animals, 0.079 ± 0.11; Aβo: n = 7 animals, 0.54 ± 0.17; ω-agatoxin IVA: n = 5 animals, 0.03 ± 0.06; Aβ + ω-agatoxin IVA: n = 6 animals, -0.13 ± 0.13) Kruskal-Wallis test with Dunn's multiple comparison test compared to Control.

3.5 Discussion

I have shown that suppressing presynaptic function by partial blockade of $Ca_V2.1$ can counteract the effects of Aβo on both LTP and LTD. This provides evidence that Aβo-driven presynaptic facilitation precedes and even actuates any postsynaptic plasticity changes. As described in the introduction, multiple studies have identified potential postsynaptic Aβo receptors that might mediate its neurotoxicity and effects on plasticity; however, my results here suggest that Aβo act upstream of the postsynaptic terminus. This could have important repercussions for future research into Aβo therapeutics, as it opens up a new area for potential intervention.

As discussed, previous studies have implicated elevated extracellular glutamate as a possible driver for Aβo-impaired plasticity, however they rule out augmented release probability as a cause (Li et al., 2009; Li et al., 2011). This could be because their sole measurement of presynaptic function is examination of PPR, which can be confounded by any rapid changes in the response of the postsynaptic terminus. Given that Aβo-incubation can cause rapid AMPAR desensitisation (Li et al., 2009), which can affect both of the required parameters (Christie et al., 2010; Heine et al., 2008; Li et al., 2009), PPR is not a reliable measurement of presynaptic function when comparing between conditions involving Aβo. Jeans *et al.* have shown, however, that blocking AMPAR desensitisation with cyclothiazide (Yamada and Tang, 1993) reveals changes in PPR upon Aβo addition, corroborating optical evidence that enhanced release is responsible for elevated synaptic glutamate (Jeans et al., Unpublished). My findings build upon this further, demonstrating that ameliorating the presynaptic effects of Aβo restores downstream plasticity processes to normal. This suggests presynaptic facilitation as an upstream pathological event in Aβo-mediated neurotoxicity and provides a potential explanation for the impaired plasticity changes that occur in the presence of oligomers. Impaired LTP and augmented LTD may be two sides of the same coin, where there is a metaplastic shift towards LTD induction. Indeed, there is evidence that some

higher frequency stimulation patterns, that usually induce LTP, can induce LTD in the presence of Aβo (Kervern et al., 2012; Moreno-Castilla et al., 2016; Sanchez-Rodriguez et al., 2019).

Enhanced presynaptic release probability turns the presynaptic bouton from a high-pass filter that preferentially transmits high frequency signals, to a low-pass filter that is weighted more towards low frequency patterns of stimulation (Abbott and Regehr, 2004). This is reflected experimentally; LTD can be induced by subthreshold (300x 1 Hz) protocols in the presence of oligomers (Chen et al., 2013; Huang et al., 2018; Kim et al., 2001; Li et al., 2009; Ondrejcak et al., 2019; Salgado-Puga et al., 2017; Shankar et al., 2008), implying that synapses are more sensitive to LTD-inducing conditions. Indeed, it could be that dysregulated LTD lies at the heart of AD pathology. Inhibitory interneurons exhibit plasticity as well as excitatory principal neurons. While our understanding of the stimuli and processes that induce plasticity in inhibitory neurons is still poor, in part due to the difficulty in isolating and characterising the numerous different types, it is clear that glutamatergic signalling can induce changes in inhibitory neuron excitability and firing, both in a heterosynaptic way, and as excitatory inputs onto inhibitory interneurons (Castillo et al., 2011; Kullmann et al., 2012). Enhanced release probability at excitatory terminals together with resultant increases in tonic extracellular glutamate could facilitate LTD at inhibitory neurons as well as excitatory principal ones, leading to a reduction in inhibitory tone, an increase in the E/I ratio, and subsequent network level hyperexcitability that is observed early on in AD progression as discussed in the introduction. As the disease progresses, however, the ongoing LTD occurring at excitatory neurons might begin to show network level effects, resulting in reduced network activity that is characteristic of later stage AD.

Although the mechanism suggested by which Aβo might bring about the pathological changes that occur both early on and further into AD progression is conjecture, it highlights the importance of Aβo-induced presynaptic changes. My results here, have established an important link between

presynaptic enhancement and postsynaptic weakening that could have important implications for our future understanding, and, our treatment of AD. Next, I sought to investigate whether these plasticity changes might be responsible for driving further, downstream pathology.

4. Synaptic activity drives pathological tau phosphorylation

4.1 Introduction

Although it is clear that Aβo are the upstream trigger of pathogenesis in AD (Mucke and Selkoe, 2012), it is becoming apparent that the presence of pathologically phosphorylated and aggregated tau is also critical for the pathophysiological progression of the disease (Rapoport et al., 2002; Roberson et al., 2011; Roberson et al., 2007), with neurons from tau knockout mice being resistant to Aβo-induced deficits (Rapoport et al., 2002). Although the roles of both Aβo and hyperphosphorylated tau in AD have been extensively studied, very little is known about the relationship between these two key players, and in particular, about how elevated Aβo levels might recruit abnormally phosphorylated tau. This represents a vital unanswered question that needs to be resolved and doing so will not only fill an important gap in our understanding of AD pathophysiology, but likely identify important candidate pathways for therapeutic intervention. In addition, understanding better the mechanisms by which aberrant tau phosphorylation can occur might also shed light on the mechanisms underlying other tauopathies.

A clue to the recruitment of pathological tau phosphorylation could lie in its non-disease state interactions. As has been discussed in the introduction, recent evidence suggests that tau appears to have a role in dendrites at the synapse (Ahmed et al., 2014; Ittner and Ittner, 2018; Ittner et al., 2010; Kimura et al., 2014; Mondragon-Rodriguez et al., 2012; Regan et al., 2015; Regan et al., 2017). In particular, evidence suggests that tau interacts with proteins important for synaptic AMPAR expression, including KIBRA (Tracy et al., 2016), PICK1, and the AMPAR subunit GluA2 (Regan et al., 2015). AMPAR endocytosis is important for the expression of LTD (Citri et al., 2010; Hanley and Henley, 2005; Penn et al., 2017; Roth et al., 2019), and recent evidence has shown that GSK-3-mediated phosphorylation of tau at serine 396 is critical for both induction of hippocampal LTD and the reversal of learning in vivo (Kimura et al., 2014; Regan et al., 2015).

Given my results of the previous chapter, that Aβo enhance LTD, together with further evidence from other studies that Aβo facilitate LTD induction (Chen et al., 2013; Huang et al., 2018; Kim et al., 2001; Li et al., 2009; Ondrejcak et al., 2019; Salgado-Puga et al., 2017; Shankar et al., 2008) it could be that there is a link between synaptic changes induced by Aβo and tau phosphorylation in AD, with LTD, or a pathological LTD-like process, serving as a potential mechanistic link between the two. This would be consistent with studies that demonstrate mechanistic overlap between LTD and Aβo-induced synaptic decline in AD (Hsieh et al., 2006), and would also align the dependence of both processes on extrasynaptic NMDARs (Li et al., 2009; Papouin et al., 2012; Talantova et al., 2013). While it is known that tau is phosphorylated at many more residues in AD pathology than during physiological LTD (Regan et al., 2015), this may arise from the prolonged activation of LTD-associated kinases, such as GSK-3β, as a result of Aβo enhancing synaptic activity, leading to non-physiological phosphorylation of additional residues.

Therefore, I set out to test this hypothesis using a variety of chronic treatments on rat organotypic hippocampal slices and measurement of tau phosphorylation by Western blotting. Developing my evidence that Aβo-enhanced release drives plasticity changes, I show that this extends to downstream AD pathology. Partially reducing presynaptic function with ω-agatoxin IVA can rescue long-term, NMDAR-dependent Aβo-enhanced pathological phosphorylation of tau. Furthermore, I expand the evidence that chronic LTD-like conditions serve as a mechanistic link between Aβo and pathological tau modification. I demonstrate that several chronic LTD-inducing stimuli, such as repeated applications of NMDA, or optogenetically-driven LTD induction, both increase the amount of pathologically phosphorylated tau. Taken together, these data support a mechanism in which Aβo enhance release probability, resulting in elevated extracellular glutamate levels, aberrant induction of synaptic LTD, and consequently elevated tau phosphorylation.

4.2 NMDAR-dependent Aβo-induced tau phosphorylation is rescued by partial suppression of presynaptic activity

Since I have shown that reducing Aβo-enhanced presynaptic function can normalise plasticity deficits, I sought to determine whether Aβo-enhanced synaptic activity might play a role in driving the downstream AD pathology of tau phosphorylation. I studied the effects of chronic Aβo incubation (over 7 days) in organotypic slices on tau phosphorylation using the antibody AT8, which specifically recognises pathologically phosphorylated tau, binding to phosphorylated serine 202 and phosphorylated threonine 205 residues (Goedert et al., 1995). Both of these are present in pathological paired helical filaments of tau in AD brains (Braak et al., 1994; Su et al., 1994) and are residues phosphorylated by GSK-3 (Liu et al., 2002; Wang et al., 1998), implicated in Aβo-associated tau phosphorylation in AD (Shipton et al., 2011; Takashima, 2006). In addition, phosphorylation of these residues is associated with tau misfolding into a pathological conformation (Bibow et al., 2011; Jeganathan et al., 2008). Phosphorylation was quantified using Western blotting, comparing the levels of pathologically phosphorylated tau (AT8) with total tau (E178), and normalising to control lanes within each blot to account for inter-experimental variation.

As expected, a 7 day incubation in Aβo led to an increase in the levels of pathologically phosphorylated tau compared to control ($p = 0.001$) (Figure 4.1a). Application of the ROUT method identified two outliers in the control group which were excluded. Chronic incubation in the NMDAR blocker 2-amino-5-phosphonopentaoic acid (APV) (50 μM) had no effect on tau phosphorylation alone, but incubation in Aβo with APV restored tau phosphorylation back to control levels (Figure 4.1b), indicating its dependence on NMDAR activation.

In order to further test the importance of Aβo-enhancement of synaptic glutamate release in driving pathological tau phosphorylation, I sought to investigate whether reducing presynaptic activity might have an effect on Aβo-induced tau phosphorylation. I partially reduced presynaptic function pharmacologically, again using ω-agatoxin IVA (50 nM). Slices were incubated in ω-agatoxin alone, or in ω-agatoxin IVA with Aβo, and tau phosphorylation was again examined. I found that incubation in ω-agatoxin IVA alone had no effect on tau phosphorylation with respect to control, but ω-agatoxin IVA was able to prevent the Aβo-induced tau phosphorylation increase, since ω-agatoxin IVA with Aβo returned tau phosphorylation to control levels (Figure 4.1c).

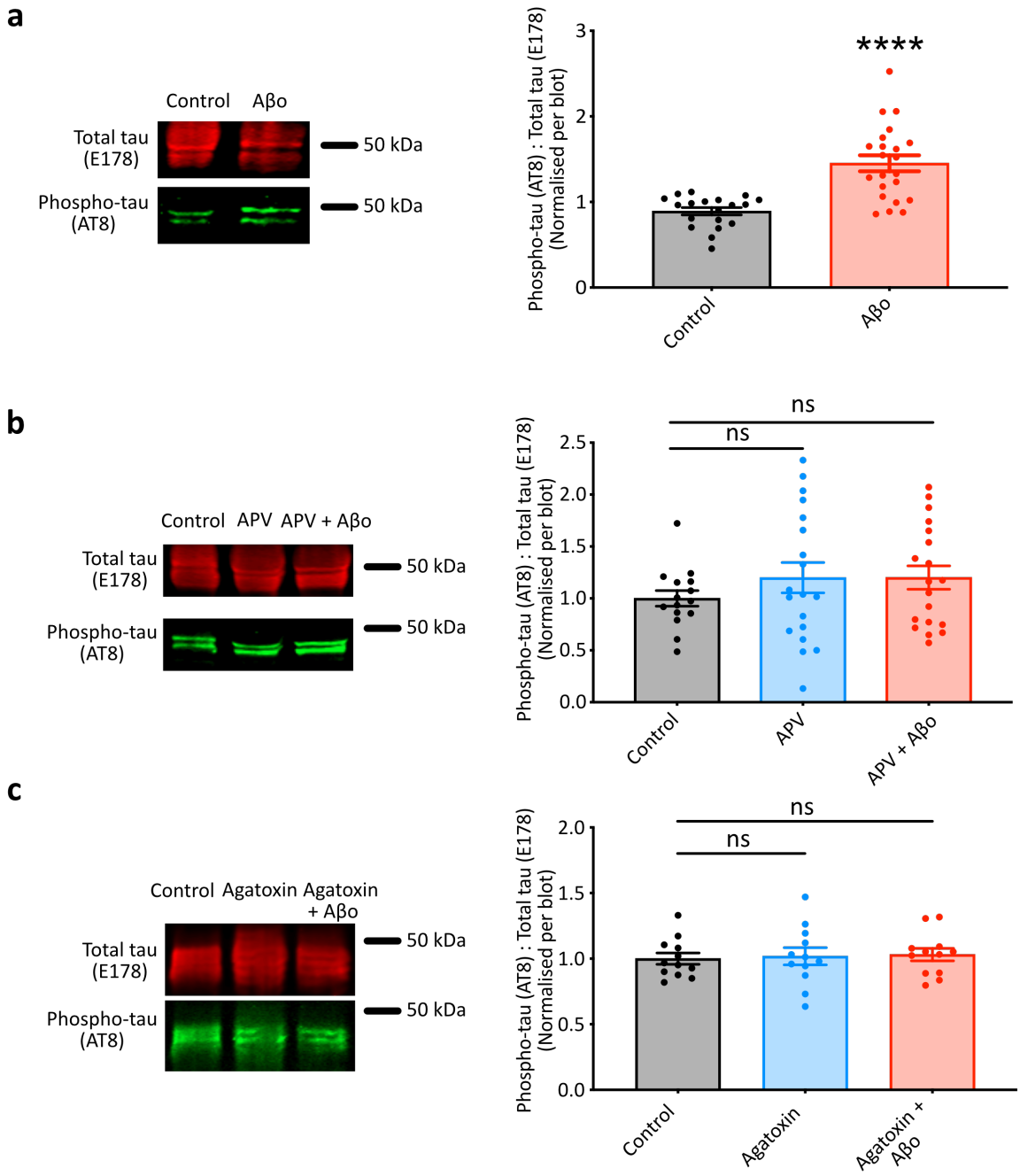

Figure 4.1: Aβo incubation enhances pathological tau phosphorylation in an NMDAR-dependent manner that can be blocked with ω-agatoxin IVA. **a)** Representative Western blot data with pooled data of Western blot analysis of Aβo-incubated slices over 7 days. Changes in tau phosphorylation are measured by the ratio of pathologically phosphorylated tau (AT8) : Total tau (E178), normalised to control per blot (Control: n = 19 slices, 0.89 ± 0.04; Aβo: n = 22 slices, 1.45 ± 0.09). Mann-Whitney test. **b)** Representative Western blot data with pooled data of Western blot analysis of APV-incubated and APV with Aβo-incubated slices over 7 days. Changes in tau

phosphorylation are measured by the ratio of pathologically phosphorylated tau (AT8) : Total tau (E178), normalised to control per blot (Control: n = 15 slices, 1.00 ± 0.08; APV: n = 19 slices, 1.20 ± 0.15; Agatoxin + Aβo: n = 19 slices, 1.20 ± 0.11) Kruskal-Wallis test followed by Dunn's multiple comparison test vs control. **c)** Representative Western blot data with pooled data of Western blot analysis of ω-agatoxin IVA (Agatoxin)-incubated and Agatoxin with Aβo-incubated slices over 7 days. Changes in tau phosphorylation are measured by the ratio of pathologically phosphorylated tau (AT8) : Total tau (E178), normalised to control per blot (Control: n = 12 slices, 1.00 ± 0.04; Agatoxin: n = 12 slices, 1.02 ± 0.07; Agatoxin + Aβo: n = 12 slices, 1.03 ± 0.05) Kruskal-Wallis test followed by Dunn's multiple comparison test vs control.

