An exploration of the impact of amyloid-β on intracellular Ca^{2+} signalling and metabolic pathways in models of Alzheimer's Disease.

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A thesis submitted for the degree of Doctor of Philosophy
Department of Pharmacology, University of Oxford
Trinity 2024

Acknowledgements

I am deeply grateful for being given the opportunity to learn and develop as a scientist during my time as a DPhil student at the University of Oxford. I would also like to acknowledge the financial support I have received from Somerville College, which enabled me to pursue my studies and allowed me to be a part of an exceptional college community. I would like to express my sincere gratitude to the following people that have supported me throughout the course of my DPhil.

Firstly, I would like to thank my supervisors, Nigel Emptage and Daniel Anthony, for their guidance, encouragement and invaluable feedback and advice throughout this journey. Their expertise has been instrumental in shaping my projects and academic development. I would also like to thank Alexander Jeans for his insightful feedback and allowing me the opportunity to contribute to a very exciting and important project. To the Emptage lab members, Sally, Maike and Yuki – thank you for all the support and making me laugh on a daily basis.

The friends that I have met in Oxford have been a constant source of happiness and will always be a big part of my life. Stefano and Vicky – there are not enough pages to express how much you two mean to me, although I hope the annual birthday cards serve as a reminder of the depth of my appreciation and love for you both. Hanson –thank you for your support during this process and the 100 daily memes. Oana – you have made my life so much brighter, thank you for all the fun nights, especially at the retreat! Lara, Shruti, Adriana, Razanne, Vasilii – our friendship is forever, I am so thankful to have met you all and have you in my life. Ash, Gaurav, Bahar, Daan and Sofija – thank you for all the love and support during these past few years, I'm so happy our DPhil journeys coincided.

Finally, I would like to thank my cats at home for all the hugs and my parents for their unwavering love, patience, and unlimited support during these years.

Abstract

In the field of Alzheimer's Disease (AD) research, there's a growing consensus that therapeutic intervention to significantly modify disease progression must begin at the preclinical stages of AD. By investigating early disease mechanisms, researchers can develop targeted therapies aimed at preventing the onset of cognitive symptoms. The amyloid hypothesis of AD suggests that the accumulation of Aβ peptides, particularly Aβ oligomers, is a central and early event in AD pathogenesis. Aβ oligomers are neurotoxic and cause synaptic dysfunction, eventually resulting in synapse loss and neuronal death, which contributes to the cognitive decline observed in AD patients. The mixed results observed in clinical trials of antibodies targeting Aβ highlight the necessity of identifying alternative molecular targets for AD therapeutics. Furthermore, the exact role of Aβ in AD pathophysiology is still under constant investigation. Extensive research efforts have focused on characterising the effects of Aβ oligomers at the pre- and postsynaptic compartment, intracellular Ca^{2+} signalling, and structural plasticity of dendritic spines. Although, there are still significant gaps in our understanding, which may delay the comprehension of the relationship between early disease mechanisms and late-stage cognitive decline in AD. In this thesis, I aim to advance the understanding of the role that Aβ oligomers play in synaptic function and Ca^{2+} signalling mechanisms in CA1 hippocampal neurons by using synthetic Aβ oligomers applied to hippocampal neurons and slice cultures, as well as in acute hippocampal slices of J20 mice, an AD model. Using a combination of electrophysiology and imaging techniques, I firstly investigated the effects of a presynaptic Ca^{2+} channel, $Ca_v2.1$, in mediating Aβ oligomer toxicity. A heterozygous knockout of $Ca_v2.1$ normalised presynaptic function and rescued Aβ-induced LTP impairment, while preserving basal neurotransmission. Secondly, I assessed lysosomal and endoplasmic reticulum (ER) activity-dependent dynamics and Ca^{2+} release, and the effects on structural plasticity in response to Aβ oligomer treatment. Aβ oligomers were able to disrupt specific features of lysosome dynamics and recruit Ca^{2+}-induced Ca^{2+} release (CICR) from the ER

in response to back-propagating action potentials in neuronal dendrites. Finally, ^{1}H Nuclear Magnetic Resonance (NMR) metabolomics was used to identify metabolite changes throughout regular aging and AD pathogenesis in tissues from wild type and J20 mice. Both regular aging and early/late stage AD induced specific metabolic signatures, which may contribute to the discovery of novel biomarkers associated with metabolites altered in AD.

Abbreviations

ACSF	Artificial Cerebrospinal Fluid
AD	Alzheimer's Disease
AICD	APP Intracellular Domain
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor
ANOVA	Analysis of Variance
APOE	Apolipoprotein E
APP	Amyloid Precursor Protein
ARIA	Amyloid-Related Imaging Abnormalities
ATP	Adenosine Triphosphate
Aβ	Amyloid β
BACE1	β-Site Amyloid Precursor Protein Cleaving Enzyme 1
BBB	Blood-Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
CAA	Cerebral Amyloid Angiopathy
CA1-3	Cornu Ammonis 1-3
cADPR	Cyclic Adenosine Diphosphate Ribose
CaM	Calmodulin
CaMK	Calmodulin Kinase
CaMKII	Ca^{2+}/calmodulin-dependent Protein Kinase II
CBP	CREB-Binding Protein
CICR	Calcium Induced Calcium Release
CLU	Clusterin
CNS	Central Nervous System
CREB	cAMP Response Element Binding Protein
CSF	Cerebrospinal Fluid
EAAT-1/2	Excitatory Amino Acid Transporter 1/2
ENaC	Epithelial Sodium Channels
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-Associated Protein Degradation
FAD	Familial Alzheimer's Disease
FDG-PET	Fluorodeoxyglucose Positron Emission Tomography
FDR	False Discovery Rate
fEPSP	Field Excitatory Postsynaptic Potential
FFA	Free Fatty Acids
FTDP-17	Frontotemporal Dementia and Parkinsonism Linked to Chromosome 17
GABA	Gamma-aminobutyric Acid
GC	Gas Chromatography
GFAP	Glial Fibrillary Acidic Protein
GPN	Glycyl-L-phenylalanine 2-naphthylamide
GSK-3β	Glycogen Synthase Kinase 3β

GWAS	Genome Wide Association Studies
HDL	High Density Lipoprotein
Iba1	Ionized Calcium Binding Adaptor Molecule 1
IP3R	Inositol Triphosphate Receptor
LAMP-2	Lysosome Associated Membrane Protein 2
LC	Liquid Chromatography
LRP1	Low Density Lipoprotein Receptor-Related Protein 1
LSD	Lysosome Storage Diseases
LTD	Long Term Depression
LTP	Long Term Potentiation
MAPT	Microtubule Associated Protein Tau
mGluR	Metabotropic Glutamate Receptor
MMP-9	Matrix Metalloproteinase 9
MNI-Glutamate	4-Methoxy-7-nitroindolinyl-caged-L-glutamate
MS	Mass Spectrometry
MTEP	3-((2-Methyl-4-thiazolyl)ethynyl)pyridine
mTORC1	Mammalian Target of Rapamycin Complex 1
NAADP	Nicotinic Acid Adenine Dinucleotide Phosphate
nAChR	Nicotinic Acetylcholine Receptor
NFT	Neurofibrillary Tangles
NMDAR	N-Methyl-D-Aspartate Receptor
NMR	Nuclear Magnetic Resonance
OGB-1	Oregon Green BAPTA 1
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
PDGF-β	Platelet Derived Growth Factor β
PET	Positron Emission Tomography
PETA	Phosphoethanolamine
PFC	Prefrontal Cortex
PiB	Pittsburgh Compound B
PKC	Protein Kinase C
PMCA	Plasma Membrane Ca^{2+} ATPase
PPF	Paired Pulse Facilitation
PPM	Parts Per Million
PPR	Paired Pulse Ratio
Pr	Probability of Neurotransmitter Release
PrPc	Cellular Prion Protein
PS1/2	Presenilin 1/2
PSD-95	Postsynaptic Density Protein 95
PUFA	Polyunsaturated Fatty Acids
qPCR	Quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptor
sAPP	Soluble Amyloid Precursor Protein

SERCA	Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase
SORL1	Sortilin-Related Receptor 1
STIM 1/2	Stromal Interaction Molecule 1/2
TBS	Theta Burst Stimulation
TCA	Tricarboxylic Acid Cycle
TFEB	Transcription Factor EB
TIMP-1	Tissue Inhibitor of Metalloproteinase 1
TIRFM	Total Internal Reflection Fluorescence Microscopy
TPC 1/2	Two Pore Channels 1/2
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
TRPA1	Transient Receptor Potential Cation Channel, A1
TRPC6	Transient Receptor Potential Cation Channel, C6
TRPML1	Transient Receptor Potential Cation Channel, Mucolipin 1
UPR	Unfolded Protein Response
VGCC	Voltage Gated Calcium Channel
VIP	Variable Importance in Projection
VLDL	Very Low Density Lipoprotein
WT	Wild Type

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1. Introduction

1.1. Alzheimer's Disease

1.1.1. Alzheimer's Disease (AD) pathology

Alzheimer's Disease (AD) is the most common cause of dementia and affects over 6% of people aged over 65 years of age (Burns and Iliffe 2009). The neurodegenerative disorder involves progressive cognitive impairment and is characterised by widespread amyloid and tau pathophysiology throughout the brain (Burns and Iliffe 2009) (Figure 1.1). Presently, there are no treatments that stop or reverse the progression of AD, and current treatments only temporarily improve symptoms (Masters et al. 2015). In 1992, the amyloid hypothesis proposed that extracellular amyloid-β (Aβ) deposits are central to the pathogenesis, specifically $A\beta_{1-42}$, the longer isoform that is more prone to aggregation (Hardy and Higgins 1992). It is currently widely accepted that soluble Aβ oligomers rather than amyloid plaques are responsible for the disease and have been more directly implicated in neurotoxic effects (Wilcox et al. 2011). Aβ accumulation begins decades before the emergence of clinical symptoms and disease (Selkoe and Hardy 2016). Hence, it is crucial to elucidate the mechanisms by which upstream pathogenic species, such as soluble Aβ oligomers, interfere with neuronal function.

1.1.1.1. Amyloid-β

Amyloid-β (Aβ) is a peptide with 40- and 42-amino acid residues as the predominant species and is the main component of amyloid plaques in AD (Burns and Iliffe 2009). It is derived through the amyloidogenic pathway by subsequential proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase (BACE1), producing soluble APP (sAPPβ) and C99; and γ-secretase, generating an APP intracellular domain (AICD) and the Aβ monomers, which then readily

aggregate and give rise to various assemblies including oligomers, protofibrils and amyloid fibrils (Glabe 2008) (Figure 1.1). In contrast, in the non-amyloidogenic pathway, APP is cleaved by α-secretase, releasing sAPPα and a C-terminal fragment (CTFα), and then γ-secretase, releasing AICD and peptide P3 (Haukedal and Freude 2021) (Figure 1.1). Aβ is produced within subcellular regions, particularly the endosomal compartment and trans-Golgi network, since these areas are enriched with the enzymes involved in Aβ production (Chen et al. 2017). Aβ is then transported extracellularly through exocytosis (Chen et al. 2017). $A\beta_{1-42}$ is predominantly prone to oligomerisation as it contains three key hydrophobic sites that promotes aggregation into oligomers (Hayden and Teplow 2013). Soluble Aβ oligomers are able to spread throughout the brain, while amyloid fibrils are insoluble and assemble into amyloid plaques, forming the lesions that are characteristic of AD (Ferreira et al. 2015). Thal Stages 1-5 reflect the progression of Aβ pathology across brain regions. Stage 1-2 is characterised by amyloid deposition in the neocortex and the association cortices of the temporal, parietal and occipital lobes. At these stages, Aβ does not correlate with clinical symptoms and cognitive impairment is minimal or absent. At Stage 3, Aβ deposition reaches the hippocampus and entorhinal cortex, and cognitive deficits become more noticeable. At Stage 4, Aβ spreads to the brainstem and diencephalon, with associated severe impairments in memory. At Stage 5, Aβ reaches the cerebellum and additional subcortical structures, and correlates with significant cognitive decline (Figure 1.2). The spread of Aβ plaques follows a pattern that relates to the anatomical connectivity of the brain; brain regions that are more interconnected are more vulnerable to Aβ deposition (Thal et al. 2002). Additionally, Aβ can be transported through neuronal pathways, spreading to neighbouring regions and triggering further Aβ deposition by causing normal Aβ to misfold and aggregate through a process referred to as 'seeding' (Olsson, Klementieva, and Gouras 2018).

1.1.1.2. Neurofibrillary tangles (NFTs)

Neurofibrillary tangles (NFTs) composed of aggregated hyperphosphorylated tau are another pathological hallmark of AD (Knopman et al. 2021). Tau is encoded by the microtubule associated protein tau (MAPT) gene on chromosome 17, and it can undergo alternative splicing to produce six distinct isoforms, which exhibit differential expression in brain development (Buée et al. 2000). In AD, tau is present as a mix of three and four-repeat isoforms, while other human tauopathies exhibit aggregates of different tau isoforms (Buée et al. 2000). Previous experiments have highlighted a role for tau in microtubule assembly, stabilisation of neuronal axons, and modulation of microtubule transport (Kent, Spires-Jones, and Durrant 2020). The accumulation of NFTs disrupt microtubule structure and axonal transport, resulting in the impaired mobilisation of essential nutrients and molecules to synapses, which may subsequently cause synapse degeneration (Adalbert et al. 2018). Although NFT formation correlates with synaptic impairment and cognitive decline in AD, the degree of the involvement of tau in instigating AD pathology has been a topic of considerable debate (Medeiros, Baglietto-Vargas, and Laferla 2011). According to the amyloid cascade hypothesis and several longitudinal studies, tau dysfunction is a downstream process that is recruited once Aβ has accumulated (Gulisano et al. 2018). The Braak stages are used to describe progression of NFT accumulation in AD (Figure 1.2). Stages I and II are characterised by NFTs in the transentorhinal region with very mild cognitive symptoms. At Stages III and IV, NFTs spread to the hippocampus and nearby limbic structures, and patients start to show early signs of mild cognitive impairment. At Stages V and VI, NFTs extend throughout the neocortex and patients exhibit severe cognitive decline (Braak et al. 2006).

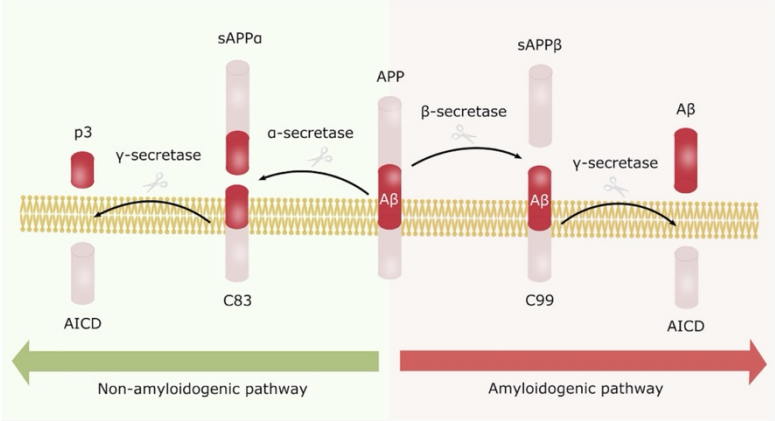

Figure 1.1: APP processing through the non-amyloidogenic or amyloidogenic pathway. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase, resulting in the production of sAPPα and C83. C83 is further processed by γ-secretase, yielding AICD and the non-toxic p3 fragment. In the amyloidogenic pathway, APP is first cleaved by β-secretase, producing sAPPβ and C99. C99 is subsequently cleaved by γ-secretase, generating AICD and Aβ peptides. Reproduced from (Haukedal and Freude 2021).

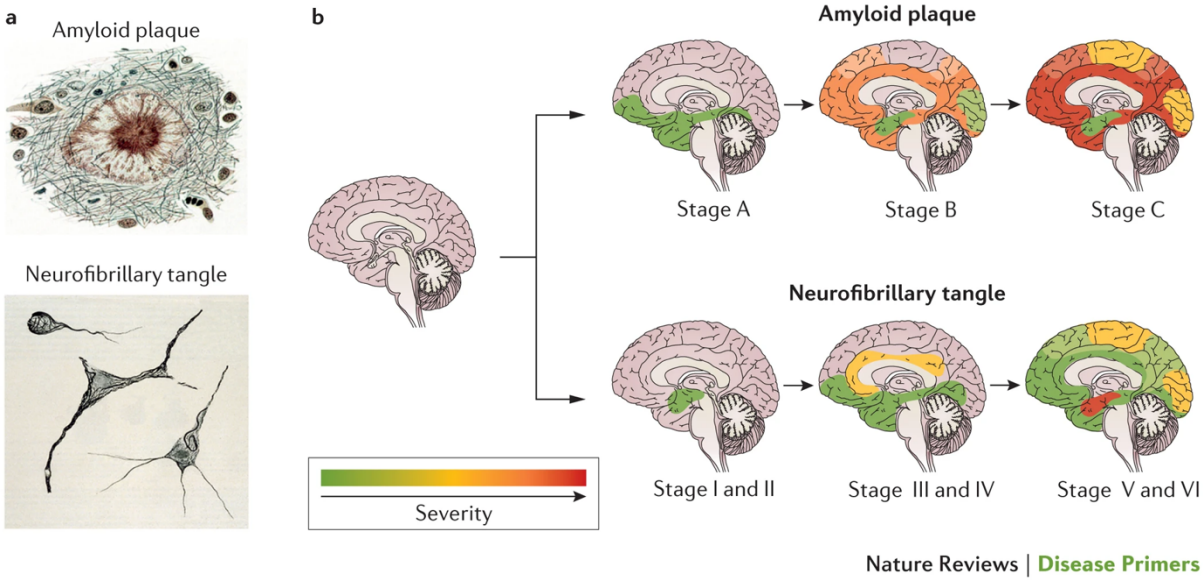

Figure 1.2: Amyloid plaques and NFTs spread throughout the brain as AD progresses. Amyloid deposition precedes NFTs and originates from the frontal and temporal lobes, hippocampus, and limbic system. NFTs originate from the medial temporal lobe and hippocampus. In later stages, both pathological species spread to other areas of the neocortex. Reproduced from (Masters et al. 2015).

1.1.1.3. AD genetic risk factors

The amyloid hypothesis came from the identification of mutations in certain genes that cause early-onset familial AD (FAD), accounting for only 5-10% of all AD cases (Burns and Iliffe 2009). Mutations in the genes encoding amyloid precursor protein (APP) and presenilins 1 and 2 (PS1 and PS2), which are proteins in the catalytic subunit of γ-secretase, responsible for generating Aβ from APP, cause an increase in the production of $A\beta_{1-42}$ (Shea et al. 2016). Studies have shown that mutations in the APP gene can either lead to an increased production of the $A\beta_{1-42}$ isoform or enhance Aβ peptide aggregation, promoting the formation of amyloid plaques (Masters et al. 2015). Additionally, the location of the gene that encodes for APP is located on chromosome 21 and individuals with Down Syndrome, who have an extra copy of chromosome 21, exhibit early symptoms of AD by 40 years of age (Hardy and Higgins 1992). FAD shares many clinical and neuropathological characteristics with sporadic AD, suggesting that Aβ plays a significant role in the pathogenesis of both sporadic and FAD (Hardy and Higgins 1992).

Genome-wide association studies (GWAS) have identified numerous polymorphisms in other genes that are associated with the risk of developing AD (Karch and Goate 2015). For example, genetic variants of clusterin (CLU), a glycoprotein that interacts with multiple protein ligands and receptors (Foster et al. 2019), and sortilin-related receptor 1 (SORL1), involved in endosomal trafficking, have been associated with AD risk (Yin, Yu, and Tan 2015). Genetic and clinical research studies identified APOE4 as a central genetic risk factor for developing sporadic AD (Corder et al. 1993). Apolipoprotein E (APOE) is produced by astrocytes in the CNS and plays an important role in cholesterol transport to neurons (Kim, Basak, and Holtzman 2009). The APOE4 allele increases AD risk through multiple processes, such as promoting neuroinflammation and disrupting glucose metabolism, mitochondrial function, and synaptic transmission (Chia Chan Liu et al. 2013). APOE is also able to regulate Aβ levels by competitively binding to low-density

lipoprotein receptor-related protein 1 (LRP1) on the surface of astrocytes and microglia, thereby preventing Aβ uptake (Castellano et al. 2011). APOE4 is associated with reduced Aβ clearance and increased seeding activity, promoting the accumulation of Aβ aggregates (Chia Chen Liu et al. 2017).

Multiple studies have provided evidence that the activation of immune mediators plays a pivotal role in AD pathology (Heneka et al. 2015). Reactive astrogliosis and microgliosis, contributing to widespread neuroinflammation, are prominent characteristics observed in AD brains (Hansen, Hanson, and Sheng 2018). Exome sequencing and targeted association studies have revealed polymorphisms in genes related to microglial function, which have also been implicated as risk factors of AD (Guerreiro et al. 2013). Triggering receptor on myeloid cells 2 (TREM2) is an AD risk gene, with a rare variation in the allele R4LH, which has been strongly linked to an elevated risk of developing AD (Jonsson et al. 2013). TREM2 is expressed in microglia, where it plays various roles such as promoting microglial phagocytosis, modulating inflammatory signalling, and enhancing microglial survival (Takahashi, Rochford, and Neumann 2005). During early stages of AD, TREM2 levels are increased and the receptor can bind and stimulate the phagocytosis of Aβ (Zhao et al. 2022; Kim et al. 2017). The variant is linked to a loss of function mutation, resulting in a microglial phenotype with disrupted energy state and reduced Aβ binding (Gratuze, Leyns, and Holtzman 2018).