4.3 Chronic elevation of synaptic glutamate enhances tau phosphorylation in an NMDAR-dependent manner

To gather further support for my hypothesis, I asked whether raising the level of extracellular glutamate by another means, independent of synaptic activity, would have the same effect on tau phosphorylation. To achieve this, slices were incubated for 7 days in a pan blocker of excitatory amino acid transporters (EAATs), DL-TBOA (20 μM), and the effects on tau phosphorylation were measured as before. I found that chronic incubation in DL-TBOA led to enhanced tau phosphorylation ($p = 0.025$), and that this could be counteracted by inhibition of NMDARs (Figure 4.2).

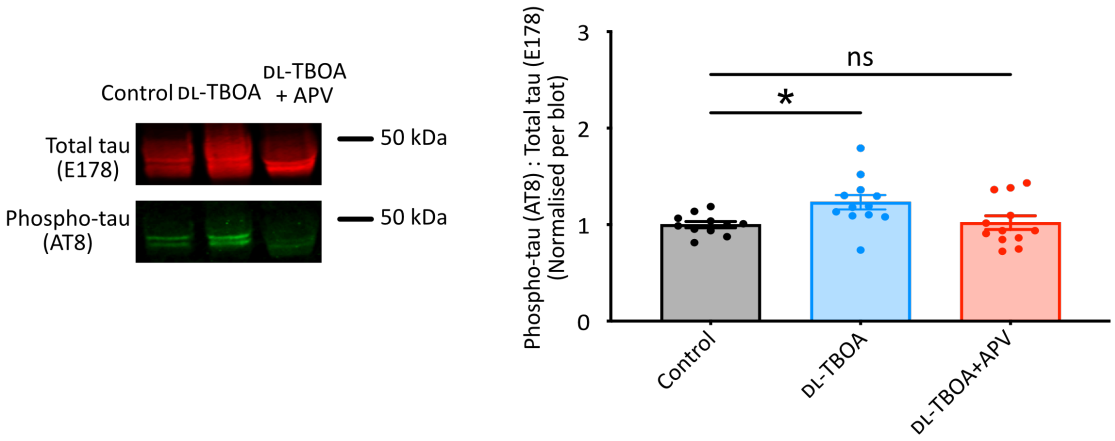

Figure 4.2: DL-TBOA incubation increases tau phosphorylation in an NMDAR-dependent manner. Representative Western blot data with pooled data of Western blot analysis of DL-TBOA-incubated, or DL-TBOA with APV-incubated slices over 7 days. Changes in tau phosphorylation are measured by the ratio of pathologically phosphorylated tau (AT8) : Total tau (E178), normalised to control per blot (Control: n = 11 slices, 1.00 ± 0.03; DL-TBOA: n = 12 slices, 1.23 ± 0.08; DL-TBOA + APV: n = 12 slices, 1.02 ± 0.07) Kruskal-Wallis test followed by Dunn's multiple comparison test vs control.

4.4 Chronic, repeated LTD protocols enhance tau phosphorylation

I have shown that both Aβo-mediated phosphorylation of tau and Aβo-mediated facilitation of LTD are dependent on enhanced presynaptic activity. Together, these results suggest that increased synaptic activity drives pathological phosphorylation of tau via LTD following exposure to Aβo. In order to ask more directly whether elevated low-frequency synaptic activity can induce LTD and drive pathological tau phosphorylation, I examined whether chronic LTD-inducing conditions would lead to increased tau phosphorylation. Initially, I used a chemical method of LTD induction (20 μM NMDA for 3 min) (Lee et al., 1998) and verified that this induced LTD in organotypic hippocampal slices, confirming a robust depression of the excitatory postsynaptic current (EPSP) following NMDA application ($p = 0.027$) (Figure 4.3a). This protocol has been shown to require tau for LTD to occur (Kimura et al., 2014).

NMDA (20 μM) was presented to organotypic slices for 3 min, once a day, for 7 days, after which tau phosphorylation was examined as before. I found that this NMDA-LTD protocol caused a slight increase that was close to significance ($p = 0.059$) increase in pathological tau phosphorylation (Figure 4.3b). It seems that much of this difference is being driven by one point, however, upon application of the ROUT method to identify any outliers, this point was not identified. Despite this, the effect is not statistically significant, and it is, therefore, not possible to say whether this chemical LTD applied once a day is enough to induce pathological tau phosphorylation.

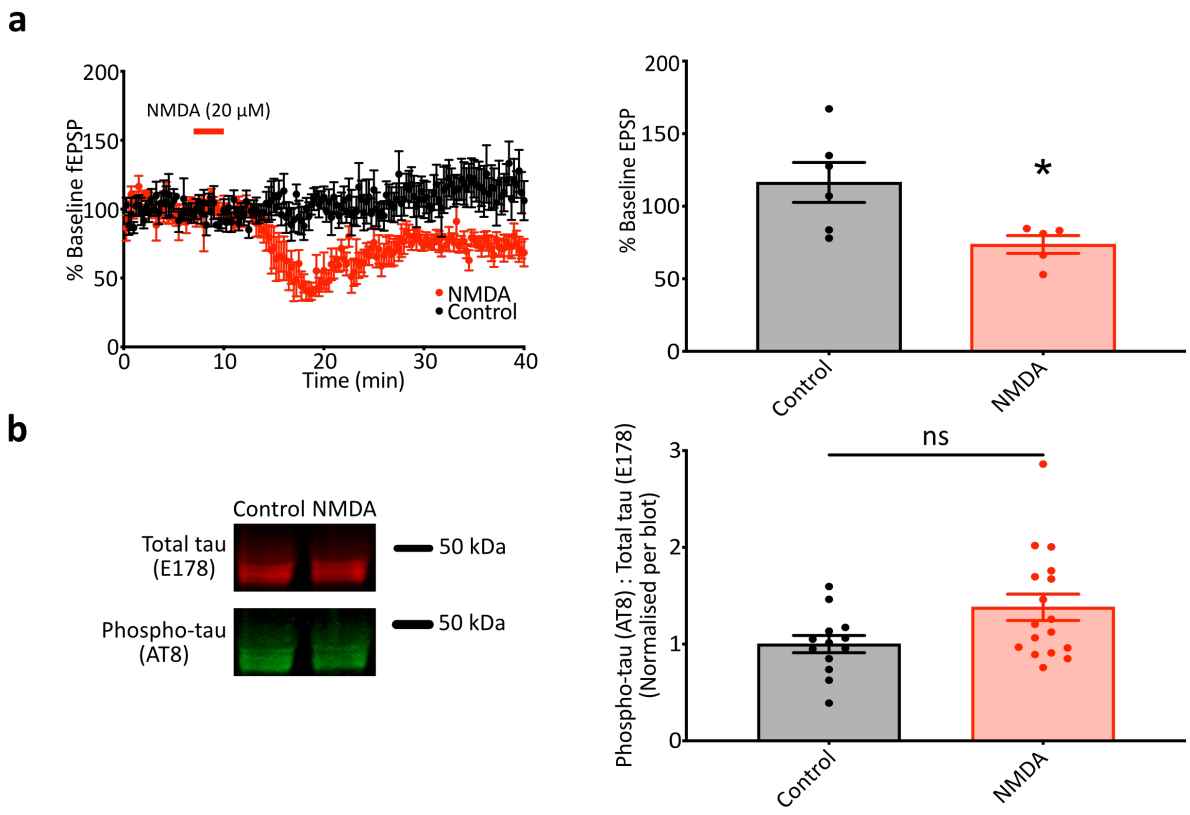

Figure 4.3: Repeated, chronic chemical LTD repeated once a day does not significantly enhance tau phosphorylation. **a)** Summary traces showing EPSP slope post addition of NMDA compared to Control conditions normalised to baseline (pre NMDA addition). Error bars ± SEM (left) and average baseline 30 min post NMDA application (Control: n = 6 slices, 116.4 ± 13.75 % Baseline EPSP; NMDA: n = 5 slices, 73.63 ± 6.12 % baseline EPSP) Unpaired t-test (right). **b)** Representative Western blot data with pooled data of Western blot analysis of NMDA-incubated slices over 7 days. Changes in tau phosphorylation are measured by the ratio of pathologically phosphorylated tau (AT8) : Total tau (E178), normalised to control per blot (Control: n = 13 slices, 1.00 ± 0.09; NMDA: n = 17 slices, 1.38 ± 0.14) Mann-Whitney test.

I next asked whether a chronic LTD induction protocol based on synaptic activity would have similar effects. CA3 hippocampal neurons in organotypic slices were transfected with ChR2 to allow for direct optical activation. I found robust expression of ChR2 at a high level, with transfected axons projecting to CA1 (Figure 2.1a) and established a minimum light intensity and pulse width that could reliably cause firing of a single action potential in CA3 neurons (Figure 2.1b, c). To establish whether I could induce LTD via optical stimulation, slices were stimulated with

blue light at either 500 x 1 Hz, or 900 x 1 Hz to elicit LTD. Both stimuli induced LTD (51.49 ± 4.91 % baseline EPSP, $p = 0.008$ (Figure 4.4a) and 57.24 ± 10.46 % baseline EPSP, $p = 0.008$ (Figure 4.4b)). The 500 x 1 Hz LTD was blocked by incubation with a pan metabotropic Glu receptor (mGluR) blocker LY341495 (Figure 4.4a), while the 900 x 1 Hz LTD was blocked by APV, indicating that it is dependent on NMDARs (Figure 4.4b).

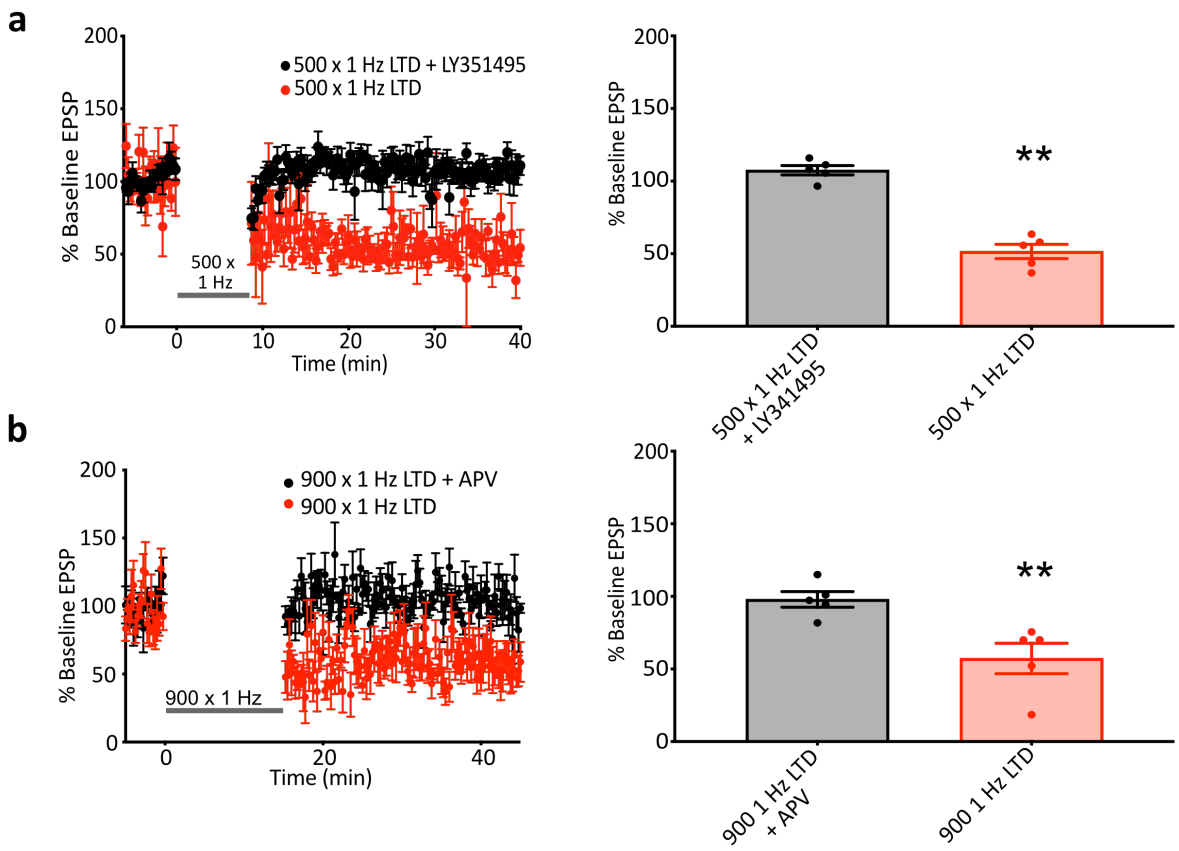

Figure 4.4: Different LFS delivered via optogenetics can induce different types of LTD. **a)** Summary traces showing EPSP slope post 500 x 1 Hz illumination of Schaeffer Collateral synapses of organotypic hippocampal slices transfected with ChR2 with and without mGluR blockade using LY341495 normalised to baseline (pre LTD stimulation). Error bars ± SEM (left) and average baseline 35–40 min post LTD induction (500 x 1 Hz + LY341495: n = 5 slices, 107.4 ± 3.22 % Baseline EPSP; 500 x 1 Hz: n = 5 slices, 51.49 ± 4.91 % baseline EPSP) Mann-Whitney test (right). **b)** Summary traces showing EPSP slope post 900 x 1 Hz illumination of Schaeffer Collateral synapses of organotypic hippocampal slices transfected with ChR2 with and without NMDAR blockade using APV normalised to baseline (pre LTD stimulation). Error bars ± SEM (left) and average baseline 35–40 min post LTD induction (900 x 1 Hz + APV: n = 5 slices, 97.91 ± 5.36 % Baseline EPSP; 900 x 1 Hz: n = 5 slices, 57.24 ± 10.46 % baseline EPSP) Mann-Whitney test (right).

To study the effects of repeated, chronic optogenetic LTD induction protocols on tau phosphorylation, I stimulated slices once a day over 7 days, either with the 500 x 1 Hz mGluR-

dependent protocol, or with the 900 x 1 Hz NMDAR-dependent protocol. Neither condition significantly increased tau phosphorylation.

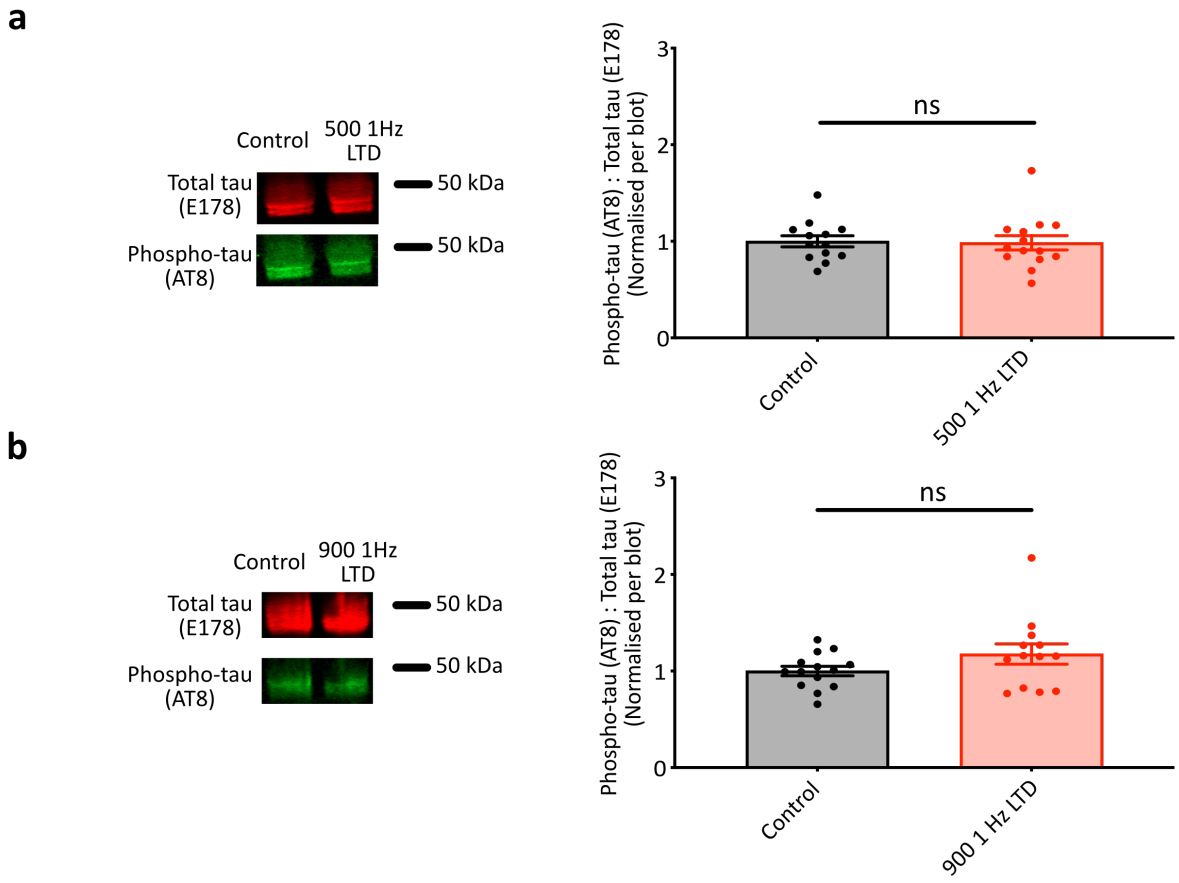

Figure 4.5: Neither mGluR-dependent LTD, nor NMDAR-dependent LTD-inducing conditions significantly increase tau phosphorylation when applied once a day over 7 days. **a)** Representative Western blot data with pooled data of Western blot analysis of slices transfected with ChR2 and stimulated with a 500 x 1 Hz protocol via light once a day over 7 days. Changes in tau phosphorylation are measured by the ratio of pathologically phosphorylated tau (AT8) : Total tau (E178), normalised to control per blot (Control: n = 13 slices, 1.00 ± 0.06; 500 x 1 Hz LTD: n = 14 slices, 0.98 ± 0.07) Mann-Whitney test. **b)** Representative Western blot data with pooled data of Western blot analysis of slices transfected with ChR2 and stimulated with a 900 x 1 Hz protocol via light once a day over 7 days. Changes in tau phosphorylation are measured by the ratio of pathologically phosphorylated tau (AT8) : Total tau (E178), normalised to control per blot (Control: n = 14 slices, 1.00 ± 0.05; 900 x 1 Hz LTD: n = 13 slices, 1.18 ± 0.11) Mann-Whitney test.

I then explored whether increasing the number of induction cycles over 7 days might increase tau phosphorylation, as this reflects a pathophysiological state, since the enhancement of synaptic activity by Aβo would be constant. Therefore, I delivered the 900 x 1 Hz protocol every 2 hours for 7 days to see whether this had any further effect, finding that it did, resulting in tau phosphorylation being significantly increased ($p = 0.014$) (Figure 4.6).

These results demonstrate that repeated, chronic induction of NMDAR-dependent LTD (either chemically, or optogenetically) enhances the pathological phosphorylation of tau at residues distinct from serine 396 and 404 that are shown to be phosphorylated during physiological LTD (Figure 4.6). Indeed, the residues probed by the AT8 antibody were explicitly shown to be unaltered during normal LTD (Regan et al., 2015). Overall, these data support a mechanism for pathological tau phosphorylation in AD that is dependent on LTD induced by Aβo-enhanced synaptic activity which is shown in Figure 4.7.

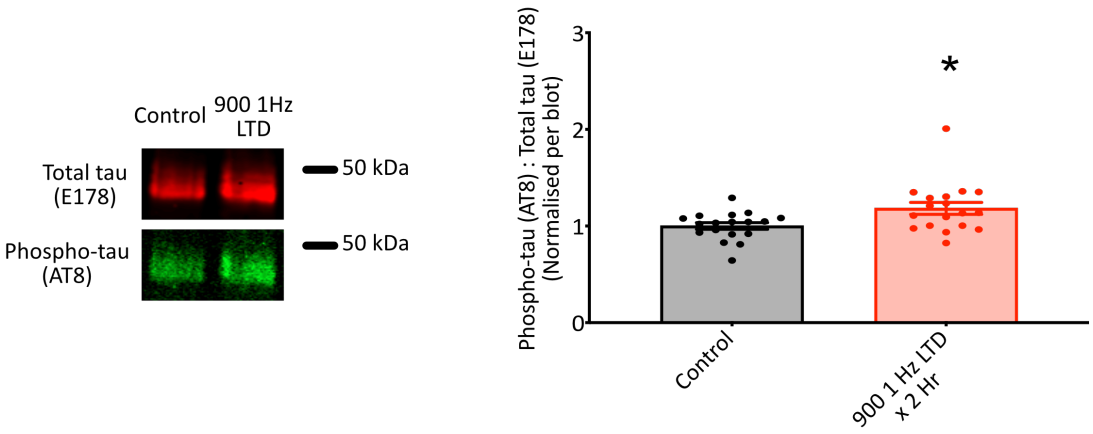

Figure 4.6: Chronic LTD protocols applied every two hours enhance tau phosphorylation. Representative Western blot data with pooled data of Western blot analysis of slices transfected with ChR2 and stimulated with a 900 x 1 Hz protocol via light every 2 h over 7 days. Changes in tau phosphorylation are measured by the ratio of pathologically phosphorylated tau (AT8) : Total tau (E178), normalised to control per blot (Control: n = 18 slices, 1.00 ± 0.03; 900 x 1 Hz LTD x 2 h: n = 18 slices, 1.18 ± 0.06) Mann-Whitney test.

4.5 Discussion

The abnormal phosphorylation of tau is a critical event in AD pathophysiology, but how this is triggered by the oligomeric assemblies of Aβ that initiate the disease process is unknown. Here, I present evidence that abnormal tau phosphorylation follows presynaptic hyperactivation, driven by Aβo, and that this pattern is capitulated in other studies (Brito-Moreira et al., 2011; Dolev et al., 2013; Kabogo et al., 2010; Russell et al., 2012). I propose that chronic increases in low frequency, uncorrelated synaptic activity, that classically drives LTD (Citri and Malenka, 2008), lead to a form of chronic, unopposed LTD that recruits and phosphorylates tau (Figure 4.7). Understanding the processes underlying the generation of hyperphosphorylated, neurotoxic tau is of great importance, not only for the AD field (Rapoport et al., 2002; Roberson et al., 2007), but also for its relevance to other neurodegenerative diseases (tauopathies), in which aberrant tau phosphorylation plays a key role (Orr et al., 2017). Despite the use of a more chronic incubation, compared to the more acute treatments that are often performed, it is important to note that AD

is a disease that takes place over decades in aged subjects. Here slices from young (P7) animals are treated over multiple days. As such, any results must be understood in terms of the limitations of this study. Despite this, they can still indicate some disease relevant processes and early links on a cellular basis between Aβo and tau phosphorylation. The relatively short time period of this chronic study could account for the relatively small size of the effect of my chronic LTD protocol, and it would be interesting to investigate over a longer period and in different systems whether the degree of phosphorylation increases.

Canonically, it is believed that the physiological role of tau is in microtubule stabilisation (Drubin and Kirschner, 1986; Lee et al., 1989), where phosphorylation of tau tunes its ability to bind to the microtubules, thereby regulating axonal transport (Bramblett et al., 1993; Butner and Kirschner, 1991). Recent evidence that tau is present in the dendrites of healthy neurons (Ittner et al., 2010; Kimura et al., 2014; Mondragon-Rodriguez et al., 2012; Swanson et al., 2017; Zempel et al., 2013), and furthermore that its phosphorylation is required for LTD (Kimura et al., 2014; Regan et al., 2015) raises the possibility that tau is also involved in trafficking of glutamate receptors at dendritic spines, a process critical for plasticity (Penn et al., 2017; Roth et al., 2019). Indeed, there is evidence for interactions between tau and several proteins involved in AMPAR endocytosis (Regan et al., 2015; Tracy et al., 2016) and I have laid out a possible mechanism for the involvement of tau in AMPAR endocytosis in response to NMDAR-dependent LTD stimuli (Figure 1.5). It could be that the pathological modification of tau is actually related to these physiological interactions. My data suggests that this process, at least in part, may be recruited by Aβo-induced chronic, low-frequency synaptic activity resulting in tau phosphorylation characteristic of AD. However, there is an important distinction between physiological phosphorylation of tau in LTD and the pathological tau hyperphosphorylation of AD, which is that tau is phosphorylated at many more residues in AD pathogenesis (Regan et al., 2015). One explanation for this is that excessive or prolonged activation of LTD-associated kinases arising from chronically Aβo-enhanced synaptic

activity might promote non-physiological phosphorylation of tau at the additional residues. Indeed, as discussed in the introduction, one such kinase, GSK-3, has been strongly implicated in both tau phosphorylation during LTD (Bradley et al., 2012; Kimura et al., 2014), and in the phosphorylation of many of the pathology-specific residues additionally involved in AD (Deng et al., 2014; Jo et al., 2011; Shipton et al., 2011; Yi et al., 2018). The tau residues recognised by the phosphorylation state-specific antibody AT8, which is used to detect pathological tau, are amongst the known substrates of GSK-3 (Liu et al., 2002; Wang et al., 1998). It has been proposed that GSK-3 plays a role in AD pathogenesis (Hooper et al., 2008; Lauretti et al., 2020; Takashima, 2006), and GSK-3 inhibitors have been shown to have beneficial effects in AD models (Griebel et al., 2019; Hu et al., 2009b; Morales-Garcia et al., 2012; Sereno et al., 2009). It may be that much of its importance in AD lies in its recruitment of tau downstream of altered synaptic activity, and my work adds further to the importance of this kinase as a potential therapeutic target.

It is possible that the processes that are involved in lysing the cells could cause some of the tau phosphorylation that I have observed here, but, since all slices from every condition were treated in the same way, comparisons between groups should still represent changes between the effects of incubation. Therefore, this work supports a mechanistic link between the upstream Aβo-induced plasticity changes and pathological tau phosphorylation. Previous studies have indicated that Aβo accumulation drives the downstream pathology of AD, including tau hyperphosphorylation (Mucke and Selkoe, 2012), an event critical for pathogenesis, but none have identified a mechanism by which these two key players of AD pathology might be linked. My candidate mechanism directly addresses this major gap in our understanding of AD and suggests that a variety of effector proteins involved in LTD induction, including the kinase GSK-3, particularly the GSK-3β subtype, may be potential targets for therapeutic intervention.

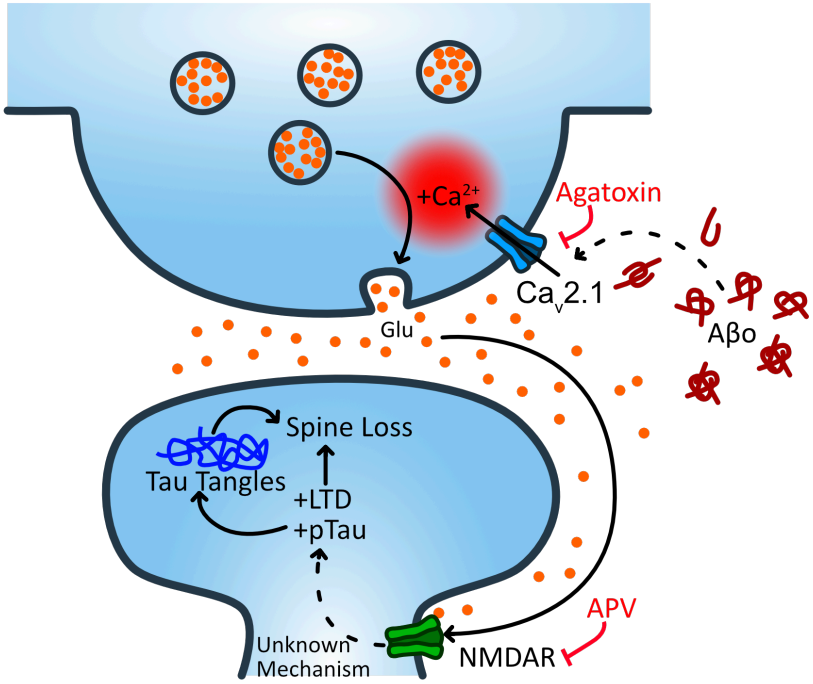

Figure 4.7: Schematic of the proposed mechanism for Aβo-induced tau phosphorylation. Aβ oligomers elevate release from the presynaptic terminal, resulting in elevated extrasynaptic Glu. Increased extrasynaptic Glu levels lead to increased induction of NMDAR-dependent LTD (possibly involving extrasynaptic NMDARs), enhanced phosphorylated tau, eventually arriving at pathologically hyperphosphorylated tau that aggregates and causes neurodegeneration.

5. A novel quantal analysis approach to study miniature events reveals enhanced frequency following Aβo exposure

5.1 Introduction

I have previously described that Aβo enhance presynaptic neurotransmitter release and my results suggest that these presynaptic changes might drive downstream AD pathology such as plasticity changes and aberrant tau processing. As discussed, there is good evidence for enhanced release of neurotransmitter, however, this has primarily been studied in response to APs (Brito-Moreira et al., 2011; Dolev et al., 2013; Jeans et al., Unpublished; Kabogo et al., 2010; Merlo et al., 2016; Russell et al., 2012; Wang et al., 2017b). Other aspects of presynaptic function remain poorly understood in the context of AD.

As well as releasing quantised amounts of neurotransmitter in response to APs, neurons spontaneously release vesicles of neurotransmitter. This spontaneous release occurs at a low frequency and is not temporally linked to an AP. Until recently, these presynaptic 'miniature release events' (minis) were widely thought to be functionally unimportant for synaptic transmission, however, it has become increasingly clear that they play an important physiological role. Miniature release mechanisms have been shown to be, in part, quite separate from those of evoked release (Deitcher et al., 1998), and minis appear to have some separate receptor targets in addition (Atasoy et al., 2008; Reese and Kavalali, 2016) and release machinery (Ramirez et al., 2012) to evoked release. In addition, there is growing evidence that minis play a role in synaptic plasticity; spontaneous miniature excitatory postsynaptic currents (mEPSC) amplitude and frequency reflect previous activity at the synapse (Bacci et al., 2001; Liu et al., 2000; Turrigiano et al., 1998), possibly having a role in metaplasticity, with increased mEPSC amplitude and frequency accompanying LTP (Oliet et al., 1996) (although due to the nature of measuring minis at this time these findings have been subject to much debate) and lowered mEPSC amplitude and frequency correlating with

the strength of LTD induction (Zhang et al., 2005). In addition to this, minis have been implicated in homeostatic synaptic scaling (Gonzalez-Islas et al., 2018; Kavalali, 2015; Pozo and Goda, 2010; Turrigiano, 2012), a process that is impaired in certain neurodegenerative diseases, including AD (Frere and Slutsky, 2018), via regulation of dendritic protein synthesis (Sutton et al., 2006; Sutton and Schuman, 2006; Sutton et al., 2004).

There is some evidence for altered minis in models of AD. Following exposure to Aβo, or upon APP overexpression, multiple studies have shown that mini frequency is reduced in rat hippocampal cultures (Kamenetz et al., 2003; Nimmrich et al., 2008; Shankar et al., 2007; Talantova et al., 2013), although, the evidence is confused, with one study showing that this reduction was preceded by a short-lived frequency increase (Parodi et al., 2010). These Aβo-induced changes in mini frequency have usually been explained by a presumed presynaptic weakening over time, although, there is no direct evidence for this, and, as discussed, several more direct studies show that the opposite occurs (Brito-Moreira et al., 2011; Dolev et al., 2013; Jeans et al., Unpublished; Kabogo et al., 2010; Merlo et al., 2016; Russell et al., 2012; Wang et al., 2017b). There is, however, a well-established process of postsynaptic weakening and depression which begins rapidly following the addition of Aβo (Sheng et al., 2012).

As with evoked release, minis have typically been studied experimentally by recording the currents that they produce (mEPSCs) from the soma of the postsynaptic neuron. The somatic recording approach may, however, introduce important constraints on the measurements. Firstly, it is not possible to attribute currents recorded at the soma of the postsynaptic cell to a specific synapse. There is, therefore, no way to study the role of minis at specific inputs, or even those arising at groups of synapses on specific dendritic branches. The latter could be particularly important as there is good evidence that dendritic branches are an important spatial unit for synaptic regulation (Branco et al., 2008) and information storage (Govindarajan et al., 2011). Secondly, the magnitude

of electrical signals from more distal synapses may well decay en-route to the soma, simply as a consequence of passive electrical attenuation along the dendrites. Where this occurs, somatic electrophysiological recordings will inevitably be subject to biased sampling of the contributions of synapses from proximal versus distal dendritic branches (Williams and Stuart, 2003). Finally, changes in the postsynaptic strength, particularly weakening, can occur as a result of physiological processes such as LTD, or as a consequence of pathology, as in Aβo addition. These will change the amplitude of mEPSCs and, if the extent of the postsynaptic weakening is large, could cause a fraction of events to fall below the detection threshold of the recording. If this happens, those events are lost, and frequency measurements will be artefactually low.