1.1.1.4. AD diagnosis and biomarkers

The prevailing challenge in AD diagnosis is to identify the disease at the prodromal stage, before clinical symptoms appear. Unfortunately, the prodromal stage is exceedingly difficult to detect with current practices, and furthermore, this stage is integrated into the general 'mild cognitive impairment' category, which is associated with many other diseases (Forlenza, Diniz, and Gattaz

2010). The development of novel diagnosis tools, such as neuroimaging and cerebrospinal fluid (CSF) biomarkers may improve the specificity of the prodromal AD diagnosis (Blennow and Zetterberg 2018). Neurological and neuropsychological assessments are required for patient screening in memory clinics prior to the use of advanced tests (Di Pucchio et al. 2018).

The introduction of Pittsburgh compound B (PiB) marked a significant advancement in AD diagnosis research. PiB is a radioactive analogue of thioflavin-T and possesses the ability to cross the blood-brain barrier and bind to fibrillar Aβ, allowing for Aβ imaging with PET scans (Mathis et al. 2002). The initial investigations into Aβ imaging in the brain has verified that Aβ deposition initiates many years prior to the onset of cognitive decline and brain atrophy (Villemagne et al. 2013). Longitudinal studies utilising Aβ PET scans have demonstrated its ability as a marker for the transition from mild cognitive impairment to AD (Ong et al. 2015). Additionally, PET ligands for tau aggregates are currently being developed for the diagnostic assessment of patients (Villemagne et al. 2015).

The CSF is able to reflect the biochemical changes occurring within the brain, and CSF collection through lumbar puncture is a routine practice in AD diagnosis (Masters et al. 2015). Several biomarkers associated with AD have been identified in the CSF, including $A\beta_{1\text{-}42}$, total tau protein, and phosphorylated tau protein (Mattsson et al. 2009). CSF levels of these biomarkers are incorporated in clinical trials to monitor the biochemical effects of candidate drugs (Masters et al. 2015). The combination of low CSF levels of $A\beta_{1\text{-}42}$ and high levels of total tau protein and phosphorylated tau is often referred to as the AD CSF profile, and is highly specific for diagnosing AD (Maddalena et al. 2003). The low levels of $A\beta_{1\text{-}42}$ may reflect impaired CSF clearance in the brain or the accumulation of $A\beta_{1\text{-}42}$ into amyloid plaques (Janelidze et al. 2016). Levels of phosphorylated tau have been shown to correlate with the rate of hippocampal atrophy and rapid disease progression (Hampel et al. 2004).

The pursuit of novel and accessible biomarkers for AD has been a large focus of AD research. Blood-based biomarkers have great potential due to their non-invasive nature, reduced costs and ease of sample collection (Hansson et al. 2023). Several types of blood-based biomarkers have been explored for their potential use in AD diagnosis and monitoring, including plasma Aβ levels, tau, inflammatory markers, and metabolomic and proteomic profiles (Teunissen et al. 2022). The utility of blood-based biomarkers remains limited due to the confounding influence of peripheral tissues, rather than solely reflecting proteins in the brain, therefore, CSF biomarkers remain the preferred choice for AD diagnosis (Hampel et al. 2023).

1.1.1.5. Current AD treatments

Although there are limited treatments available for AD, recently, there has been some progress due to the accelerated approval of two disease-modifying therapies (Belder, Schott, and Fox 2023). Previous drugs merely offered temporary symptomatic relief for patients and did not affect disease progression (Masters et al. 2015). Degeneration of cholinergic neurons in the basal forebrain cholinergic system in AD results in a reduction of cholinergic neurotransmission (Auld et al. 2002). Acetylcholinesterase inhibitors, such as donepezil, galantamine, and rivastigmine, are used as the primary treatment and delay the development of clinical symptoms (McGleenon, Dynan, and Passmore 1999). Acetylcholine plays an important role in learning and memory, and therefore, decreased acetylcholine is associated with cognitive deficits observed in AD patients (Auld et al. 2002). Acetylcholinesterase inhibitors block the action of acetylcholinesterase, the enzyme responsible for degrading acetylcholine, leading to increased acetylcholine levels and promoting cholinergic neurotransmission to alleviate cognitive symptoms in patients (Grossberg 2003). In addition to the impairment in cholinergic neurotransmission, the glutamatergic system is also disrupted in AD (Conway and Conway 2020). Glutamatergic synapses are the main excitatory synapses in the brain and have been widely studied in the context of synaptic plasticity, a process

underlying learning and memory (Liu et al. 2019). Memantine, a drug approved for use in moderate to severe AD, works by uncompetitively antagonising the N-methyl-D-aspartate receptor (NMDAR), consequently normalising glutamatergic excitotoxicity and the increased Ca^{2+} influx via NMDARs due to the excessive glutamate levels in AD (Parsons et al. 2013). It is common to use a combination of a cholinesterase inhibitor and memantine in therapy, which provides additional benefits through their combined effects (Tariot et al. 2004). These drugs offer a limited clinical response, although, a minority of patients do experience cognitive enhancement and cognitive decline is delayed by approximately 6-9 months (Takeda et al. 2006).

Monoclonal antibodies directed against the Aβ peptide have been extensively tested, and several have moved on to clinical trials, with a number having advanced to stage III (Musiek, Gomez-Isla, and Holtzman 2021). Thus far, the outcomes have been indecisive and the majority of these antibodies have not shown much promise (Musiek, Gomez-Isla, and Holtzman 2021). Although, more recently, Lecanemab, an antibody targeting a protofibrillar type of Aβ, has been shown to reduce markers of Aβ in early AD and prevent cognitive deterioration; however, longer trials are needed to determine the efficacy and safety profile (van Dyck et al. 2022). Donanemab, the latest anti-amyloid antibody, clears amyloid plaque and showed a reduction in the rate of cognitive decline (Sims et al. 2023). In contrast to lecanemab, which is given as a long-term treatment, patients using donanemab are able to stop treatment after achieving amyloid-negative status (Sims et al. 2023). Both drugs produced similar side effects, particularly related to amyloid-related imaging abnormalities (ARIA), including brain swelling and haemorrhages (Sims et al. 2023; van Dyck et al. 2022). ARIA is thought to occur due to removal of amyloid plaques from the brain and tends to be more frequent in patients with cerebral amyloid angiopathy (CAA), a condition characterised by amyloid deposits in the walls of blood vessels (Sperling et al. 2011) Monoclonal antibodies that clear the amyloid deposits may cause transient changes in the blood-brain barrier, leading to symptoms associated with ARIA (Sperling et al. 2011).

Although the approach of monoclonal antibodies in AD therapy to date has been unsatisfactory, significant progress has been made in this research area.

1.1.2. The physiological role of Aβ

Aβ plays an important role in various physiological processes that are vital to neuronal function (Kent, Spires-Jones, and Durrant 2020). Enzymes responsible for Aβ production are present within the synaptic compartment, indicating a role for Aβ in synaptic function (Chen et al. 2017). Importantly, Aβ concentration is positively regulated by neuronal activity (Cirrito et al. 2005), and physiological concentrations of Aβ have been shown to enhance neurotransmitter release probability and long-term potentiation (LTP) (Puzzo et al. 2011; Abramov et al. 2009). Evidence from animal models supports the importance of Aβ in synaptic function and cognition. APP knockout mouse models display impaired LTP and cognitive deficits (Dawson et al. 1999). Additionally, studies involving in vivo administration of anti-Aβ antibodies caused disturbances in short-term memory related to contextual fear conditioning, while the infusion of picomolar concentrations of Aβ was able to prevent the memory impairment (Puzzo et al. 2011). Aβ peptides also promote the survival of primary neurons (Whitson, Selkoe, and Cotman 1989), and hippocampal neurons from APP knockout mice exhibit reduced synapse formation, aberrant synaptic network organisation, and decreased levels of synaptic proteins (Southam et al. 2018; Seabrook et al. 1999). sAPP also plays important roles in various physiological functions, including modulation of synaptic transmission and plasticity (Rice et al. 2019), neuronal growth, and neuroprotection (Chasseigneaux and Allinquant 2012).

Evidence from animal studies have also shown that Aβ displays antimicrobial properties (Moir, Lathe, and Tanzi 2018). Aβ can capture and perforate the membranes of microbes, suggesting that it may limit various types of infections in cells (Soscia et al. 2010). Furthermore, Aβ is linked to

the integrity of the blood-brain barrier, and studies have demonstrated that Aβ may help to seal leaks in the barrier (Atwood et al. 2003). Clinical trials targeting Aβ have provided support for this observation, as trials have reported side effects of brain oedema and/or micro-haemorrhages following Aβ reduction (Penninkilampi, Brothers, and Eslick 2017). The occurrence of these adverse effects suggests that modulation of Aβ levels may disrupt blood-brain barrier function and vascular integrity (Mann et al. 2018).

These studies suggest that while dysregulated Aβ production is associated with AD pathology, Aβ also serves important physiological functions in a range of physiological processes, highlighting the need for a better understanding of the function of Aβ in both health and disease.

1.1.3. Mechanisms of Aβ-induced toxicity

1.1.3.1. Synaptic alterations

Aβ oligomers are known to rapidly inhibit LTP, a critical mechanism of synaptic plasticity essential for learning and memory (Lambert et al. 1998). Aβ oligomers exert their neurotoxic effects by binding at synapses, particularly targeting excitatory synapses (Wilcox et al. 2011). Prior to synapse loss, Aβ induce changes in synaptic membranes, including the downregulation of surface glutamate NMDA and AMPA receptors (Wang et al. 2004). This reduction likely contributes to the impairment of LTP, although the exact mechanisms by which Aβ oligomers target neurons remains unclear. Studies have proposed that synaptic toxicity may be initiated by Aβ oligomers binding to specific sites on the surface of neurons (Viola and Klein 2015). This suggestion has led to the identification of several receptors for Aβ oligomers. These receptors may include various cell surface proteins that interact with Aβ oligomers and mediate their toxic effects on synaptic function and neuronal viability (Mroczko et al. 2018). Aβ oligomers modulate both pre- and postsynaptic structures (Mucke and Selkoe, 2012).

Over the years, numerous studies have investigated the effects of Aβ on postsynaptic function, revealing significant impacts on synaptic transmission and plasticity (Mucke and Selkoe 2012). It is well-established that Aβ production is influenced by action potential-dependent synaptic activity, leading to elevated levels of extracellular Aβ and alterations in postsynaptic function (Cirrito et al. 2005). Several mechanisms have been proposed as to how Aβ oligomers disrupt postsynaptic function. Some studies suggest that Aβ oligomers may indirectly impair postsynaptic function by partially blocking NMDARs, leading to induction of long-term depression (LTD) and synapse loss (Shankar et al. 2007). Other studies propose that Aβ affects synaptic function by activating extra-synaptic NMDARs and metabotropic glutamate receptors (mGluRs), initiating signalling cascades associated with LTD (Talantova et al. 2013; Li et al. 2009). Aβ oligomers are able to induce clustering of mGluR5 at synapses, further affecting mechanisms of synaptic plasticity (Renner et al. 2010).