As discussed in the introduction, direct imaging of presynaptic terminals offers the opportunity to circumvent these confounds. There have been successful attempts to optically measure quantal release events, both evoked and spontaneous, using the fluorescent dye FM 1-43 (Ryan et al., 1997; Tokuoka and Goda, 2008), but no attempts have been made to look specifically at miniature release. Here I demonstrate a novel, optical method of directly measuring the miniature presynaptic release events using the genetically encoded fluorescent probe SypH 2x. I show that this bypasses confounds of postsynaptic weakening and allows true changes in the frequency of miniature release to be unmasked following Aβo exposure. Given the importance of minis in the regulation of synaptic strength via multiple processes, this technique should not only facilitate AD research, but also studies in several areas of neuronal and synaptic biology.

5.2 SypH 2x allows direct measurement of miniature release in the presence of folimycin

In order to directly probe presynaptic release, I used SypH 2x, a genetically encoded reporter of vesicle fusion (Zhu et al., 2009) that fluoresces at neutral pH, and is quenched at the low pH observed within acidified synaptic vesicles (Figure 5.1a, b). SypH 2x and variants have been used

to observe responses to single AP stimulation (Jeans et al., 2017; Zhang et al., 2009) given their enhanced signal-to-noise ratio due to the inclusion of two pHluorin moieties (Zhu et al., 2009). Despite this, it is still difficult to resolve single release events from the background signal, even more so when they are not time-locked to a known stimulus. Therefore, I used the cell-impermeable vesicular ATPase inhibitor folimycin (Figure 5.1a, b), which blocks reacidification of synaptic vesicles (Ertunc et al., 2007), and therefore allows the summation of multiple individual events over time, each of which individually may produce a signal change at or below detection threshold, into a single, larger signal change that is readily quantified (Figure 5.1c, d).

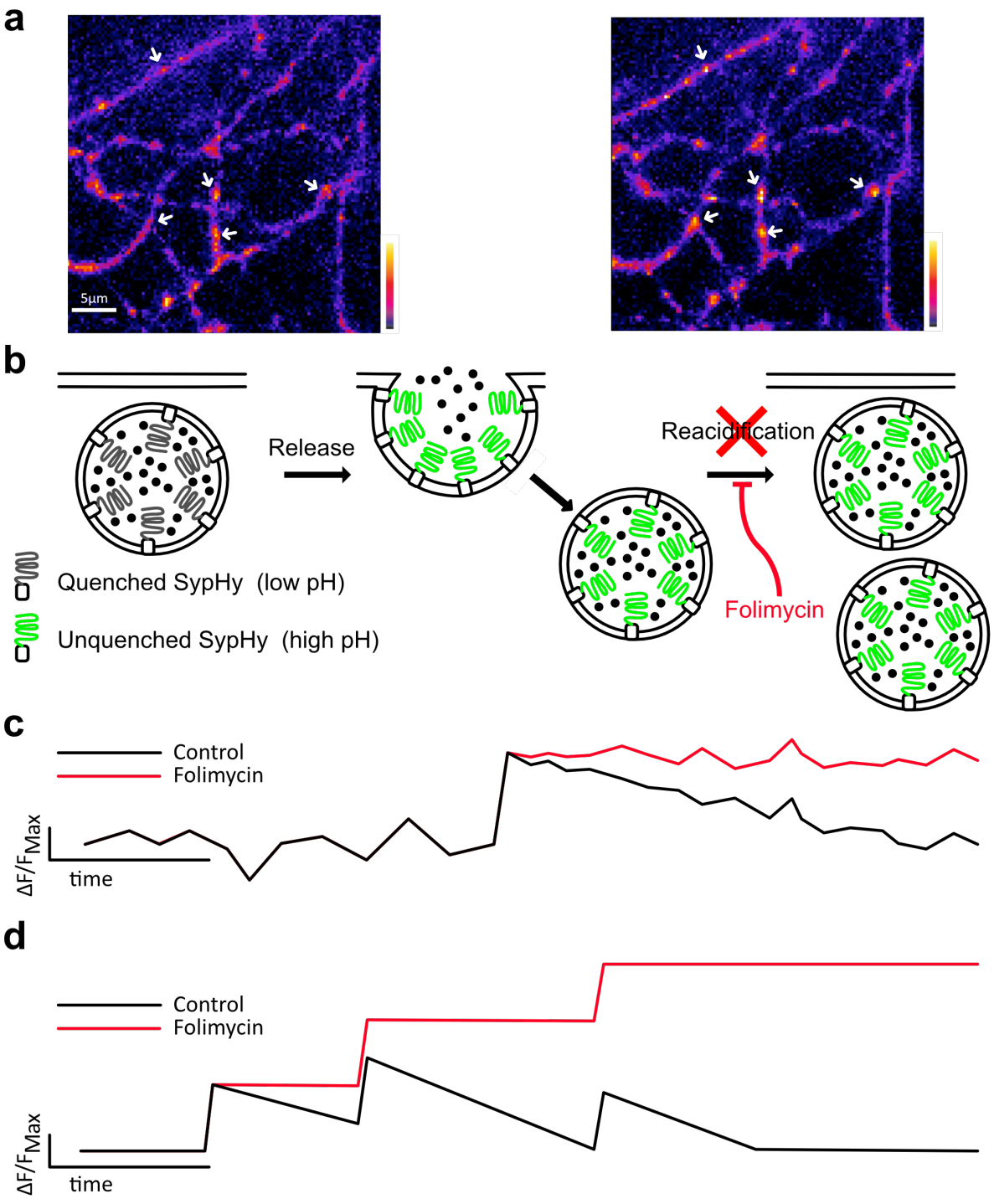

Figure 5.1: Folimycin allows summation of SypH 2x signals from multiple miniature release events. **a)** Representative fields from a typical control SypH 2x experiment in the presence of TTX, left: baseline image prior to folimycin addition, right: response image after incubation in folimycin for 10 min. Arrows highlight some boutons that have released. Image shown using the Image J Fire look up table. **b)** Schematic diagram illustrating phases depicted in a). Addition of folimycin blocks the reacidification of endocytosed vesicles, preserving them in the unquenched, fluorescing state. **c)** Cartoon of a theoretical fluorescence trace from a single release event with

(red) and without (black) folimycin to demonstrate how preventing vesicle reacidification prevents SypH 2x fluorescence from being quenched. **d)** Over time, signal in the bouton will build up in the presence of folimycin as all released vesicle continue to fluoresce and events are therefore summed.

I imaged cultured hippocampal neurons expressing SypH 2x in the presence of tetrodotoxin (TTX) (1 μM) to isolate spontaneous miniature release events. Images were taken at 1 Hz over 10 s to give a baseline signal before addition of folimycin (10 nM). After waiting for 10 min to allow spontaneous miniature release to occur, a series of images was again acquired at 1 Hz for 30 s. Over the last 10 s, SypH 2x was unquenched by alkalisation using a buffer containing NH_4Cl to reveal the maximal SypH 2x response at each terminal (Figure 5.2a). All responses were normalised to this maximal response in order to control for inter-terminal differences in pHluorin expression level (Jeans et al., 2017). Background adjusted responses were then plotted as a frequency distribution. Using the control dataset, the AIC was determined for Gaussian mixture models with varying numbers of Gaussian distributions (1-10 Gaussians) fitted to the data. The lowest (optimal) AIC value corresponded to a fit of four Gaussians which were fitted using an objective curve fitting algorithm (Figure 5.2b). The mean values of these distributions were equally spaced, as would be anticipated if events were quantal. Indeed, the resultant plot fitted well with a linear relationship (R^2= 0.98), with the interpeak distance representing the fluorescence change upon the release of one quantum (q) (q= 0.037 $\Delta F/F_{NH4Cl}$) (Figure 5.2c).

In order to validate my calculated value for q I studied evoked release events following single action potential (AP) stimuli. I serially imaged SypH 2x-expressing boutons over 10 s while giving a single stimulus, subjecting each to 23 trials with 30 s rest between each one. I plotted a frequency distribution of the NH_4Cl-normalised responses and fitted this with two Gaussian distributions (corresponding to either no release, or one release event). I again derived q as the interpeak distance (Figure 5.2d). This yielded a q estimate of similar magnitude to that derived from the mini

experiment ($q= 0.028\ \Delta F/F_{NH4Cl}$). The proportion of events that showed successful release was 21%, corresponding to an average probability of release (Pr) of 0.21 for the population of boutons that I measured (Figure 5.2e). Next, I examined Pr at individual boutons, discarding silent boutons, and plotted the values as a frequency distribution (Figure 5.2g). This gave a mean Pr of 0.22 with a median of 0.14 (Figure 5.2f), which is highly consistent with that observed by others in cultured hippocampal neurons (Branco et al., 2008; Tokuoka and Goda, 2008). Overall, these results indicate that my method is able to detect quantal miniature release events with high sensitivity.

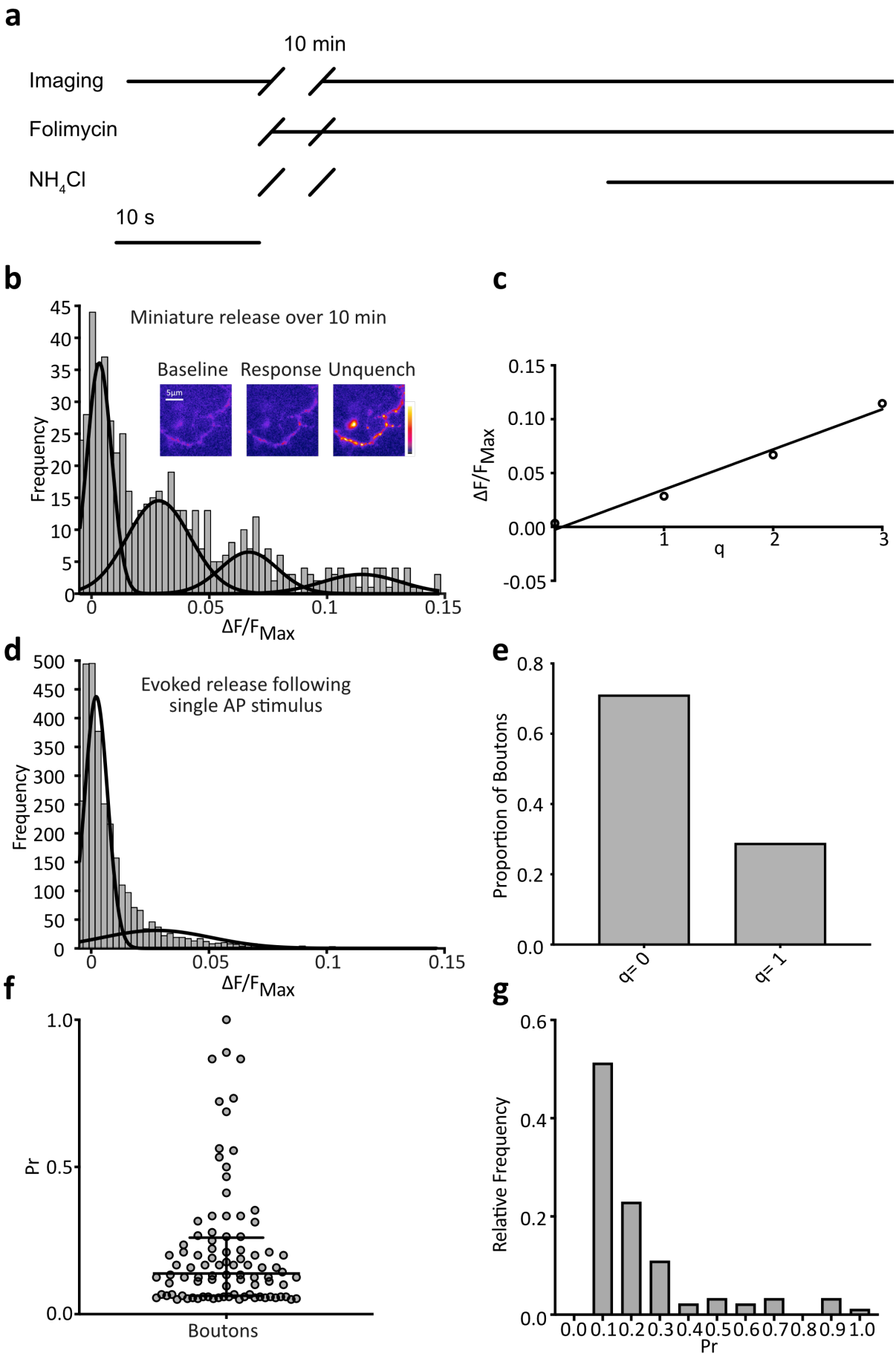
a
10 min
Imaging
Folimycin
NH4Cl
10 s
b
Miniature release over 10 min
Baseline
Response
Unquench
5µm
Frequency
ΔF/FMax
c
ΔF/FMax
q
d
Evoked release following
single AP stimulus
Frequency
ΔF/FMax
e
Proportion of Boutons
q= 0
q= 1
f
Pr
Boutons
g
Relative Frequency
Pr

Figure 5.2: Detection of quantal spontaneous miniature release. **a)** Experimental scheme with baseline images taken prior to addition of folimycin and 10 minute wait, before further image acquisition and unquenching using basic NH_4Cl-containing buffer. TTX is present throughout. **b)** Following a 10 min incubation in TTX and folimycin, a frequency distribution of responses was plotted (n= 574 synapses from 31 coverslips) and multiple Gaussian curves were fit as described in the main text. Insert: representative images at different points of an experiment; look up table: Image J Fire. **c)** Calculating the interpeak distance using linear regression allowed estimation of quantal size (q) as the slope of the plot of the mean of each Gaussian versus number of release events it represents (q= 0.037 $\Delta F/F_{NH4Cl}$). **d)** Responses of boutons to 1 AP stimuli with a frequency distribution of responses (n= 4297 observations from 3 coverslips) and an objective Gaussian curve fitting algorithm was used to model the distribution with mean of the second Gaussian giving q = 0.028 **e)** Proportion of observations contributing to each Gaussian (q = 0: 0.79; q = 1: 0.21) **f)** Pr of boutons over the experiment shows median and interquartile range (n = 92 boutons from 3 coverslips, mean Pr = 0.22 ± 0.02, median Pr = 0.14 interquartile range: 0.06–0.26). **g)** frequency distribution of Pr.

5.3 Aβo incubation increases frequency of miniature release events

As a first application of this new method for mini detection, I asked whether there are changes in mini frequency following application of Aβo. This is an important question as miniature transmission may regulate synaptic strength (Sutton et al., 2006), which is reduced via poorly-understood mechanisms following Aβo treatment (Sheng et al., 2012). Most studies that have examined the effects of Aβo on minis have done so electrophysiologically and concluded that mini frequency in cultured hippocampal neurons is reduced by Aβo treatment (Kamenetz et al., 2003; Nimmrich et al., 2008; Parodi et al., 2010; Shankar et al., 2007; Talantova et al., 2013). However, as has been discussed, Aβo cause rapid postsynaptic depression and weakening, which will tend to reduce the apparent number of events as some fall below the detectable threshold. I wondered whether this could confound the interpretation of electrophysiological data, and therefore whether a true effect may have been masked.

Cultured neurons expressing SypH 2x were incubated in media containing Aβo (200 nM) for over an hour and miniature release events were then measured as described above with the Aβo concentration being present also in the experimental buffer. Responses were again plotted as a frequency distribution in the same range as the control, and four Gaussians were fitted (Figure 5.3a). The expected values of each Gaussian were plotted to find an evenly spaced distribution (R^2= 0.95) with q= 0.037 $\Delta F/F_{NH4Cl}$ (Figure 5.3b), identical to that of control.

In order to investigate changes in the frequency of minis between conditions, the fraction of boutons undergoing different numbers of release events was assessed. The intersections of each pair of Gaussians were calculated, and boutons within each range were assigned as corresponding to that number of release events (Figure 5.3e). This revealed that the Aβo condition was right shifted compared to the control dataset (Figure 5.2b), with more boutons having released over 10 minutes, and more release events from those that did. Chi squared analysis of the two treatments confirmed that these differences were highly significant (Figure 5.3e). An analysis of the variation between each coverslip showed a similar standard variation (Figure 5.3c) and distribution of mean responses between each condition (Figure 5.3d).

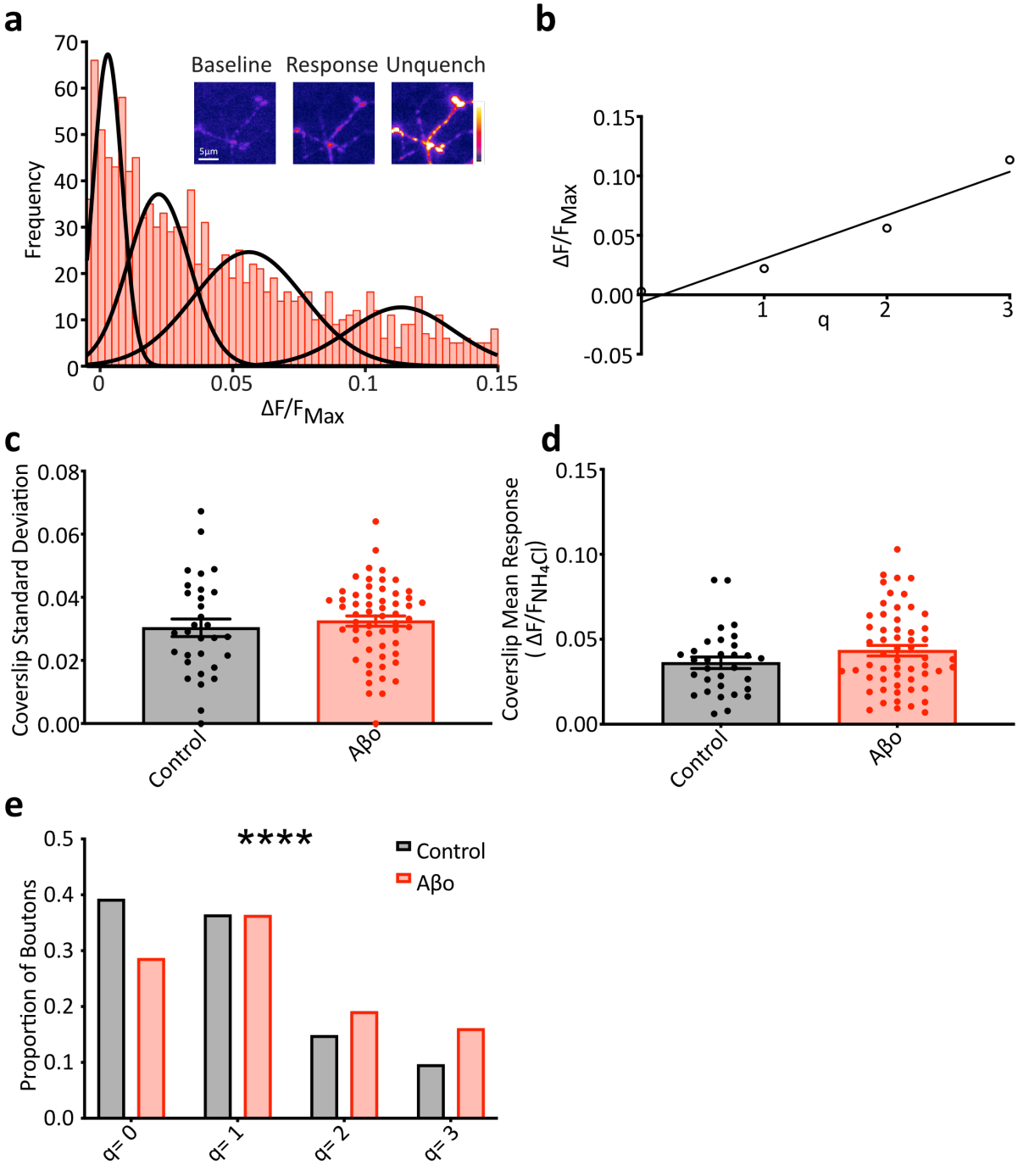

Figure 5.3: Aβo treatment increases the frequency of optically detected miniature events. **a)** Aβo-incubated cells were subjected to the same protocol as control (10 minutes in TTX and folimycin), and a frequency distribution of responses was plotted (n= 1217 synapses from 57 coverslips). Multiple Gaussians were fit as for control. Insert: representative images at different points of an experiment; look up table: Image J Fire. **b)** Calculating the interpeak distance using linear regression allowed estimation of quantal size (q) as the slope of the plot of the mean of each Gaussian vs. number of release events (q= 0.037 $\Delta F/F_{NH4Cl}$). **c)** Standard deviation per coverslip with mean standard deviation ± SEM (Control: n = 31 coverslips, 0.030 ± 0.003; Aβo: n = 57

coverslips, 0.032 ± 0.002. **d)** Mean response per coverslip with overall mean ± SEM (Control: n = 31 coverslips, 0.036 ± 0.003; Aβo: n = 57 coverslips, 0.043 ± 0.003). **e)** Proportion of boutons contributing to each Gaussian, control in black/grey, Aβo-incubated in red (Control: 39.2% of boutons not releasing, 36.4% of boutons releasing 1 quantum, 14.8% of boutons releasing 2 quanta, 9.6% of boutons releasing 3 quanta, n= 574 synapses; Aβo: 28.6% of boutons not releasing, 36.3% of boutons releasing 1 quantum, 19.1% of boutons releasing 2 quanta, 16.0% of boutons releasing 3 quanta, n = 1217 synapses). Chi-squared test.

5.4 Electrophysiological recording of miniature release shows a decrease in frequency upon Aβo incubation

The above result conflicts with several published electrophysiological studies that show reduced mini frequency with Aβo, and I wanted to ensure that this unusual result was not a reflection of the individual properties of my experimental system. I therefore measured mEPSCs in hippocampal neurons cultured and treated exactly as above using patch-clamp electrophysiology. Recordings were made in voltage clamp at -70 mV and all reasonable precautions were taken to increase the sensitivity of detection, including the use of a high impedance (low noise) amplifier and a caesium-containing internal solution in the recording electrode; this is used in order to block K^+ channels, increasing the resistivity of the cell and thereby improving the strength of the signal from inputs occurring far from the patch electrode (Fleidervish and Libman, 2008; Spruston et al., 1993). In contrast to the increase in mini frequency that I observed using direct optical measurements, I found that there was a small reduction in mEPSC frequency between the conditions (Figure 5.4b). In addition, I found a decrease in mEPSC amplitude, in keeping with the expected Aβo-induced synaptic depression (Figure 5.4c). These data confirm that my optical approach to mini analysis was able to reveal a change in mini frequency induced by Aβo treatment that is masked by conventional electrophysiological measurements.

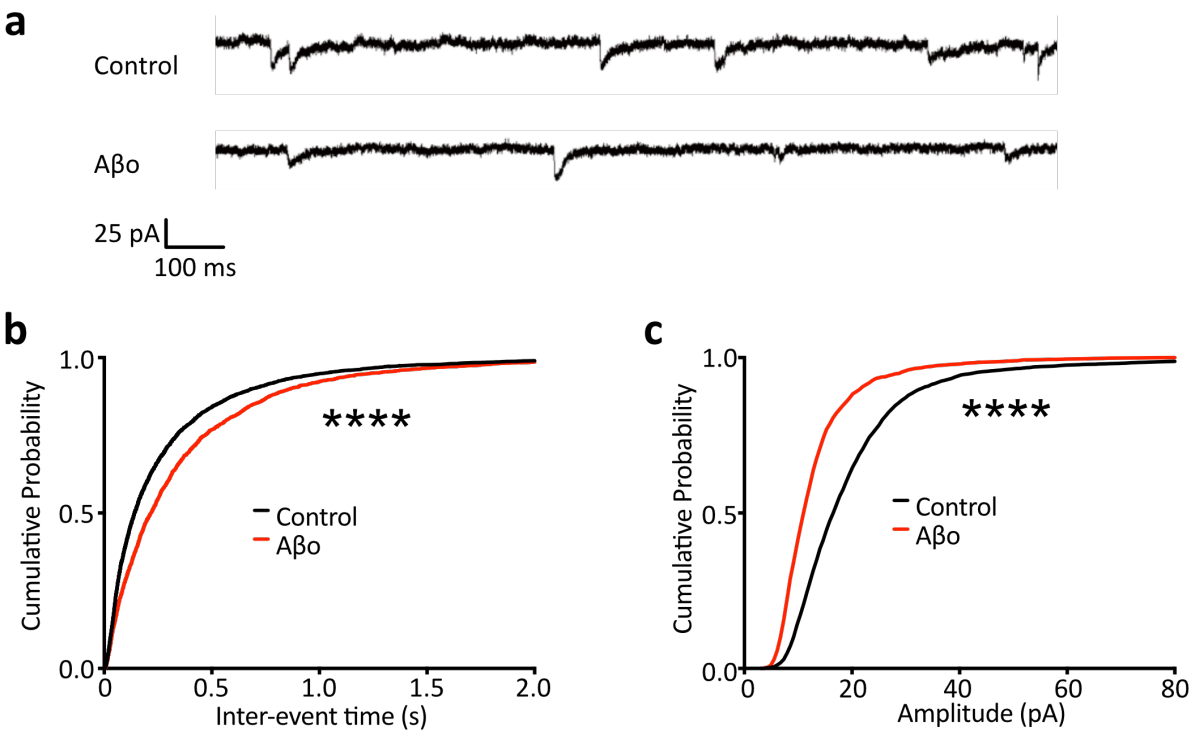

Figure 5.4: Electrophysiological recordings of mEPSCs show a reduction in frequency and amplitude between control and Aβo-treated conditions. **a)** Representative traces of mEPSC recordings **b)** Cumulative frequency distribution of mEPSC (control, n = 10 coverslips; Aβo, n = 7 coverslips); axes were cut short, with 76 data points outside the limits. Kolmogorov-Smirnov test. **c)** Cumulative frequency distribution of mEPSC amplitude mEPSC (control, n = 10 slices; Aβo, n = 7 slices); axes were cut short with 41 data points outside the limits. Kolmogorov-Smirnov test.

5.5 Discussion

In order to investigate minis more thoroughly, particularly in the context of AD, where postsynaptic weakening is a major hallmark, I have developed a new optical technique for observing spontaneous miniature release events that allows direct monitoring of release, rather than relying on traditional measurements of synaptic currents arising from the postsynaptic terminus recorded at the soma of the postsynaptic neuron which can be affected by changes in postsynaptic strength, or numbers and types of synapses on the postsynaptic neuron. I found that the method gave a consistent $\Delta F/F_{NH4Cl}$ value for q across experimental conditions that was similar

to that found with a 1 AP evoked stimulus, arguing in favour of its ability to detect quantal release events. To further validate the new method, I investigated Pr in response to a 1 AP stimulus and found that it was comparable to that of previous studies (Branco et al., 2008). It is possible that I have also measured some miniature release from inhibitory interneurons. The SypH 2x probe is not specific to excitatory or inhibitory vesicles. The prevalence of interneurons in primary hippocampal cultures is low, however (Benson et al., 1994). Despite this, in order to improve my method, it would be useful to be able to specify that terminals being observed were glutamatergic. To do this, the SypH 2x probe could be improved to use a more specific promoter, or coverslips could be counter stained with a reporter for a glutamatergic-specific protein such as a vesicular glutamate transporter.

In addition to pHluorin probes such as SypH 2x, several other methods of directly observing release at the presynaptic terminal have been developed. In particular, the fluorescent FM dyes have an inherently high signal and have been used to good effect to image presynaptic release in several systems (Kavalali and Jorgensen, 2014), including optical quantal analysis in cultured neurons (Tokuoka and Goda, 2008). New, optimized variants of the genetically encoded glutamate reporter iGluSnFR are also promising, as they allow rapid visualization of glutamate release both *in vitro* and *in vivo* (Hefendehl et al., 2016; Helassa et al., 2018; Hires et al., 2008; Marvin et al., 2013; Marvin et al., 2018; Xie et al., 2016). While either of these techniques could, in principle, also be used to develop an optical quantal analysis of minis, they both have disadvantages: FM dyes are not genetically encoded, and therefore cannot be used to label genetically defined populations of neurons as SypH 2x can. The iGluSnFR probe, on the other hand, while genetically encoded, is a glutamate sensor, so may be compromised by any change in glutamate concentration, such as alterations to the rate of clearance by synaptic transporters. Nonetheless, it would be desirable to apply these techniques for optical analysis of minis to give a means of cross validation for each result set, including my work with pHluorin probes.

Having established that I could use my optical approach to measure minis, I applied it to investigate changes in mini dynamics in the presence of Aβo. I demonstrated that the technique can be used to show alterations in spontaneous miniature release in cultured neurons, and even to reveal a change that is not detected electrophysiologically. Previous studies have shown that addition of the pathogenic Aβo leads to postsynaptic weakening at an early stage in *in vitro* AD models, possibly via mechanisms of LTD leading to AMPAR internalisation (Li et al., 2009; Shankar et al., 2007; Shankar et al., 2008). Similar postsynaptic weakening seems to be evident at an early stage *in vivo* (Chang et al., 2006; Whitcomb et al., 2015). Therefore, I looked at changes in mini frequency in the presence of Aβo, both because of its potential pathological significance, and to establish whether electrophysiological measurements had been confounded by the postsynaptic changes. My results confirm that there is an increase in mini frequency that is not observed when recording mEPSCs. Aside from the significance of this result as a validation of the new technique, it represents a potentially important addition to our understanding of presynaptic regulation in AD. Previously, the evidence that Aβo enhance evoked presynaptic release (Brito-Moreira et al., 2011; Lazarevic et al., 2017; Russell et al., 2012) has been seemingly contradicted by evidence that Aβo reduce mEPSC frequency (Kamenetz et al., 2003; Nimmrich et al., 2008; Shankar et al., 2007; Talantova et al., 2013). These results appear to be in conflict, since evoked and miniature release are typically tightly correlated; indeed, for this reason, mini frequency is often used as an indicator of overall presynaptic strength (Prange and Murphy, 1999). My result helps to resolve this contradiction and suggests that Aβo do indeed enhance presynaptic function globally. This is important for understanding the actions of Aβo at the synapse, and in particular the role of Aβo in synaptic weakening, since mini release is implicated in mechanisms of homeostatic plasticity (Kavalali, 2015), and has been shown to be a key regulator of postsynaptic strength (Sutton et al., 2006).

It is increasingly becoming apparent that regulation of presynaptic function is critical in mechanisms of learning and memory, and that presynaptic dysregulation can play an early and critical role in a host of neurological disorders, including neurodegenerative diseases such as Parkinson's disease and AD (Waites and Garner, 2011). My findings, not only expand the repertoire of techniques available to probe these important processes, but also provide specific insight into the dysregulation of miniature neurotransmission in AD. This supports the model outlined in this thesis for enhanced release driving downstream pathology and could even lead to more detailed mechanistic insight into how this happens, hopefully advancing us towards new therapeutic treatments.

6. Altering the dynamics of presynaptic release dynamics as a potential therapeutic intervention in AD

6.1 Introduction

Although Jeans *et al.* have identified $Ca_V2.1$ as a mediator of Aβo-induced enhanced release, as described in the introduction (Jeans et al., Unpublished), and here I have demonstrated that partial suppression of this VGCC can alleviate downstream AD pathology, $Ca_V2.1$ is not a wholly suitable therapeutic target. $Ca_V2.1$ is essential for normal presynaptic function, as demonstrated by evidence from mouse models; mice with a global knock-out of the α1 subunit gene of $Ca_V2.$, *Cacna1a*, show ataxia, dystonia, and postnatal lethality (Fletcher et al., 2001; Jun et al., 1999; Kaja et al., 2007; Pietrobon, 2005). So, while partial suppression of $Ca_V2.1$ seems to offer benefit, functional titration *in vivo* is likely challenging, with risk of excessive blockade being harmful.