Several lines of evidence have suggested that soluble Aβ oligomers bind to the extracellular cysteine-rich domain of the frizzled receptor, important for the Wnt signalling pathway that modulates synaptic and morphological plasticity (Magdesian et al. 2008). Aβ oligomers have also been proposed to bind to the p75 neurotrophin receptor and induce apoptosis, as it was observed that antibody-based inhibition of these receptors prevents cell death in the presence of high levels of Aβ oligomers (Yaar et al. 1997). Some evidence implies that the interaction between Aβ oligomers and the cellular prion protein (PrP^c) is necessary for many of the synaptotoxic effects, including suppression of LTP (Barry et al. 2011). Furthermore, a study has reported that PrP^c may participate in clustering Aβ oligomers with other receptors, permitting a greater response to low concentrations of Aβ (Laurén et al. 2009). Other studies have challenged the notion of PrP^c as a mediator of Aβ toxicity, reporting no change in impairment of synaptic plasticity with removal or overexpression of PrP^c in a transgenic model of AD (Calella et al. 2010).

Some of the studies that have focused predominantly on the effects of Aβ oligomers on presynaptic function have generated conflicting results, reporting either no change in neurotransmitter release probability (Li et al., 2009), proof of presynaptic enhancement (Abramov et al. 2009), or depression, featuring a specific inhibition of presynaptic Ca^{2+} currents (Nimmrich et al. 2008). However, there is significant evidence that Aβ acts as a positive regulator at the presynaptic level (Abramov et al. 2009), and studies have stated that Aβ may facilitate glutamatergic release in neurons, resulting from a build-up of Ca^{2+} in the presynaptic terminal (Palop and Mucke 2010). In the hippocampus, both P-/Q-type and N-type voltage-gated calcium channels (VGCCs) are involved in mediating presynaptic Ca^{2+} influx (Wheeler, Randall, and Tsien 1994). Aβ oligomers have been shown to directly increase the current through P/Q-type channels, leading to excitotoxic effects (Mezler et al. 2012). The elevated glutamate release probability induced by Aβ enhances NMDAR-dependent LTD, and consequently induces pathological tau hyperphosphorylation, a potential link connecting Aβ and LTD to tau pathology in AD (Taylor, Emptage, and Jeans 2021). Aβ can also interact with nicotinic acetylcholine receptors (nAChRs), particularly the α7-contaning nAChRs on presynaptic terminals (Snyder et al. 2005). Aβ is able to increase presynaptic Ca^{2+} levels via these receptors, and is linked to synaptic dysfunction associated with AD (Jeans et al., Unpublished).

Inhibitory neurons, also referred to as GABAergic neurons, release the neurotransmitter GABA, which is known to hyperpolarize postsynaptic neurons and cause inhibition of neuron firing (Wu and Sun 2015). Emerging evidence implies that GABAergic dysfunction might be important in the pathogenesis of AD, particularly in relation to network dysfunction (Busche et al. 2008). Initial evidence came from a study using a transgenic mouse model of AD, which showed that hyperactive neurons in cortical circuits are associated with decreased GABAergic inhibition rather than increased glutamatergic transmission (Busche et al. 2008). Mouse models of APOE4, the main genetic risk factor for AD, also present with significant GABAergic dysfunction and impaired

GABA release in the hippocampus (Li et al. 2009). Another report indicated that APP expression may regulate GABAergic function by modulating L-type Ca^{2+} channels expressed by GABAergic neurons, reducing Ca^{2+} currents in vivo, and therefore, weakening GABAergic neuron function (Yang et al. 2009). However, this may represent only one aspect of Aβ-mediated synaptotoxic effects as there is abundant evidence that implicates direct pro-excitatory effects on glutamatergic neurons as a mechanism underlying enhanced network activity (Mucke and Selkoe, 2012).

1.1.3.2. Synaptic Ca^{2+} signalling dysregulation

The calcium hypothesis of AD theorises that Aβ peptides disrupt neuronal Ca^{2+} homeostasis, leading to synaptic failure, spine loss, neural network dysfunction, and cognitive deficits (Khachaturian 1994). In vitro studies have also demonstrated that elevated Ca^{2+} levels can stimulate the formation and aggregation of Aβ into protofibrils, implicating Ca^{2+} signalling dysregulation as a potential cause of AD (Etcheberrigaray et al. 1998). Evidence suggests that Ca^{2+} alterations may be an early neuropathological event related to mild cognitive impairment in AD patients (Tong et al. 2018). Aβ has been linked to dysregulations in Ca^{2+}-dependent molecular cascades, LTP impairment, and memory deficits (Hermes, Eichhoff, and Garaschuk 2010). Furthermore, advancements in Ca^{2+} sensors and imaging have enabled evaluation of Ca^{2+} alterations in AD models, revealing a significant increase in basal Ca^{2+} levels in neurons surrounding amyloid deposits (Kuchibhotla et al. 2008). In studies that utilised the application of exogenous synthetic Aβ oligomers, it was shown that the acute application of Aβ dramatically elevated resting Ca^{2+} levels (Arbel-Ornath et al. 2017). Since MK-801, an NMDAR antagonist, was able to block the increase in Ca^{2+} following Aβ incubation, this study concluded that the Aβ-induced Ca^{2+} increase was mediated by NMDAR activation (Arbel-Ornath et al. 2017). Multiple mechanisms contribute to the Ca^{2+} elevation induced by Aβ oligomers, including glutamate release by astrocytes and the downregulation of excitatory amino acid transporters (EAAT-1 and 2), which results in excess

glutamate in the perisynaptic domain (Acosta, Anderson, and Anderson 2017). The elevated glutamate impacts NMDARs, and to a lesser extent, AMPARs, leading to increased Ca^{2+} influx and excitotoxicity (Alberdi et al. 2010). Furthermore, Aβ can interact and disrupt lipid membranes, creating perforations in the lipid bilayer to allow for Ca^{2+} influx (Demuro et al. 2005). It is currently unclear whether Aβ binds directly to glutamate receptors or if these receptors are indirectly affected by membrane tension induced by Aβ oligomers inserted in the lipid bilayer (Fani et al. 2021). Other studies have provided conflicting evidence on several Ca^{2+} channels presumed to be involved in mediating the effects of Aβ, such as VGCC and α7-nAChR (Ueda et al. 1997; Lasala et al. 2019; Fani et al. 2021).

Given the significance of neuroinflammation in AD, research has examined Ca^{2+} signalling in astrocytes as well. Astrocytes play a crucial role in safeguarding synaptic function, and emerging evidence suggests that the loss of synapses in AD may also reflect a functional decline in astrocytes (Hulshof et al. 2022). The transient receptor potential A1 (TRPA1) channels are specifically expressed in astrocytes and are absent from neurons within the hippocampus (Shigetomi et al. 2011). In a particular study, TRPA1 channels were shown to a play a minor role in astrocytic Ca^{2+} signalling, however, they are rapidly recruited in the presence of Aβ oligomers (Bosson et al. 2017). Astrocytic Ca^{2+} hyperactivity was shown to be dependent on TRPA1 channel activation by Aβ, and this may contribute to the hyperactivity observed in glutamatergic synapses (Bosson et al. 2017).

The excessive influx of Ca^{2+} through NMDARs and AMPARs triggered by Aβ oligomers can also lead to the disruption of intracellular Ca^{2+} stores. In one study, chronic exposure to Aβ oligomers resulted in the overactivation of glutamate receptors, leading to mitochondrial Ca^{2+} overload and subsequent mitochondrial damage, culminating in oxidative stress and cell death (Alberdi et al. 2010). The mitochondria, endoplasmic reticulum (ER) and lysosomes, all serve as intracellular Ca^{2+}

storage organelles and are affected in AD (Supnet and Bezprozvanny 2010). ER Ca^{2+} dysregulation is a prominent feature of AD pathophysiology, and enhanced Ca^{2+} release from the ER has been documented in many AD models (LaFerla 2002). Additionally, exaggerated ER Ca^{2+} release has been shown to increase Aβ production and phosphorylation of tau, due to Ca^{2+}-dependent activation of kinases (Pierrot et al. 2006). The ER is notably overfilled with Ca^{2+} in AD mouse models (Nelson et al. 2007), therefore, there is a reduction in store-operated Ca^{2+} entry, which functions to refill ER Ca^{2+} stores (Leissring et al. 2000). The downregulation of Ca^{2+} sensing proteins in AD models, which participate in store-operated Ca^{2+} entry, impact downstream signalling processes and gene transcription related to LTP expression (Sun et al. 2014). Mitochondrial functions rely on Ca^{2+} transport from the ER into the mitochondrial matrix, and elevated Ca^{2+} transfer can lead to suppression of normal mitochondrial function (Lin and Beal 2006). A sustained increase in mitochondrial Ca^{2+} levels suppresses ATP production and impairs cellular energy metabolism, which may affect essential neuronal processes such as neurotransmitter release and synaptic plasticity (Tong et al. 2018). The mitochondrial Ca^{2+} dysregulation also leads to elevated levels of reactive oxygen species (ROS), contributing to oxidative stress and apoptosis (Hidalgo and Arias-Cavieres 2016). Deficits in the autophagy pathway in AD are closely related to lysosomal Ca^{2+} dysregulation (Pickford et al. 2008). Lysosomal Ca^{2+} efflux is increased in models of AD, reducing lysosomal Ca^{2+} content (Coen et al. 2012). In a study using PS1-deficient mice, the optimal acidic pH required for lysosomal function was disrupted, shifting towards a more alkaline environment (Lee et al. 2010). The alteration in pH may also disrupt lysosomal Ca^{2+} storage and other essential lysosomal functions (McBrayer and Nixon 2013).

Therapeutic strategies targeting abnormal Ca^{2+} levels are currently under investigation in animal studies, with some showing potential for improving AD symptoms (Tong et al. 2018). For example, nilvadipine, an L-type VGCC blocker used primarily for hypertension treatment, has shown therapeutic potential in preclinical studies with its ability to decrease Aβ levels (Kennelly et

al. 2011). Additionally, hyperforin, a compound that activates transient receptor potential cation channel 6 (TRPC6), a Ca^{2+}-permeable channel that participates in store-operated Ca^{2+} entry (Leuner et al. 2007), has been shown to promote autophagic clearance of APP and improve memory in an AD mouse model (Inestrosa et al. 2011). While targeting Ca^{2+} channels or handling proteins may hold therapeutic potential for AD, it's important to consider that Ca^{2+} is an essential second messenger involved in numerous cellular functions, therefore, inhibition of these proteins may have adverse effects on normal neuronal function, possibly leading to unwanted side effects or neurotoxicity. It is crucial to develop therapeutics that selectively regulate Ca^{2+}-signalling pathways dysregulated in AD pathology, such as state-dependent antagonists, without disrupting normal Ca^{2+} homeostasis (Nimmrich and Eckert 2013).