Therefore, I decided to investigate whether presynaptic function could be modulated by manipulating a different, non-VGCC target, and whether this could normalise the effects of Aβo on presynaptic release. Sphingolipids are a class of membrane lipids that are abundant in neurons and have a variety of important signalling roles in cells. Although originally their role was thought to be mainly as components of lipid rafts (Simons and Ikonen, 1997), they are now implicated in a plethora of cellular functions, including growth regulation, cell migration, apoptosis, senescence, inflammatory responses (Hannun and Obeid, 2018), and interestingly synaptic vesicle cycle underlying neurotransmitter release (Rohrbough and Broadie, 2005); specifically, the sphingolipid ceramide has been shown to be a key regulator of synaptic vesicle exocytosis, with blockade of ceramidase disrupting release (Rohrbough et al., 2004), while sphingosine has been identified as a direct modulator of release through effects on SNARE complex assembly (Darios et al., 2009). Sphingosine-1-phosphate (S1P) also impacts release (Kajimoto et al., 2007) via control of presynaptic synapsin 1 localisation (Figure 6.1) (Riganti et al., 2016).

Importantly, this class of lipids has also been established as viable and promising drug targets, with their levels being readily modulated by small molecule inhibitors of enzymes in the various sphingolipid synthetic pathways (Wymann and Schneiter, 2008). It has been shown that sphingolipids are dysregulated in the brains of AD patients, with increased levels of both ceramide and sphingosine (Haughey et al., 2010). This sphingolipid profile would be expected to have a facilitatory effect on evoked release; therefore, although P-/Q-type VGCC dysfunction has already been implicated, sphingolipid dysregulation is a promising candidate for further enhancement of pathological Aβ-mediated enhancement of release. Manipulation of sphingolipid levels could represent a valuable therapeutic approach to alleviating synaptic toxicity in AD, partly because of their potential involvement, but mainly since they represent a potential route to the manipulation of presynaptic function that is readily addressable by pharmacological means, since small molecules, targeting the synthetic enzymes can rapidly modulate levels of individual sphingolipids and, consequently, cellular functions.

Accordingly, I set out to test two important hypotheses with direct therapeutic implications: i) the enhancement of synaptic glutamate release by Aβo can be rescued by manipulation of sphingolipid levels; ii) and that this rescue can ameliorate Aβo-mediated synaptic toxicity.

I pharmacologically manipulated three separate enzymes involved in sphingolipid metabolism: Sphingomyelinase (SMase), Ceramidase, and Sphingosine kinase (SphK) and measured changes in evoked release with and without Aβo incubation (Figure 6.1). I confirm that Aβo incubation alone significantly enhances release. In addition, I show that all three manipulations that I tried elevated release, but that additional incubation with Aβo did not significantly change release further. This

is somewhat surprising, since at least one of the manipulations that I performed, blockade of SMase, was expected to reduce release.

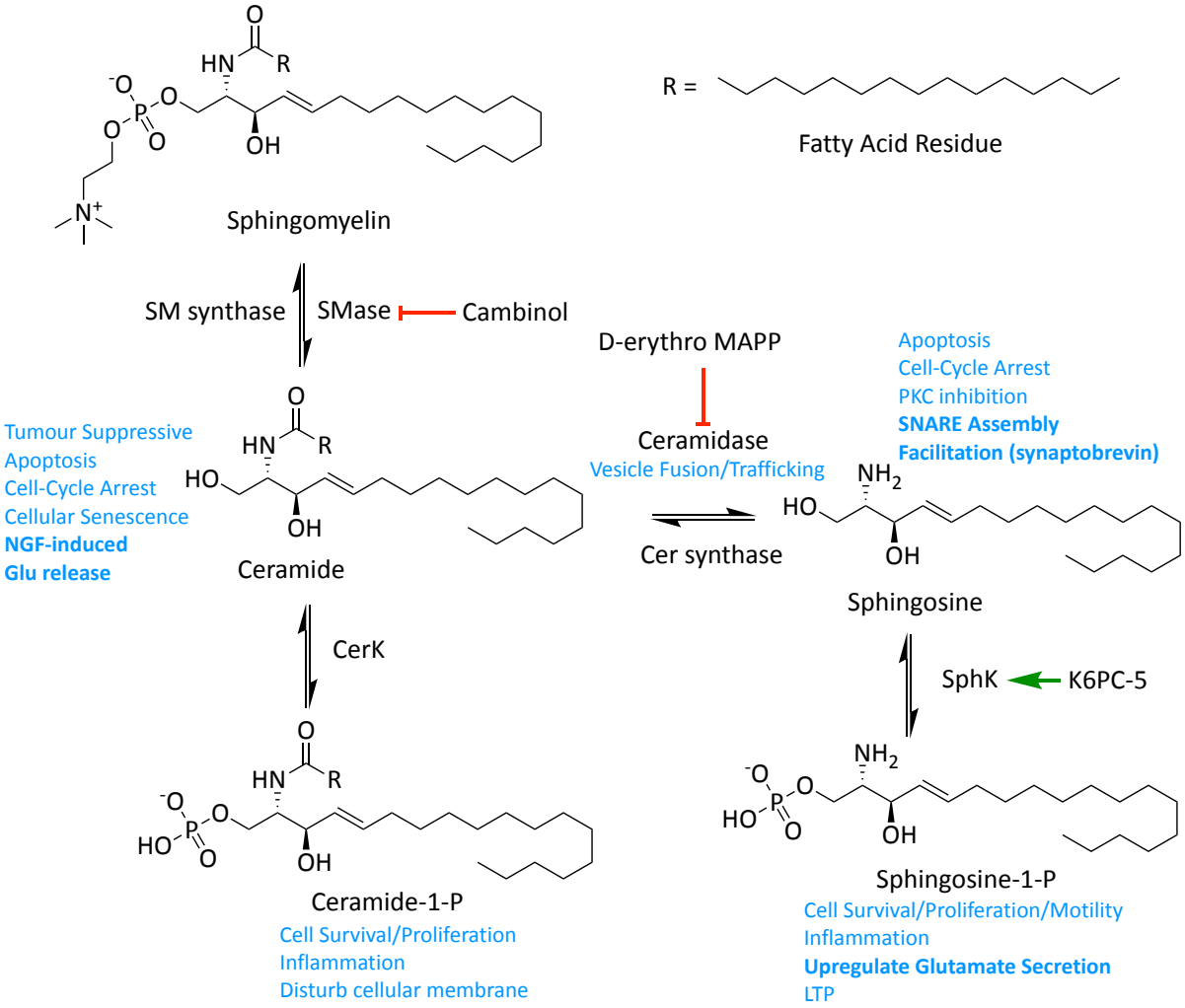

Figure 6.1: Scheme representing sphingolipid metabolism and some physiological effects of the sphingolipid metabolites. SM synthase – sphingomyelin synthase, SMase – sphingomyelinase, Cer synthase – Ceramide synthase, CerK – ceramide kinase, SphK – sphingosine kinase. Physiological effects shown in blue, those pertaining to release are in bold (Darios et al., 2009; Hannun and Obeid, 2018; Kajimoto et al., 2007; Riganti et al., 2016; Rohrbough and Broadie, 2005; Rohrbough et al., 2004).

6.2 Aβo incubation enhances evoked release

First, I set out to confirm that Aβo can enhance release in my system. I incubated primary dissociated hippocampal neurons transfected with SypH 2x in Aβo for over an hour, and measured

changes in release compared to control. After imaging over 10 s at 1 Hz to acquire a baseline signal, 100 action potentials were delivered at 10 Hz via field stimulation. It has been shown that over this period, the signal almost wholly represents exocytosis, as the kinetics of endocytosis and reacidification are too slow to impact measurement on this time scale (Kim and Ryan, 2009). The fluorescence was then recorded for a further 120 s before unquenching by alkalisation with a solution high in NH_4Cl to establish the maximal SypH 2x response at each terminal. As before, this was done to correct for a variation in expression levels, with responses normalized to this signal at each bouton. Treatment with Aβo (50 nM) potentiated release compared to control (Figure 6.2 and Figure 6.6) (Data from Figure 6.2 repeated from Figure 6.6). This result is consistent with those previously found by Jeans *et al.* who showed that Aβo enhanced release (Jeans et al., Unpublished).

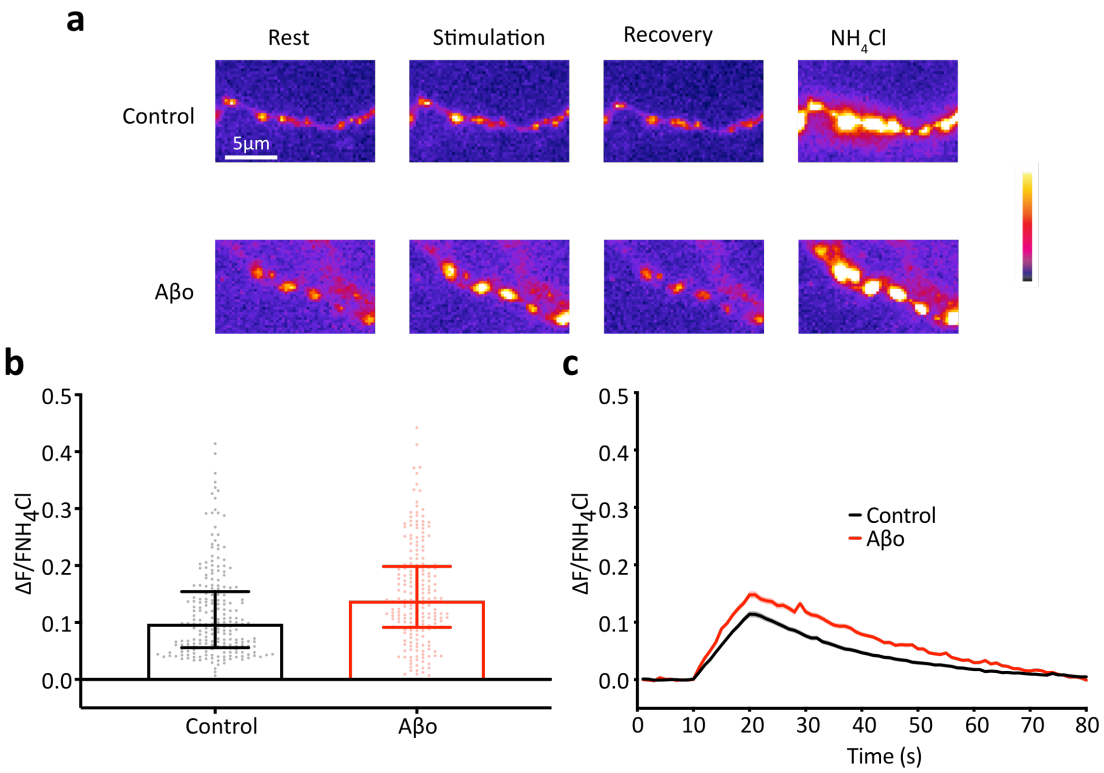

Figure 6.2: Summary of control and Aβo treated pHluorin experiments. **a)** Representative images of SypH 2x at different stages of the experiment. **b)** Median peak amplitudes of responses with interquartile range. Control: 0.096, n= 215 synapses from 7 coverslips; Aβo: 0.137, n= 209 synapses from 6 coverslips. **c)** Average SypH 2x fluorescence traces. Traces show mean ± SEM.

6.3 D-e-MAPP enhances release

Next, I investigated whether altering activity of ceramidase might affect release. Previous work in *Drosophila* has shown that ceramidase function is important for regulating neurotransmitter release, as well as vesicle trafficking dynamics (Rohrbough et al., 2004), however there is also evidence that ceramide facilitates neurotransmitter release (Inokuchi et al., 1998; Jeon et al., 2005; Numakawa et al., 2003).

I incubated neuronal cultures in the ceramidase inhibitor (1S, 2R)-D-erythro-2-(N-Myristoylamino)-1-phenyl-1-propanol (D-e-MAPP) (5µM) (Bielawska et al., 1996), or D-e-MAPP with Aβo for over an hour and measured the extent of evoked release as before. I found that D-

e-MAPP potentiated release, and that incubation with Aβo did not significantly change release (Figure 6.3 and Figure 6.6) (Data from Figure 6.3 repeated from Figure 6.6).

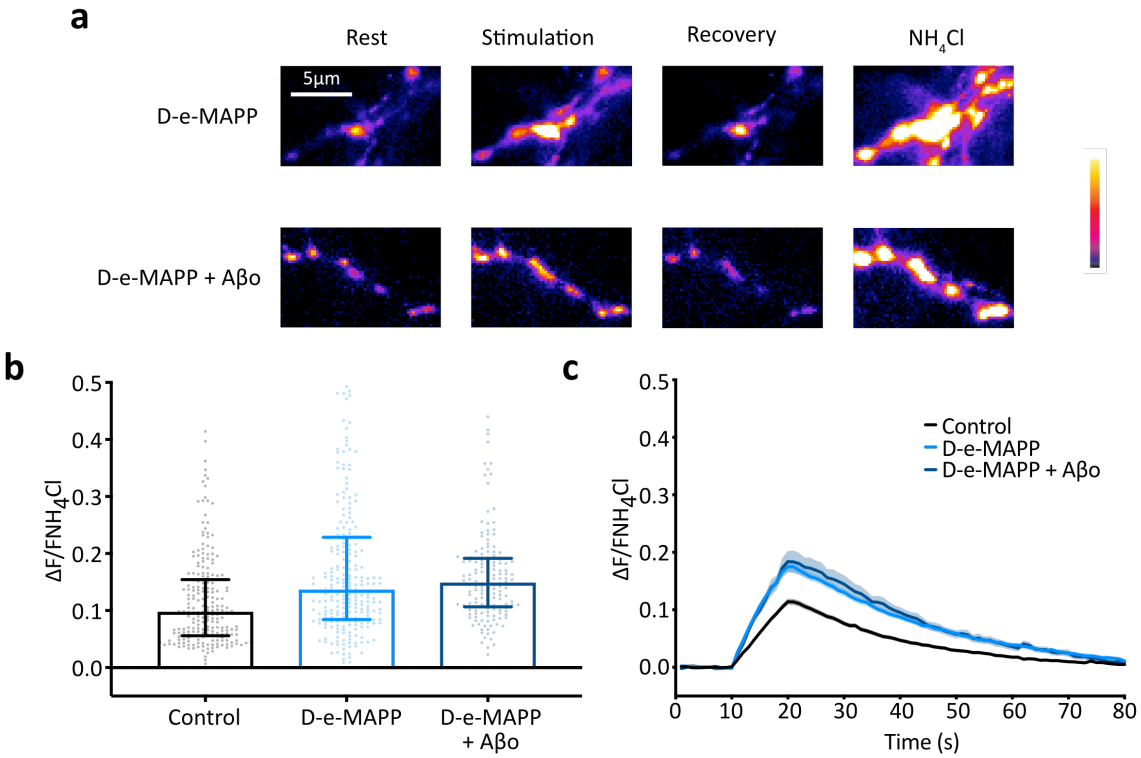

Figure 6.3: Summary of control (repeated from Figure 6.2), D-e-MAPP, and D-e-MAPP and Aβo treated pHluorin experiments. **a)** Representative images of SypH 2x at different stages of the experiment. **b)** Median peak amplitudes of responses with interquartile range: control (repeated from Figure 6.2): 0.114 ± 0.005, n= 218 synapses; D-e-MAPP: 0.135, n= 240 synapses from 8 coverslips; D-e-MAPP + Aβo: 0.148, n=165 synapses from 7 coverslips. **c)** Average SypH 2x fluorescence traces. Traces show mean ± SEM.

6.4 K6PC-5 enhances release

As well as ceramide, S1P has shown glutamate release enhancing effects (Kajimoto et al., 2007; Kanno et al., 2010). In order to test whether I could enhance release by interfering with sphingolipid metabolism and whether this would be additive with the effects of Aβo, so as to give potential insight into mechanistic effects of Aβo-enhanced release, I used the SphK activator,

K6PC-5 (Ji et al., 2015), to investigate whether enhancing SphK activity could potentiate release. Incubation with K6PC-5 (10 μM) for over an hour potentiated release, and incubation with Aβo did not significantly change this K6PC-5-enhanced release (Figure 6.4 and Figure 6.6) (Data from Figure 6.4 repeated from Figure 6.6).