1.1.3.3. Synaptic and structural plasticity impairments

AD pathogenesis involves a loss of synaptic plasticity, which is the process by which synapses dynamically regulate their strength, forming new connections with other neurons and reshaping neural circuits (Parihar and Brewer 2010). Synaptic plasticity encompasses mechanisms such as LTP and LTD, and these mechanism are believed to underlie learning and memory by enabling the encoding and storage of information in neural networks (Sheng and Kim 2002). Synapse loss is one of the strongest correlates of the degree of cognitive decline in AD (Terry et al. 1991). This loss is accompanied by the degeneration of dendritic spines, which are key structures involved in excitatory synaptic transmission (Moolman et al. 2004). Several groups have provided evidence that Aβ causes loss of dendritic spines, and the accumulation of Aβ in the vicinity of amyloid plaques has been correlated to the loss of synapses (Jacobsen et al. 2006; Spires et al. 2005). While it's clear that accumulation of Aβ plays a central role in synaptic loss, the mechanisms by which it leads to synaptic dysfunction remain poorly understood. Soluble Aβ oligomers can disrupt synaptic morphology and impair LTP by their actions on synaptic receptors and Ca^{2+} signalling, as

previously mentioned. Aβ oligomers can downregulate NMDARs, by increasing their endocytosis (Snyder et al. 2005), and activate extra-synaptic NMDARs, which are linked to excitotoxicity and synaptic weakening, leading to the inhibition of LTP (Li et al. 2011). The activation of calcineurin by Aβ also inhibits NMDAR function and contributes to LTP impairment (Zhang et al. 2022). In addition, Aβ oligomers can rapidly activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and this aberrant activation leads to the destabilisation of AMPARs at synapses, contributing to the weakening of excitatory synapses (Zhang et al. 2022). Dysregulated Ca^{2+} signalling can affect Ca^{2+}-dependent proteases and kinases in AD and destabilise the cytoskeleton, contributing to synapse loss (Penzes and VanLeeuwen 2011). Postsynaptic density protein 95 (PSD-95), a scaffolding protein that plays a critical role in anchoring and stabilising glutamate receptors at the postsynaptic density, has also been reduced by Aβ, disrupting the molecular components essential for maintaining synaptic integrity (Roselli et al. 2005).

Aβ oligomers may additionally act on signalling pathways related to synaptic plasticity. Under physiological conditions, the Wnt signalling pathway causes the inactivation of glycogen synthase kinase-3β (GSK-3β), elevation of β-catenin levels, and transcription of Wnt target genes (Cerpa et al. 2011). These processes are essential for dendrite development, synaptic plasticity, and the regulation of glutamate receptor trafficking (Beaumont et al. 2007). Aβ oligomers disrupt Wnt signalling in AD, leading to impaired Wnt target gene transcription and decreased levels of β-catenin, resulting in synaptic dysfunction (Chia Chen Liu et al. 2014; Magdesian et al. 2008). It is plausible that Aβ-induced synaptic changes may be crucial contributors to early alterations in AD, while tau pathology becomes more predominant as the disease progresses, as it has been shown that pathogenic tau can exacerbate synaptic degeneration and cognitive impairment (Ittner and Götz 2011).

Gliosis, a characteristic feature of AD, can also contribute to synaptic dysfunction and neurodegeneration (Merluzzi et al. 2018). Aβ accumulation causes microglial chronic activation and recruitment, which can contribute to synapse loss (Floden, Li, and Combs 2005). Furthermore, increased accumulation of microglia surrounding hippocampal amyloid plaques may induce specific gene expression in microglia associated with phagocytic and degradative processes (Wood et al. 2022; Mallach et al. 2024).

Most studies have focused on extracellular Aβ, although, intracellular accumulation of Aβ also plays an important role in synaptic dysfunction (Ripoli et al. 2014). Studies have demonstrated that intracellular Aβ can contribute to tau hyperphosphorylation, diminished synaptic protein expression, and impaired organelle transport (Umeda et al. 2015). By preventing the transport of vital organelles and proteins, such as brain-derived neurotrophic factor (BDNF), Aβ impairs synaptic maintenance (Umeda et al. 2015). Furthermore, Aβ can disrupt mitochondrial function, inducing cytochrome c release, and activation of caspases, which is associated with dendritic spine collapse (Cai and Tammineni 2017).

The molecular mechanisms underlying synapse loss in AD are multifaceted and complex, involving interactions between Aβ and a wide range of neuronal mechanisms. An abundance of evidence has proposed that Aβ leads to Ca^{2+} level increases, which eventually causes synapse degeneration. However, the precise molecular mechanisms linking Aβ to synapse loss require further elucidation, as this process undoubtedly involves numerous other neuronal molecular components operating within similar pathways.

1.2. Neuronal Ca^{2+} stores and signalling

1.2.1. Ca^{2+} influx and signalling in neurons

Ca^{2+} serves as a universal second messenger that plays a central role in regulating a range of cellular processes in eukaryotic cells (Carafoli and Krebs 2016). In neurons, Ca^{2+} signalling is particularly significant due to its involvement in various aspects of neuronal function, including synaptic transmission, plasticity and survival (Brini et al. 2014). Neurons have developed elaborate Ca^{2+} signalling pathways to synchronise the Ca^{2+} signal to biochemical machinery (Brini et al. 2014). The maintenance of low basal levels of Ca^{2+} within neurons is crucial for ensuring that Ca^{2+} signalling remains dynamic and receptive to extracellular stimuli. The concentration of intracellular free Ca^{2+} in neurons is around 100 nM, while the extracellular Ca^{2+} concentration is typically 1.2 mM (Gleichmann and Mattson 2011).

Plasma membrane Ca^{2+} channels are separated into three groups: VGCCs, receptor-operated Ca^{2+} channels, and store-operated Ca^{2+} entry channels, which will be discussed in the section describing ER Ca^{2+} signalling. VGCCs are important for transducing electrical signals at the plasma membrane into intracellular Ca^{2+} influx (Brini et al. 2014). These channels are activated in response to changes in membrane potential, allowing Ca^{2+} to enter neurons when the membrane potential becomes depolarised (Catterall 2011). VGCCs are formed of five specific subunits and divided into groups and classes, based on the physiological properties and the specific toxins that inhibit the channels (Catterall 2011). For example, L-type VGCCs mediate long-lasting Ca^{2+} currents, and are involved in generating Ca^{2+} transients in cell bodies and dendrites (Morton et al. 2013). These Ca^{2+} transients control processes such as synaptic plasticity and gene expression (Turner, Anderson, and Zamponi 2011). The N-, P/Q-, and R-type VGCCs are mainly responsible for initiation of synaptic transmission, as they regulate neurotransmitter release by controlling Ca^{2+} influx into the presynaptic terminal (Catterall 2011). Receptor operated Ca^{2+} channels are activated

by the binding of particular ligands, such as glutamate, the principal excitatory neurotransmitter in the brain (Brini et al. 2014). Glutamate activates AMPARs, NMDARs and mGluRs. AMPARs facilitate rapid excitatory synaptic transmission and are permeable to Na^+, K^+, and Ca^{2+} when specific subunits are incorporated into AMPARs (Traynelis et al. 2010). NMDARs are permeable to Ca^{2+} and Na^+, and these receptors have a more gradual activation to glutamate binding compared to AMPARs (Traynelis et al. 2010). The activation of NMDARs requires both glutamate binding and membrane depolarisation to remove the internal Mg^{2+} that normally obstructs the channel (Blanke and VanDongen 2009). NMDARs are crucial in learning and memory mechanisms, such as gene expression during long-term memory formation (Blanke and VanDongen 2009). mGluRs are linked to G proteins and produce Ca^{2+} signals by activating specific downstream signalling pathways, including the activation of phospholipase C and modulation of adenylyl cyclase activity (Niswender and Conn 2010).

The plasma membrane Ca^{2+} ATPase (PMCA) and Na^+/Ca^{2+} exchanger are the two main systems involved in removing Ca^{2+} ions from the intracellular to extracellular environment (Brini et al. 2014). The Na^+/Ca^{2+} exchanger functions in removing Ca^{2+} by exploiting the electrochemical gradient of Na^+, and each time three Na^+ ions enter the cell, one Ca^{2+} ion is removed against its concentration gradient (Brini and Carafoli 2011). The PMCA pump utilises energy derived from ATP hydrolysis to transport Ca^{2+} against the concentration gradient and into the extracellular space (Brini et al. 2013). Numerous Ca^{2+} binding proteins are also expressed in neurons, such as Ca^{2+} buffers, which cooperate with Ca^{2+} clearance mechanisms and regulate the temporal and spatial properties of Ca^{2+} signals, and Ca^{2+} sensors that transform changes in Ca^{2+} levels into molecular signals (Brini et al. 2014). Certain proteins can act as both Ca^{2+} buffers and sensors, such as calmodulin, which chelates Ca^{2+}, and the conformational changes resulting from Ca^{2+} binding allow calmodulin to interact with target proteins involved in several processes including synaptic plasticity and gene transcription (Chin and Means 2000).

Ca^{2+} signals are essential for neuronal function and regulate a range of processes. Ca^{2+} is critically involved in neurotransmitter release; the influx of Ca^{2+} triggers the fusion of synaptic vesicles with the presynaptic membrane (Südhof 2012). This process involves proteins from the SNARE family; synaptotagmin functions as a Ca^{2+} sensor with two Ca^{2+} -binding domains, and can interact with other SNARE proteins, such as synaptobrevin and SNAP-25, to release neurotransmitter contents into the synaptic space (Sauvola and Littleton 2021). LTP and LTD are also highly dependent on Ca^{2+} signals, with the activation of several Ca^{2+}-dependent kinases and phosphatases important for learning and memory (Yang, Tang, and Zucker 1999). Ca^{2+} signals induced by synaptic activity are also able to spread to the nucleus and activate gene transcription pathways (Bading 2013). In the nucleus, Ca^{2+} signalling activates the transcription factor cAMP response element-binding protein (CREB) and CREB binding protein (CBP), leading to the transcriptional activation of genes essential for neuronal survival and memory consolidation (Brini et al. 2014).

1.2.2. Endoplasmic reticulum (ER)

In the early 1970s, the important role of the ER in the regulation of muscle contraction and the concepts of calcium-induced calcium release (CICR) became accepted (Ebashi and Endo 1968). Following these initial studies, the ER became established as an intracellular Ca^{2+} store, considerably involved in neuronal signalling (Karagas and Venkatachalam 2019). The ER is considered to be the largest intracellular organelle, characterised by a complex network of microtubules and cisternae (Terasaki et al. 1994). In neurons, the ER extends throughout the neuronal structure as a continuous membrane system (Terasaki et al. 1994). The ER is commonly known as the site of protein and lipid synthesis, in addition, the ER Ca^{2+} store is tightly linked to intracellular Ca^{2+} levels (Karagas and Venkatachalam 2019). The Ca^{2+} release channels found on the ER membrane are ryanodine receptors (RyRs) and inositol triphosphate receptors (IP_3Rs) (Furuichi et al. 1989; Takeshima et al. 1989). The earliest observations of Ca^{2+} release from

intracellular stores were attributed to the discovery that caffeine can activate RyRs and affects neuronal Ca^{2+} concentration (Rousseau et al. 1988). RyRs are activated by Ca^{2+} and the second messenger, cyclic ADP ribose (cADPR) (Mészáros, Bak, and Chu 1993), and the expression of these receptors has been observed particularly in dendritic spines of hippocampal neurons (Miyazaki and Ross 2013). IP_3Rs are sensitive to cytosolic Ca^{2+} and inositol triphosphate (IP_3) to induce Ca^{2+} release through these receptors (Woll and Van Petegem 2022). To transport Ca^{2+} from the cytosol back into the ER, the sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase (SERCA) uses energy derived from ATP hydrolysis to move Ca^{2+} against its concentration gradient (Lytton et al. 1992). The ER also contains several Ca^{2+} buffer proteins, such as calreticulin, which functions as a low-affinity Ca^{2+} buffer and regulates SERCA activity (Verkhratsky 2005). Furthermore, store-operated Ca^{2+} entry channels present on the plasma membrane are able to detect ER Ca^{2+} levels and are activated in response to depletion of ER Ca^{2+} as a way of refilling ER Ca^{2+} stores (Prakriya et al. 2006; Roos et al. 2005). This refilling mechanism is also able to regulate various processes, such as gene expression linked to the growth and plasticity of dendritic spines (Wegierski and Kuznicki 2018; Zhang et al. 2015).