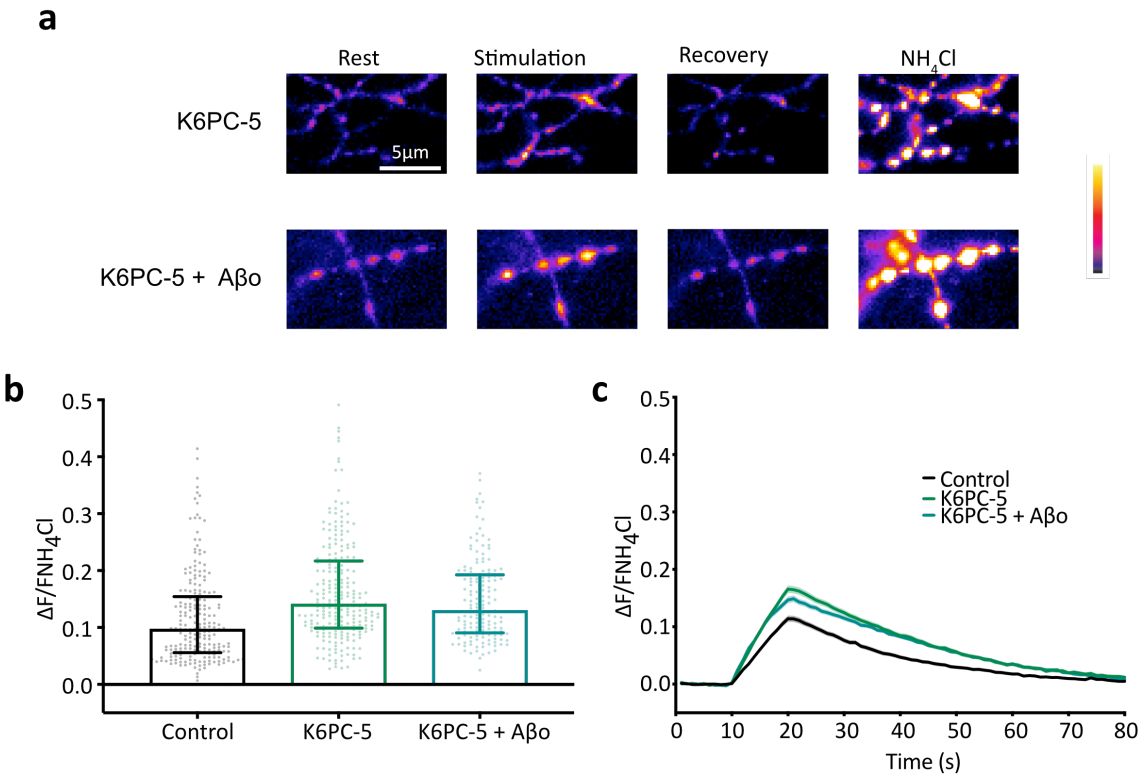

Figure 6.4: Summary of control (repeated from Figure 6.2), K6PC-5, and K6PC-5 and Aβo treated pHluorin experiments. **a)** Representative images of SypH 2x at different stages of the experiment. **b)** Median peak amplitudes of responses with interquartile range: control (repeated from Figure 6.2): 0.096, n= 215 synapses from 7 coverslips; K6PC-5: 0.140, n= 241 synapses from 7 coverslips; K6PC-5 + Aβo: 0.129, n=154 synapses from 8 coverslips. **c)** Average SypH 2x fluorescence traces. Traces show mean ± SEM.

6.5 Cambinol enhances release

The final manipulation that I investigated was inhibition of SMase. There are three different types of SMase, categorised by the pH at which their activity is optimal, acidic, neutral, alkaline. SMases

convert Sphingomyelin into ceramide, which appears to be a key regulator of synaptic vesicle exocytosis (Rohrbough et al., 2004). Given its wide expression in neurons, I decided to block neutral SMase 2 using the drug cambinol, which has shown some neuroprotective properties (Figuera-Losada et al., 2015). As before, neurons were incubated in cambinol (10 μM) for over an hour with and without Aβo, and 100 APs were delivered. Surprisingly, incubation in cambinol potentiated release, and incubation with Aβo did not significantly alter release from this (Figure 6.5 and Figure 6.6) (Data in Figure 6.5 repeated from Figure 6.6).

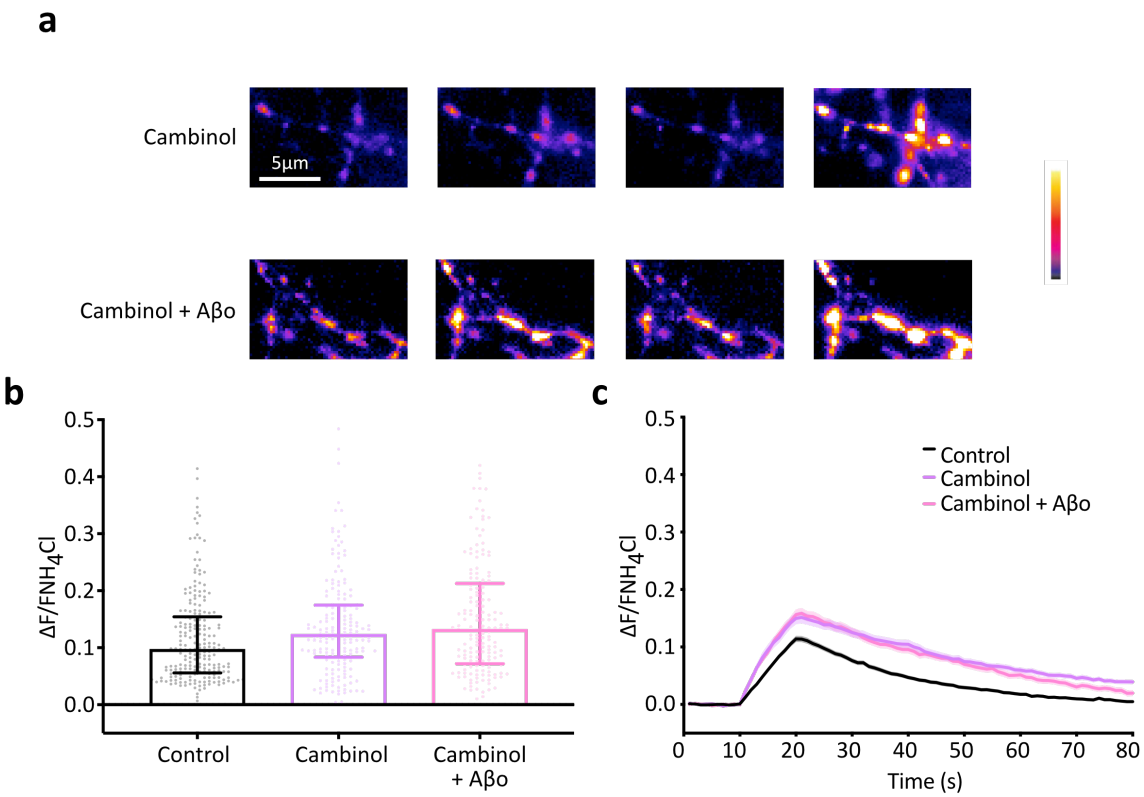

Figure 6.5: Summary of control (repeated from Figure 6.2), cambinol, and cambinol and Aβo treated pHluorin experiments. **a)** Representative images of SypH 2x at different stages of the experiment. **b)** Median peak amplitudes of responses with interquartile range: control (repeated from Figure 6.2): 0.096, n= 215 synapses from 7 coverslips; cambinol: 0.123, n= 185 synapses from 9 coverslips; cambinol + Aβo: 0.131, n=166 synapses from 10 coverslips. **c)** Average SypH 2x fluorescence traces. Traces show mean ± SEM.

6.6 Summary

I set out to investigate whether altering sphingolipid metabolism could bidirectionally modulate neurotransmitter release, and furthermore whether any manipulations could normalise the effects of Aβo. Although, I corroborate evidence that Aβo enhances release, and also show that, by interfering with sphingolipid metabolism, neurotransmitter release can be enhanced, I was unable to reduce neurotransmitter release with any manipulation, with all treatments significantly enhancing release in response to 100 APs (Figure 6.6). In addition, incubation with Aβo alongside sphingolipid metabolism altering drugs did not significantly alter the extent of release, suggesting that the release-enhancing effects of Aβo are being occluded by those of the sphingolipid metabolites (Figure 6.6) (Data shown here was collected together and interleaved).

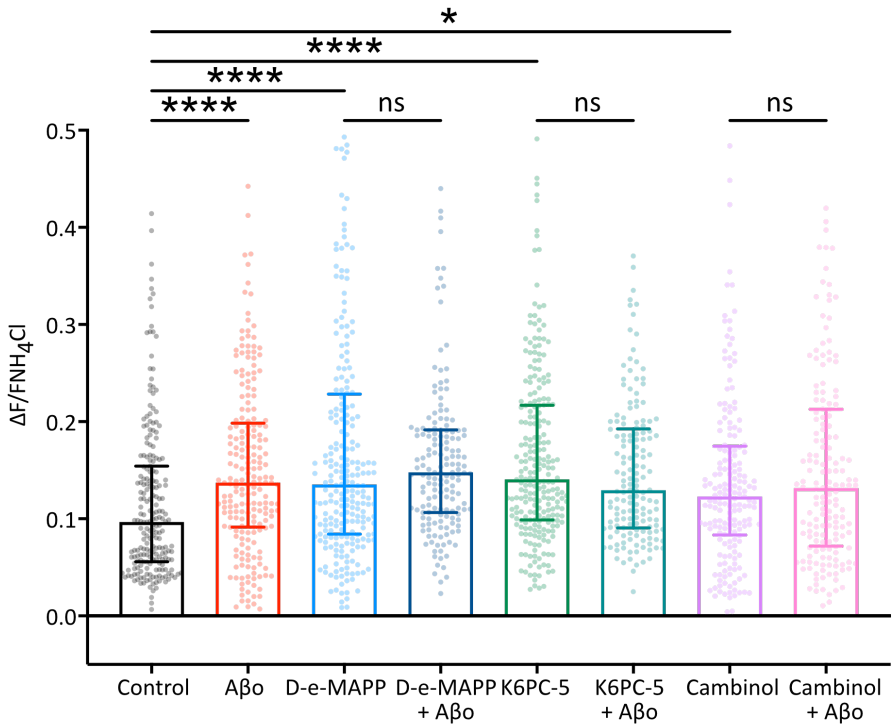

Figure 6.6: Summary of treatments, showing that Aβo and sphingolipid metabolism altering drugs enhances release, but do not have additive effects. All groups are repeated from previous figures, and significance was determined by taking into account all treatments together. Shown with median and interquartile range. Control: 0.096, n= 215 synapses from 7 coverslips; Aβo: 0.137, n= 209 synapses from 6 coverslips; D-e-MAPP: 0.135, n= 240 synapses from 8 coverslips; D-e-MAPP + Aβo: 0.148, n=165 synapses from 7 coverslips; K6PC-5: 0.140, n= 241 synapses from 7 coverslips; K6PC-5 + Aβo: 0.129, n=154 synapses from 8 coverslips; cambinol: 0.123, n= 185 synapses from 9 coverslips; cambinol + Aβo: 0.131, n=166 synapses from 10 coverslips. 21 data points outside axis limits. Kruskal-Wallis test followed by Dunn's multiple comparisons.

6.7 Discussion

I have shown that manipulating elements of sphingolipid metabolism can increase evoked release. In addition, I have confirmed previous studies that demonstrate that Aβo incubation enhances evoked release (Brito-Moreira et al., 2011; Jeans et al., Unpublished; Lazarevic et al., 2017; Russell et al., 2012), however, I also find that the increased release resulting from altered sphingolipid metabolism and Aβo enhanced release are not additive. This mechanistic occlusion suggests that both Aβo and the drugs used here are acting to augment release by mechanisms with conserved

elements, although I have not ruled out that the non-additive nature of these two effects is not the result of a ceiling effect. Further experimentation would be required to confirm this. If this occlusion is due to a conserved mechanism, its exact nature is unknown, but given that many of the sphingolipid metabolites and enzymes involved in sphingolipid metabolism are implicated in synaptic vesicle exocytosis and handling (Darios et al., 2009; Inokuchi et al., 1998; Jeon et al., 2005; Kajimoto et al., 2007; Kanno et al., 2010; Numakawa et al., 2003; Riganti et al., 2016; Rohrbough and Broadie, 2005; Rohrbough et al., 2004), it is possible that this occurs at the point of release.

Predicting the effects of altering the activity of sphingolipid-related enzymes is difficult. All sphingolipids and their metabolites exist in a complex dynamic equilibrium and altering the activity of one enzyme could lead to wider changes than simply modulating the levels of one sphingolipid relative to others (Figure 6.7a). In addition, different steps in sphingolipid metabolism occur in different parts of the cell (Figure 6.7b), and so, depending on which enzyme is targeted, the location of any changes will differ. This could explain the surprising effect of cambinol, the neutral SMase 2 blocker. SMases catalyse the conversion of sphingomyelin to ceramide, and so their blockade would be expected to reduce ceramide levels. Given that ceramide appears to promote vesicle fusion with the plasma membrane (Jeon et al., 2005; Numakawa et al., 2003) as well as promote curvature and budding of the plasma membrane as part of biogenesis of vesicles (Verderio et al., 2018), reduced ceramide levels are expected to diminish release. Furthermore, neutral SMase 2 activity is particularly involved in the release related actions of ceramide; neutral SMase 2 is important for exosome release (Trajkovic et al., 2008) and has been shown to aid dopamine release by producing ceramide in a Ca^{2+}-dependent manner at the plasma membrane (Jeon et al., 2005). Ceramide does, however, exist in equilibrium at the plasma membrane with sphingosine and S1P (Verderio et al., 2018) (Figure 6.7b), both of which also promote neurotransmitter release (Darios et al., 2009; Kajimoto et al., 2007; Kanno et al., 2010). It could be that my manipulation has inadvertently altered the relative levels of sphingosine and S1P as well,

resulting in the enhanced release that I have observed here. In addition, cambinol is not just specific to neutral SMase 2, and has also been shown to exert anti-tumour properties via inhibition of silence information regulator 1 and 2 (SIRT1/2) (Heltweg et al., 2006). Given this lack of specificity, it could be that cambinol enhances release via other off-target effects.

Although I have not succeeded in normalising the effects of Aβo on release, the results that I have described here do highlight that targeting sphingolipid metabolism could be effective in modulating neurotransmitter release. In particular, I show that I am able to enhance neurotransmitter release by manipulating the activity of sphingolipid-metabolising enzymes. In future studies it would, of course, be important to show that the reverse can be achieved. Inhibition of SphK would be a promising target, as would attempting a range of SMase inhibitors, including further, more selective neutral SMase inhibitors such as GW4869 (Wheeler et al., 2009). In addition, inhibition of acid SMases could be a viable strategy, since they also appear to play a role in microvesicle release (Verderio et al., 2018).