ER Ca^{2+} release has been shown to be important in mediating crucial neuronal functions (Karagas and Venkatachalam 2019). The temporal dynamics of synaptic vesicle release are partially influenced by ER Ca^{2+} release (Chanaday and Kavalali 2022). Enhanced RyR-mediated Ca^{2+} release can promote spontaneous synaptic vesicle release and presynaptic facilitation (Lee et al. 2010; Emptage, Reid, and Fine 2001). The spatiotemporal patterns of intracellular Ca^{2+} signals have distinct impacts on neuronal gene expression (Karagas and Venkatachalam 2019). In the context of synaptic plasticity, an increase in nuclear Ca^{2+} levels developing from passive diffusion from the cytosol or through activation of IP_3Rs located in the nuclear envelope, stimulates CREB-dependent gene transcription (Chamero et al. 2008; Hardingham et al. 1997; Humbert et al. 1996). Synaptic plasticity mechanisms originate from synaptic neurotransmission at dendritic spines and

subsequently, CICR from the ER may contribute to the propagation of Ca^{2+} waves in neurons, resulting in the increased Ca^{2+} concentration in the nucleus (Figure 1.2) (Hardingham, Arnold, and Bading 2001; Carrasco et al. 2004).

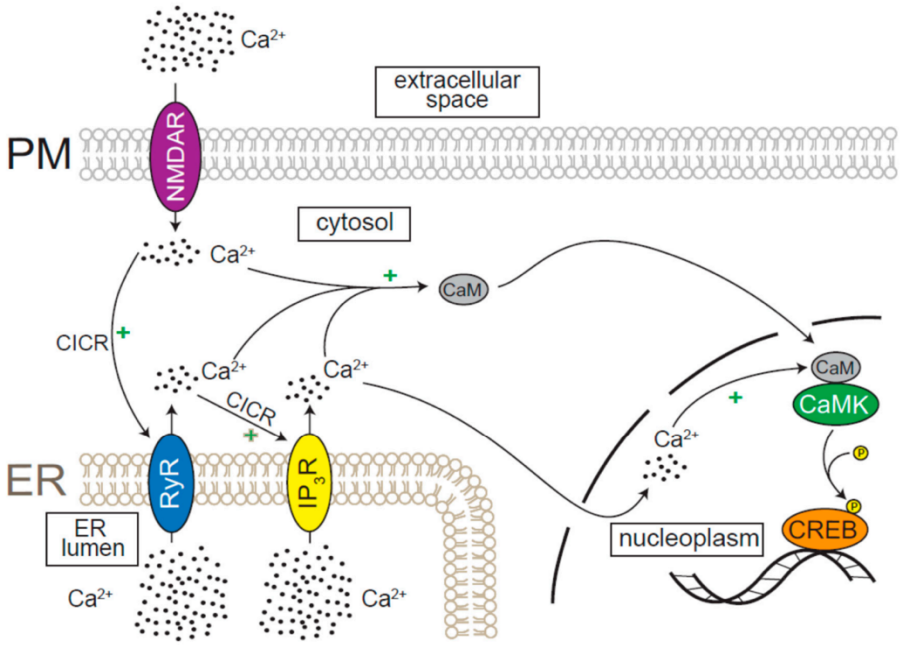

Figure 1.3: CICR activation of gene expression. In synaptic transmission, NMDARs are activated, allowing for Ca^{2+} influx, which is amplified by CICR via RyRs and IP_3Rs. Elevated intracellular Ca^{2+} levels activate calmodulin (CaM), which subsequently translocates to the nucleus to activate Ca^{2+}/CaM-dependent protein kinases (CaMK), which then phosphorylate CREB, allowing it to bind to DNA and induce gene transcription. Reproduced from (Karagas and Venkatachalam 2019).

ER Ca^{2+} signalling is intricately involved in neuronal function, however, further research is needed to elucidate the complex mechanisms by which ER Ca^{2+} dynamics contribute to physiological neuronal function as well as the role it plays in pathology.

1.2.3. Lysosomes

Lysosomes serve as the primary waste system of the cell, degrading and recycling macromolecules, and supporting the nutrient regulation of cells (Settembre et al. 2013). The organelles are characterised by an acidic environment and contain hydrolytic enzymes, capable of digesting a range of biological molecules (Saftig and Klumperman 2009). Lysosomes also function as an important Ca^{2+} signalling organelle, as they are the second largest store of intracellular Ca^{2+} (Settembre et al. 2013). In alkaline conditions, Ca^{2+} tends to precipitate with proteins, whereas in acidic environment, such as in lysosomes, reduced protein binding results in increased levels of free Ca^{2+}(Wang et al. 2002) .

The vATPase proton pump produces an appropriate proton gradient to promote an exchange of Ca^{2+} for H^+ with the Ca^{2+}/H^+ exchanger on lysosomal membranes to refill the Ca^{2+} store (Pittman 2011). Additionally, P-type Ca^{2+} ATPases are also present for Ca^{2+} uptake using an energy-dependent mechanism (Pittman 2011). Studies have also provided evidence that the ER may be involved in lysosomal Ca^{2+} store filling (Garrity et al. 2016). Contact sites between the ER and lysosomes still need to be fully characterised, although, they have been implicated in enhancing lysosomal Ca^{2+} release, leading to global Ca^{2+} release, and in supplying Ca^{2+} to replenish the lysosomal Ca^{2+} store (Morgan et al. 2013). Ca^{2+} concentration dynamics in the vicinity of lysosomes control lysosomal transport and fusion events, and it is evident that preserving physiological Ca^{2+} homeostasis within lysosomes is necessary for the normal function of the endolysosomal system, which is involved in trafficking and degradation in neurons (Lloyd-Evans and Waller-Evans 2020; Pryor et al. 2000). Many channels have been identified related to Ca^{2+} release from lysosomes, such as two-pore channels (TPC1/TPC2) and transient receptor potential cation channels of the mucolipin 1 family (TRPML1) (Di Paola, Scotto-Rosato, and Medina 2018; Ruas et al. 2015). Lysosomal Ca^{2+} release requires the second messenger nicotinic acid adenine dinucleotide

phosphate (NAADP), produced by ADP-ribosyl cyclases (Lee 2012). NAADP activates TPCs, presumably by interacting with an NAADP-associated binding protein (Pitt et al. 2010).

TRPML1 demonstrates permeability to Ca^{2+}, K^{+}, and Na^{+}, and is activated and inhibited by a group of signalling lipids known as phosphatidylinositols (Waller-Evans and Lloyd-Evans 2015). TRPML1 functions in regulating autophagy, which involves the engulfment of defective organelles and macromolecules by autophagosomes that firstly fuse with endosomes and then lysosomes for degradation of their contents (Di Paola, Scotto-Rosato, and Medina 2018). Autophagy is crucial for maintaining cellular quality control and enables the cell to utilise itself as a source of nutrients (Onyenwoke et al. 2015). TRPML1 can also interact with regulators of autophagy, including the mammalian target of rapamycin complex 1 (mTORC1) and transcription factor EB (TFEB) (Di Paola, Scotto-Rosato, and Medina 2018). Under basal conditions, mTORC1 acts as a lysosomal membrane amino acid sensor and suppresses autophagy (Lloyd-Evans and Waller-Evans 2020). Ca^{2+} release from TRPML1 is involved in mTORC1 activation, promoting biosynthesis of new cellular materials (Li et al. 2016). During starvation conditions, mTORC1 is inhibited and autophagy is induced to replenish the nutrient supply (Wang et al. 2015). TRPML1 activity is also essential for the translocation of TFEB to the nucleus during autophagy (Medina et al. 2015). This process is regulated by calcineurin that is activated by lysosomal Ca^{2+} release, leading to the dephosphorylation of TFEB, which allows for the nuclear translocation of TFEB (Palmieri et al. 2011). TFEB acts as a master regulator of lysosomal and autophagy related genes, promoting lysosome biogenesis and autophagy induction (Palmieri et al. 2011). Lysosomal exocytosis is also impacted by the activation of TFEB, which transports lysosomes closer to the plasma membrane and enhances lysosomal Ca^{2+} release, necessary for lysosomal fusion with the plasma membrane, by upregulating TRPML1 (Medina et al. 2011).

Research investigating lysosomal Ca^{2+} release and storage has expanded in recent years, particularly concerning its implications in human disease (Cao et al. 2021). Studies have continued to uncover new functions of lysosomes in neurons, such as regulating long-term structural plasticity, which expand beyond the traditional functions previously attributed to lysosomes (Padamsey et al. 2017). Although, there are still important questions to be addressed regarding the interaction of lysosomes with other intracellular Ca^{2+} stores, such as the ER, and the impact of lysosomes on intracellular Ca^{2+} signalling mechanisms in neurons.

1.3. Synaptic plasticity

1.3.1. Synaptic plasticity, learning and memory

The concept of synaptic plasticity, as proposed by Santiago Ramón y Cajal and later elaborated by Donald Hebb, has significantly shaped our understanding of how the brain encodes and stores information through activity-dependent modifications of synaptic strength (Cajal 1909; Hebb 1949). Synaptic plasticity refers to the changes in synaptic transmission strength at existing synapses, which encode transient experiences into lasting memory traces in neural circuits (Citri and Malenka 2007). Modifications of the synaptic strengths within a circuit underlie the encoding and storage of new information (Pastalkova et al. 2006). Many forms of synaptic plasticity have been observed, and these have often been separated into presynaptic and postsynaptic plasticity mechanisms (Citri and Malenka 2007). However, literature has also provided evidence that plasticity mechanisms can result as a combination of both presynaptic and postsynaptic alterations (Costa et al. 2015). Cajal was the first to suggest that neurons are interconnected and may communicate through synapses (Cajal 1909), while Hebb postulated that if two neurons fire simultaneously, the neuronal connections become strengthened (Hebb 1949).

1.3.1.1. Presynaptic plasticity

Short-term synaptic plasticity, which may last from milliseconds to several minutes, has been widely investigated at the presynaptic compartment (Zucker and Regehr 2002). When two stimuli are delivered in close succession, the response to the second stimulus can either be enhanced or depressed compared to the response from the first stimulus (Katz and Miledi 1968; Zucker and Regehr 2002). Paired-pulse depression likely results from the temporary depletion of the vesicle pool ready for release at presynaptic terminals (Debanne et al. 1996). During paired-pulse facilitation, residual Ca^{2+} from the first action potential contributes to additional release during the second stimulation (Debanne et al. 1996). The expression of paired-pulse depression or facilitation is influenced by the activation history of the synapse (Dobrunz and Stevens 1997). Synapses with initial high probability of transmitter release (P_r) exhibit depression in response to the second stimulation, while those with low initial P_r show an increase in release probability during the second stimulation (Dobrunz and Stevens 1997). Longer-lasting forms of plasticity occur after repetitive or tetanic stimulation of synapses with longer trains of high frequency stimulation, and are characterised by an enhancement of transmitter release in response to an action potential (Magleby and Zengel 1982; Zucker and Regehr 2002). The presynaptic terminal contains a range of different types of metabotropic G-protein-coupled receptors and ionotropic receptors (MacDermott, Role, and Siegelbaum 1999). P_r can also be controlled by receptor signalling pathways upon ligand binding (Miller 1998). Neuromodulators, such as dopamine, glutamate, nitric oxide, and BDNF, can also be released from the postsynaptic terminal and act as retrograde messengers to influence P_r (Kombian, Mouginot, and Pittman 1997; Nagappan and Lu 2005). Modulating the presynaptic proteins involved in synaptic vesicle exocytosis represents another key mechanism for enhancing neurotransmitter release (Citri and Malenka 2007).

In the brain, short-term synaptic plasticity can impact information processing at synapses, allowing them to act as filters (Citri and Malenka 2007). Synapses with a low P_r act as high-pass filters, facilitating during high-frequency stimulation and not transmitting low-frequency stimulation with the same efficiency (Abbott and Regehr 2004). Conversely, synapses with high P_r act as low-pass filters, and exhibit depression during high-frequency stimulation but effectively transmit low-frequency activity (Abbott and Regehr 2004). Short-term synaptic plasticity has been proposed to be involved in several cognitive functions, including sensory memory and attention (Deng and Klyachko 2011).

In response to LTP induction, presynaptic changes include increase in neurotransmitter release probability (Schulz 1997). It is now widely agreed upon that presynaptic and postsynaptic plasticity mechanisms are distinct and rely on different receptors and signalling pathways (Citri and Malenka 2007).

1.3.1.2. Postsynaptic plasticity

The most extensively researched forms of synaptic plasticity are LTP expressed in the CA1 region of the hippocampus, induced by the activation of NMDARs (Malenka 1991). In the 1970s, Bliss and Lomo demonstrated that repetitive activation of excitatory synapses in the hippocampus induces a potentiation of synaptic strength, commonly known as LTP (Bliss and Lømo 1973). LTP exhibits cooperativity, associativity, and input specificity (Nicoll, Kauer, and Malenka 1988). Cooperativity suggests that LTP can be triggered by the coincident activation of a sufficient number of synapses (Blitzer 2005). Associativity refers to its ability to strengthen a weak input when activated with a strong input (Blitzer 2005). Input specificity indicates that LTP occurs only at activated synapses, which allows for different synapses to encode specific information (Citri and Malenka 2007).

LTP in the CA1 region of the hippocampus requires the activation of NMDARs and influx of Ca^{2+} to initiate the biochemical mechanisms essential for LTP (Malenka and Nicoll 1993). The strong depolarisation of the postsynaptic terminal is initially mediated by AMPARs to remove the Mg^{2+} block of NMDARs, which then, upon glutamate binding, results in a large Ca^{2+} influx into the postsynaptic terminal, initiating downstream signalling pathways (Nowak et al. 1984). Ca^{2+} influx associated with LTP activates CaMKII and several other kinases, leading to the phosphorylation of AMPARs, increasing channel conductance associated with long-lasting synaptic potentiation (Barria et al. 1997; Benke et al. 1998). AMPARs are also transported to the plasma membrane to increase synaptic strength (Citri and Malenka 2007). LTD is characterised by low frequency stimulation and a decrease in Ca^{2+} influx, which activates calcineurin, a phosphatase that dephosphorylates AMPAR and causes endocytosis of synaptic AMPARs (Figure 1.3) (Cummings et al. 1996; Lisman 1989; Malinow and Malenka 2002). Another form of LTD, in many brain regions, is dependent on the activation of mGluRs (Anwyl 2006). This form of LTD also consists of AMPAR endocytosis, however, it is also reliant on mGluR-induced protein synthesis (Huber, Kayser, and Bear 2000; Snyder et al. 2001).

The threshold of neurons to LTP and LTD induction can change to favour one over the other (Citri and Malenka 2007). This invokes the idea of metaplasticity, wherein synapses reside on a spectrum according to their tendency to potentiate or depress in response to any given stimulus, their position on the spectrum being influenced by the history of activity at that synapse, as well as certain signalling pathways (Abraham and Bear 1996; Bear, Cooper, and Ebner 1987).

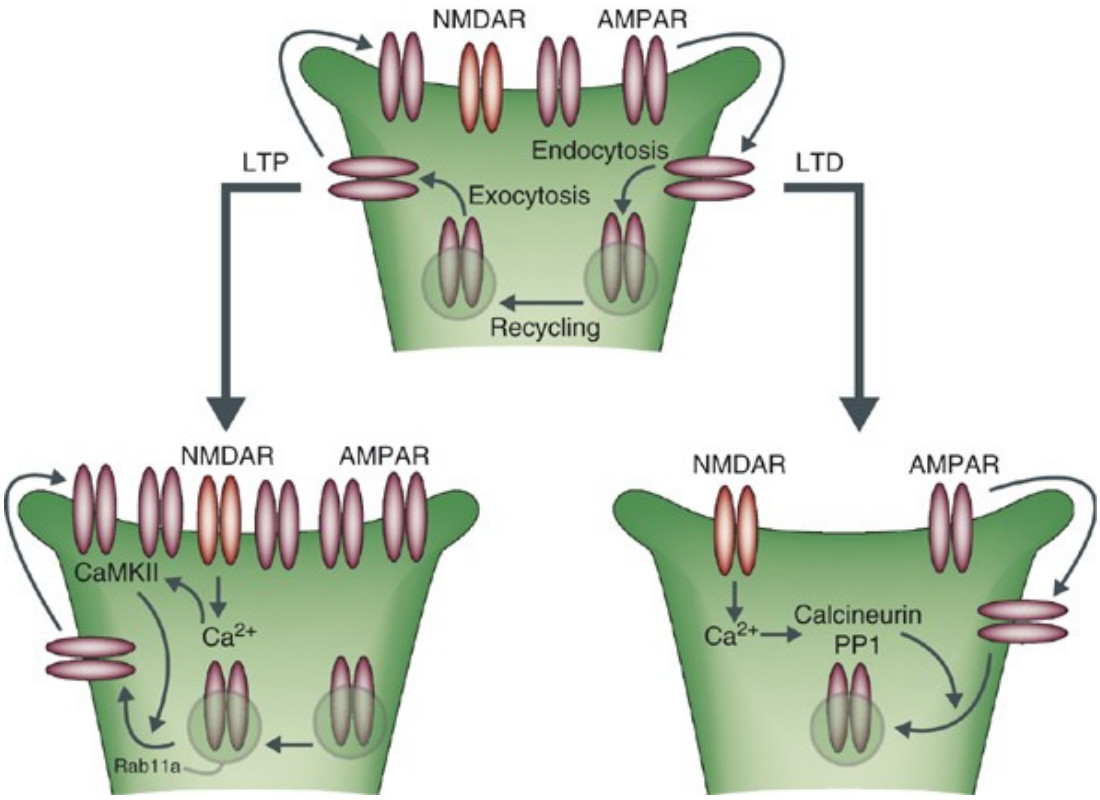

Figure 1.4: Model of LTP and LTD. In the basal state, receptors are cycled between being endocytosed, transferred to recycling endosomes and returned to the plasma membrane. In LTP, an increase in AMPAR exocytosis is observed that involves CaMKII and Rab11a, which regulates recycling endosomes. In LTD, enhanced endocytosis of AMPARs occurs in a process involving calcineurin and protein phosphatase 1 (PP1). Reproduced from (Citri and Malenka 2007).

1.3.1.3. Learning and memory

Numerous studies have been able to correlate synaptic plasticity with behavioural consequences (Martin, Grimwood, and Morris 2000). The hippocampus plays a major role in declarative memory, the ability to store and retrieve information (Squire, Stark, and Clark 2004). Animal studies have observed defective spatial learning following infusion of NMDAR antagonists into the hippocampus (Tsien, Huerta, and Tonegawa 1996; Morris and Frey 1997). Furthermore, electrophysiological recordings in vivo during an inhibitory avoidance task exhibited patterns of activity that were sufficient to induce LTP (Whitlock et al. 2006). In other studies, fear conditioning

has been shown to induce synaptic potentiation at synapses in the amygdala, with the insertion of AMPARs into the plasma membrane (Rumpel et al. 2005; McKernan and Shinnick-Gallagher 1997). Preventing the incorporation of AMPARs at synapses inhibited the associative memory linked to fear conditioning (Rumpel et al. 2005). Synaptic plasticity is also associated with drug addiction in the dopaminergic system (Citri and Malenka 2007). Drug-induced plasticity in dopamine circuits in the ventral tegmental area and nucleus accumbens may partially contribute to drug addiction (Hyman, Malenka, and Nestler 2006). Addictive drugs can utilise synaptic plasticity mechanisms underlying reward-based learning to produce an increase in synaptic strength at excitatory synapses in dopaminergic neurons (Ungless et al. 2001; Saal et al. 2003).

While LTP and LTD provide insight into how synaptic strength can be modulated by patterns of neuronal activity, establishing a direct link between different forms of synaptic plasticity and behavioural outcomes has been challenging (Mateos-Aparicio and Rodríguez-Moreno 2019). Behaviours underlying learning and memory are the result of complex interactions between multiple brain regions and neural circuits (McDonald, Devan, and Hong 2004). Furthermore, the link between synapses and memory storage still needs to be elucidated. Research into engram cells, neuronal ensembles activated during a specific memory, has advanced memory research (Tonegawa et al. 2015). It has been shown that optogenetically activating an ensemble of hippocampal neurons that contribute to a memory engram is sufficient for memory recall (Liu et al. 2012). Furthermore, the ablation of these neurons reduces memory expression (Liu et al. 2012). For a memory to persist and be retrieved, it must undergo a process of systems consolidation, which enhances synaptic connectivity in the engram cell network and the redistribution to the neocortex for long-term storage (Rao-Ruiz et al. 2021; Squire et al. 2015). It is currently unclear what the role of synapses is in relation to engram cells and the storage of memory. It has been suggested that individual synapses may not be able to store memories, however, synapses within

engram networks may be modulated to contribute to memory consolidation, storage and retrieval (Tonegawa et al. 2015).