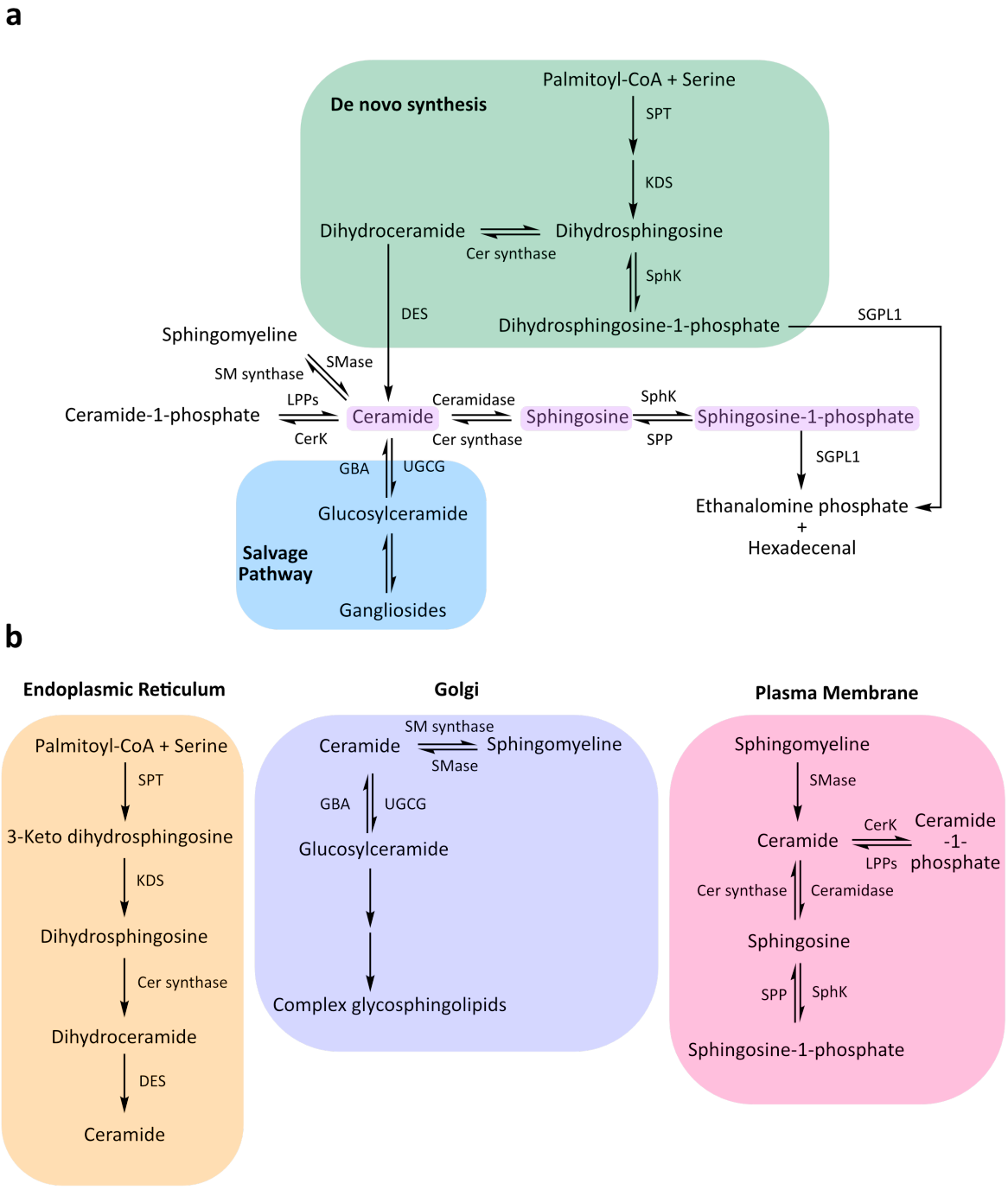

Figure 6.7: In depth sphingolipid metabolism pathway. **a)** Schematic of sphingolipid metabolism, including the initial biosynthesis of ceramide (Figure adapted from (Di Pardo and Maglione, 2018)). **b)** Schematic of sphingolipid production highlighting cell-compartment-specific areas. SPT – Serine palmitoyl transferase, KDS – 3-keto-dihydrosphingosine reductase, SphK – Sphingosine kinase, DES – Ceramide desaturase, SMase – Sphingomyelinase, LPP – Lipid phosphate phosphatase, CerK – Ceramide kinase, GBA – Glucosylceramidase, UGCG – Ceramide glucosyl transferase, Cer synthase – Ceramide synthase, SGPL1 – S1P lyase.

7. Conclusions

Although AD was first characterised over a century ago by Alois Alzheimer (Alzheimer et al., 1995), our current understanding of AD is still limited, particularly with regards to the early stages of the disease that set in motion the eventual clinical presentations. Current advances in neuroscience suggest that we are still far from being able to restore memories that have been lost to neurodegeneration, and so, in order to find an acceptable and effective treatment, the disease must be targeted early, before excessive neuronal damage has occurred. In this thesis I have endeavoured to shed more light on the early pathological events of AD and connect them to later clinical symptoms.

Numerous studies point to the neurodegenerative phase of AD originating at the synapse, and it is widely agreed that synaptic changes lead to network wide dysfunction and behavioural and cognitive deficits. More recent evidence has also suggested a role for dysfunctions in the immune system and vasculature in AD pathogenesis (De Strooper and Karran, 2016). These seemingly disparate aspects of the disease are unified by the protein that is central to AD, Aβ (Hardy and Selkoe, 2002; Mucke and Selkoe, 2012; Viola and Klein, 2015). In a non-disease state, Aβ appears to act at the synapse to promote neural growth (Bishop and Robinson, 2004; Giuffrida et al., 2010; Lopez-Toledano and Shelanski, 2004; Yankner, 1996) and neurotransmitter release (Abramov et al., 2009; Garcia-Osta and Alberini, 2009; Morley et al., 2010; Mura et al., 2012; Puzzo et al., 2011; Zucker and Regehr, 2002), however a build-up, either due to altered processing or impaired clearance that can occur with age, drives the synaptic dysfunction that develops into neurodegeneration, cognitive decline, and death. It has been postulated that the beneficial effects of exercise, diet (Ngandu et al., 2015; Scarmeas et al., 2009) and sleep (Ju et al., 2014; Lucey and Bateman, 2014) on the risk of developing AD reflect the benefits for enhancing Aβ clearance. Prevention of AD is, of course, a vital area of research, however, here, I focus on further cellular

characterisation of the disease. I have concentrated on Aβ, and in particular the active toxic form, Aβo (Viola and Klein, 2015), tracing its actions to the presynaptic terminus, and suggest that changes here occur upstream of other early pathogenic events that have been observed postsynaptically, such as impaired plasticity and reduced glutamate receptor expression (Mucke and Selkoe, 2012; Selkoe, 2008).

Presynaptic facilitation of neurotransmitter release could be expected to enhance synaptic transmission, however, in AD, the reverse is observed (Sheng et al., 2012). A clue to this could be in the evidence that mEPSC frequency has been observed to increase briefly, before then falling after acute addition of Aβo (Parodi et al., 2010). My results here suggest that this phenomenon is likely due to Aβo-induced presynaptic enhancement followed by swift postsynaptic weakening, leading to a loss of observable mEPSC, and so, an artefactually lowered mini frequency result. As discussed, elevated presynaptic release probability turns the presynaptic bouton into a low-pass filter (Abbott and Regehr, 2004), transmitting more low frequency, uncorrelated activity onto the postsynaptic dendrite. Given that postsynaptic weakening seems to depend on low level increases in Ca^{2+} influx (Cummings et al., 1996), arising from repeated, low frequency glutamate binding to postsynaptic receptors (Dudek and Bear, 1992; Mulkey and Malenka, 1992), this provides a possible mechanism for elevated presynaptic function driving postsynaptic depression. An alternative mechanism could be that altered mini release drives the postsynaptic depression via processes of homeostatic plasticity; mini release plays an important role in homeostatic plasticity (Kavalali, 2015), and has been shown to be a key regulator of postsynaptic strength (Sutton et al., 2006). It is possible that Aβo enhances presynaptic Pr, leading to elevated mini frequency, which in turn drives homeostatic weakening of the postsynaptic terminus.

It is still unclear as to how Aβo enhance presynaptic activity. Many people have attempted to identify an Aβ receptor, however this has proven elusive. It appears that Aβ is a very "sticky" peptide, as exemplified by its high binding affinities with a variety of proteins (Table 1.1) (Jarosz-Griffiths et al., 2016; Mroczko et al., 2018; Viola and Klein, 2015). Recent work from Jeans *et al.* has highlighted that elevated activity of $Ca_V2.1$ drives enhanced release via increased presynaptic Ca^{2+} influx (Jeans et al., Unpublished). Changing $Ca_V2.1$ activity is responsible for tuning neurotransmitter release probability as a homeostatic plasticity mechanism (Jeans et al., 2017), and $Ca_V2.1$ activity and neurotransmitter release probability are tuned by alternative splicing of the pore forming α_1 subunit (Thalhammer et al., 2017). Aβo could be involved in a pathway that controls the relative synaptic abundance of the two different splice variants (EFa and EFb), and a build-up of Aβo could shift the balance towards expression of the isoform with enhanced activity. Interestingly, the expression of the two splice isoforms of $Ca_V2.1$ varies with development (Vigues et al., 2002), and also in response to neural network changes in a homeostatic manner (Thalhammer et al., 2017). Impaired homeostatic plasticity has already been observed in AD models, and numerous proteins related to proper homeostatic function are altered in AD and AD models (Styr and Slutsky, 2018) (Table 1.3). It could be that the presynaptic alterations that occur with pathological levels of Aβo represent an impairment of an aspect of homeostatic plasticity, and that this is what underlies the pathology of AD.

How these synaptic plasticity changes relate to other, downstream symptoms of AD is an important connection that is still not clear. Without establishing this link, it is not possible to understand the significance of the synaptic changes induced by Aβo, and how they might be prevented or rescued. Pathological changes in tau phosphorylation and conformation are vital to the progression of AD (Rapoport et al., 2002; Roberson et al., 2011; Roberson et al., 2007), without which much of the disease progression is halted. Understanding the processes that can trigger this

is crucial, not only to AD research, but also to other neurodegenerative diseases where tau misfolding alone drives neurodegeneration (Brunden et al., 2009). I have made a step towards uncovering a link between altered synaptic function and aberrant tau phosphorylation. As with Aβ, I have found that the pathogenic action of this protein is related to its physiological function. I propose that tau hyperphosphorylation occurs as a result of increased LTD that is facilitated by Aβo-enhanced presynaptic release. Although I have yet to establish the details of the mechanism by which augmented LTD leads to pathological tau phosphorylation, there are numerous clues in the literature. In the introduction, I discuss a potential mechanism for physiological LTD, involving NMDARs, which I show to be necessary for tau phosphorylation in a variety of conditions, GSK-3β activation and phosphorylation of tau, and subsequent AMPAR internalisation resulting from an interaction between phosphorylated tau and PICK-1 (Figure 1.5 and Figure 7.1a). This physiological process could become pathological when LTD inducing conditions occur repeatedly over a longer time period (Figure 7.1b). GSK-3β becomes active when it is dephosphorylated at serine 9 by PP1 (Bennecib et al., 2000), which in turn is activated in an NMDAR-dependent manner (Mulkey et al., 1994). The conditions of enhanced uncorrelated presynaptic release in the presence of Aβo could lead to constitutively activation of PP1, and so GSK-3β, and therefore, further phosphorylation of tau at residues that are less favourably phosphorylated when GSK-3β is only transiently active. Another important kinase implicated in hyperphosphorylation of tau is CDK5 (Martin et al., 2013). This kinase also regulates tau activity by phosphorylation at serine 9, deactivating it (Morfini et al., 2004). CDK5 could conceivably be upregulated in response to elevated activity of GSK-3β as a form of negative feedback loop. Indeed, there are suggestions that CDK5 acts to maintain neuronal network stability in response to plasticity (Shah and Lahiri, 2014). Elevated activity of CDK5, in turn, could lead to further phosphorylation of tau (Figure 7.1b). Although this mechanism is largely untested, it demonstrates a possible mechanistic pathway for activity driven tau hyperphosphorylation and combines many

of the key proteins that have previously been implicated in AD and I intend to investigate this further in future work.

I have, therefore, established a link between synaptic changes, originating at the presynaptic terminus, and the later histopathological presence of tau hyperphosphorylation. This link has long been sought for, and by establishing a more causative role for Aβo-induced synaptic changes for downstream AD pathology, I highlight the importance of these early events. Although we are still far from developing an effective treatment for AD, the aspects of the disease that I have identified here will hopefully advance the field and help towards developing a therapeutic intervention that can make AD a disease of the past.

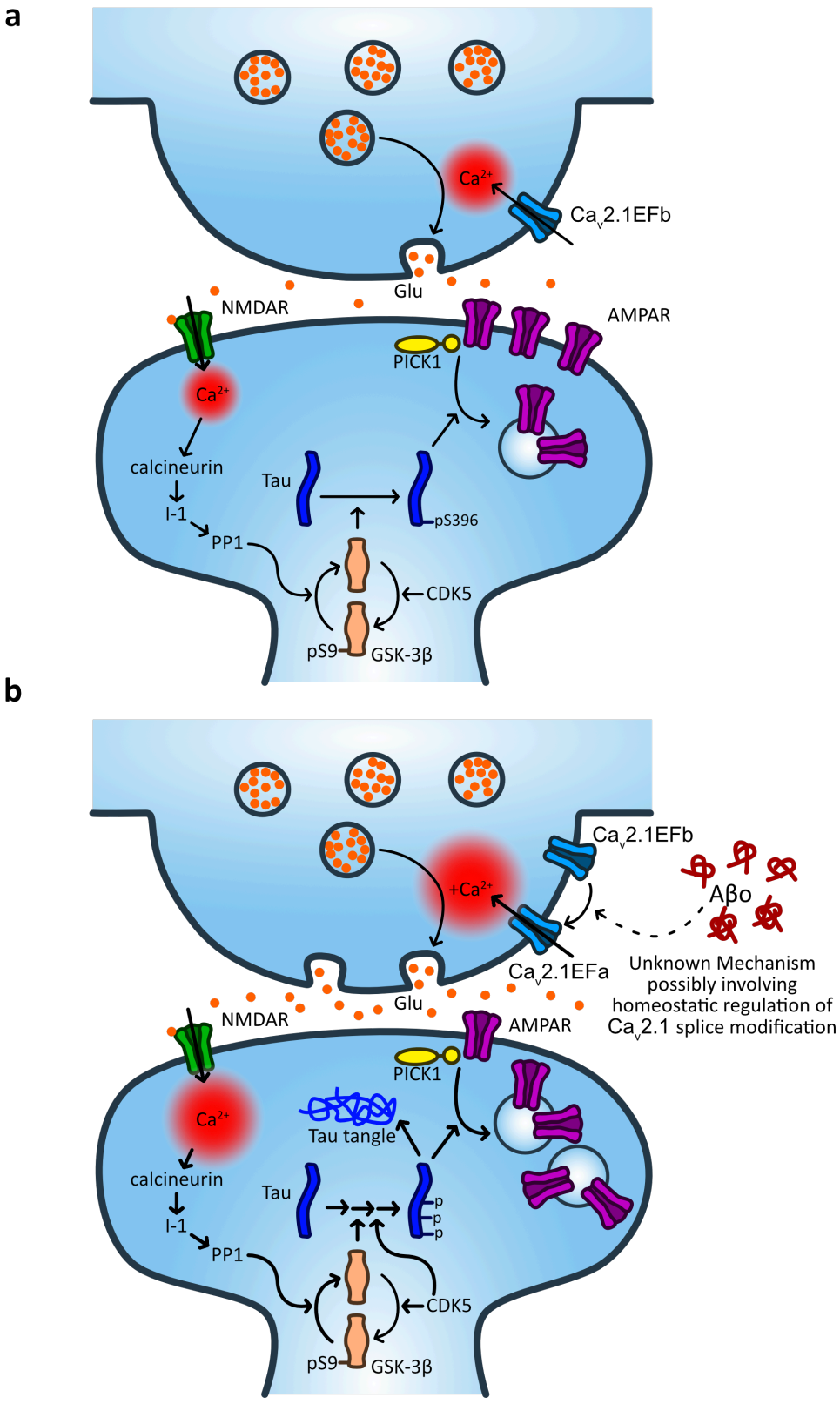

Figure 7.1: Possible mechanism for the involvement of tau in LTD, and how Aβo changes to the presynapse can lead to pathologically modified tau. **a)** under physiological conditions, LTD inducing stimuli activate NMDARs on the postsynaptic dendrite, causing low level Ca^{2+} influx,

activation of calcineurin, I-1, and PP1. PP1 dephosphorylates GSK-3β at Serine 9, activating it. GSK-3β phosphorylates tau at serine 396, allowing an interaction between tau and PICK-1 to cause AMPAR endocytosis and so LTD expression. **b)** Aβo change the expression of $Ca_V2.1$ splice isoforms from predominantly the EFb isoform to the EFa isoform that enhances $Ca_V2.1$ activity. Resultant increased Ca^{2+} entry in response to a stimulus enhance release probability and turns the bouton into a low-pass filter. The resultant increase in uncorrelated glutamate release facilitates LTD inducing conditions. Enhanced LTD leads to elevated activation of GSK-3β, and possibly CDK5 in response, causing enhanced phosphorylation of tau and AMPAR endocytosis. The increased phosphorylation of tau eventually leads to formation of intracellular tau tangles.

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