1.3.2. Structural plasticity

In LTP, the incorporation of AMPARs into the postsynaptic membrane is accompanied by structural changes in the dendritic spines, small protrusions in neuronal dendrites associated with the postsynaptic component of excitatory synapses (Citri and Malenka 2007). To allow LTP to persist for long periods of time, the 'late phase' of LTP is characterised by local protein synthesis (Sutton and Schuman 2006). The signalling pathways required for maintaining the synaptic enhancement involve the activation of several protein kinases and transcription factors that promote the expression of effector genes encoding proteins involved in synaptic structure and function (Thomas and Huganir 2004; Zhou et al. 2006). The concept of a 'synaptic tag' provides an interesting basis for understanding the mechanisms underlying LTP maintenance at synapses (Frey and Morris 1997). This concept proposes that the synaptic activity that induces LTP creates a molecular tag at the potentiated synapse, which marks the synapse as undergoing activity-dependent plasticity, and allows it to capture plasticity-related proteins that contribute to promoting changes in synapse structure (Frey and Morris 1997). The identity of synaptic tags and the function of newly synthesised proteins delivered to dendritic spines are still being investigated (Reymann and Frey 2007; Young et al. 2006).

The structural remodeling of synapses is associated with LTP expression, and may be initiated by the exocytosis of AMPARs to the synapse (Lüscher et al. 2000). Enlargement of the postsynaptic density (PSD) is linked to actin cytoskeleton remodelling and the local synthesis of PSD proteins (Figure 1.4) (Abraham and Williams 2003; Yuste and Bonhoeffer 2001). CaMKII is also necessary for dendritic spine enlargement (Okamoto, Bosch, and Hayashi 2009). In response to the growth

of the PSD, the presynaptic active zone becomes enlarged to match the PSD, and this process involves direct protein interactions between the two synaptic compartments (Lisman and Harris 1993). Synaptic parameters, such as dendritic spine volume, are directly correlated to synaptic strength, and the use of glutamate uncaging at single spines has demonstrated specific and rapid spine growth, which is initially independent of protein synthesis (Matsuzaki et al. 2004). LTD is associated with the shrinkage of dendritic spines, which may reflect the loss of AMPARs (Zhou, Homma, and Poo 2004). The morphology of spines, therefore, is an accurate depiction of synapse function, making it relevant to examine spine volume changes during synaptic plasticity.

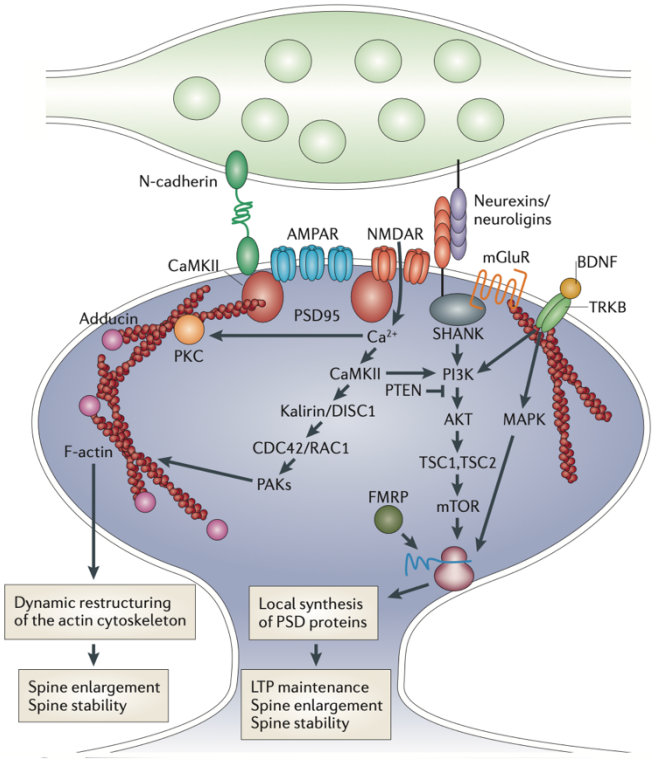

Figure 1.5: Structural plasticity mechanisms in the postsynaptic terminal. The induction of structural plasticity at dendritic spines involves rapid enlargement of the spine head and increased synaptic potentiation. Protein kinases, PKC and CaMKII, function in LTP maintenance, spine stability and enlargement. Local protein synthesis of PSD proteins also contributes to spine enlargement, and proteins regulating the actin cytoskeleton are also involved in this process. Additionally, adhesion molecules, such as N-cadherin and neurexins/neuroligins, interact with PSD proteins to assist in the dynamic restructuring of the dendritic spine. Reproduced from (Caroni, Donato, and Muller 2012).

1.3.3. The role of the ER and lysosomes in synaptic plasticity

The dynamic nature of the ER in synapses plays an important role in synaptic plasticity (Chanaday and Kavalali 2022). Only a subset of dendritic spines contain ER at a given time, however, the transport of ER into spines is positively regulated by neuronal activity (Harris 2020; Perez-Alvarez et al. 2020). The presence of ER within spines provides a source of intracellular Ca^{2+} that can regulate various signalling pathways related to synaptic plasticity (Chanaday and Kavalali 2022). The ER can also provide local translation of synaptic proteins in dendritic spines undergoing structural changes (Biever, Donlin-Asp, and Schuman 2019). In addition, the ER can become enlarged and form part of the spine apparatus in potentiated spines, which involves actin cytoskeleton remodelling and Ca^{2+} release (Chirillo et al. 2019; Spacek and Harris 1997).

It has previously been demonstrated that only ER-containing spines can undergo mGluR-dependent LTD, since the activation of mGluR triggers Ca^{2+} release through the production of IP_3 and activation of IP_3Rs on the ER (Holbro, Grunditz, and Oertner 2009). Large synaptic potentiation is able to induce LTP expression despite ER entry, although, the ER may function in limiting potentiation by exploiting a mGluR-dependent mechanism (Perez-Alvarez et al. 2020). ER entry into spines may also regulate Ca^{2+} dynamics through the release of ER Ca^{2+} or the uptake of Ca^{2+} by SERCA, contributing to structural plasticity mechanisms (Chanaday and Kavalali 2022). The ER is extensively present in axons and contributes to short-term plasticity mechanisms, as observed in the CA3-CA1 synapse (Singh et al. 2021). Ca^{2+} uptake by SERCA reduces the overall availability of SERCA binding sites and diminishes its buffering capacity, leading to elevated levels of cytosolic Ca^{2+}, causing a lower P_r and resulting in high facilitation; IP_3Rs and RyRs are involved to a lesser extent in this form of presynaptic facilitation (Singh et al. 2021).

Lysosomes have also been studied in the context of synaptic plasticity, and have been shown to function in dendritic spines (Goo et al. 2017). Lysosomal trafficking is influenced by neuronal activity to enable local protein degradation, specifically of AMPAR, important for homeostatic downscaling (Schwarz, Hall, and Patrick 2010; Rodrigues et al. 2016). Lysosomes are strategically located at dendritic spines in response to activity to facilitate synaptic remodelling through protein degradation (Goo et al. 2017). Another study demonstrated that lysosomes in dendritic spines enhance NMDAR-dependent LTP through lysosomal Ca^{2+} release and promoting ER entry into spines (Chen et al. 2023). Lysosomal fusion with the plasma membrane is also essential for maintaining long-term dendritic spine growth (Padamsey et al. 2017). Lysosomal exocytosis results in the release of Cathepsin B, which increases the activity of matrix metalloproteinase 9 (MMP-9), an enzyme involved in extracellular matrix remodelling that allows for the expansion of dendritic spines undergoing structural plasticity (Padamsey et al. 2017). In contrast, inhibiting lysosomal Ca^{2+} release causes changes in spine morphology characteristic of LTD (Padamsey, McGuinness, and Emptage 2017).

Lysosomes observed in presynaptic compartments serve as an important Ca^{2+} signalling store (Sambri et al. 2017). In one study, disrupting the structural integrity of lysosomes and the resulting focal elevation of Ca^{2+} produced an increased frequency of spontaneous neurotransmitter release, suggesting that lysosomes contribute to the action potential-evoked Ca^{2+} signal in hippocampal boutons (McGuinness, Bardo, and Emptage 2007). Interestingly, inhibiting the ER eliminated the lysosomal contribution to activity-evoked Ca^{2+} transients (McGuinness, Bardo, and Emptage 2007), suggesting that these organelles often act in concert.

1.4. Models of AD

1.4.1. Application of exogenous synthetic Aβ oligomers

Aβ peptides were first isolated from the brains of AD patients in the 1980s (Glenner and Wong 1984). It became evident that Aβ has the capacity to aggregate and form insoluble amyloid plaques in the brain (Burdick et al. 1992). Early attempts to model Aβ toxicity in vitro demonstrated that soluble Aβ monomers did not induce neurotoxicity, however, as the incubation time increased to favour peptide aggregation, the neurotoxic species was produced (Pike, Cummings, and Cotman 1992; Pike et al. 1991). The toxic species of Aβ include oligomers, protofibrils, and fibrils, with oligomers being considered the primary and most potent neurotoxic species (Mucke and Selkoe 2012). Initial studies demonstrated that endogenous and synthetic Aβ produce specific forms of oligomers, which can also be detected in the brains of AD patients (Podlisny et al. 1995). These studies challenged the view that plaques are the pathogenic species of AD by providing strong evidence of the neurotoxic effects of Aβ oligomers (Fontana et al. 2020). Currently, it is accepted that both endogenously produced and synthetic preparations of Aβ oligomers are neurotoxic in animal models and in vitro cell cultures (Fontana et al. 2020).

Aβ oligomers can be isolated from cells or prepared from synthetic Aβ, with the most commonly used synthetic Aβ consisting of 40 or 42 amino acids, reflecting the most abundant peptide species in AD pathology (Pike et al. 1993). Aβ rapidly aggregates in an aqueous medium, and eventually, the level of monomers decreases over time and oligomers are formed (Nag et al. 2011). Aβ applied in vitro in cell cultures can interact either directly or indirectly with several neuronal receptors, as previously described. These interactions stimulate downstream signalling pathways that lead to a wide range of neurotoxic effects such as neuroinflammation, oxidative stress, and disruption of synaptic plasticity processes (Fontana et al. 2020). Synthetic Aβ oligomers may also initiate a

positive feedback response, increasing Aβ release and facilitating neuronal dysfunction (Tabner et al. 2005).

Many studies typically use synthetic Aβ oligomers applied to hippocampal or cortical neuronal cultures, the most commonly used in vitro model for studying AD-related neuronal effects in the brain regions typically impacted by amyloidosis (Calvo-Rodríguez et al. 2017; Fontana et al. 2020). This in vitro model has offered important insights into neuronal signalling pathways and downstream mechanisms relevant to AD pathology. Aβ oligomers induce neurotoxicity at the presynaptic and postsynaptic compartments, and initiate impairments in hippocampal synaptic plasticity, such as the inhibition of LTP, associated with early events in AD (Lambert et al. 1998). Furthermore, intracerebroventricular injections of synthetic Aβ oligomers in mice impair consolidation of long-term recognition memory (Balducci et al. 2010). Aβ oligomers also contribute to synapse loss in primary neuronal cultures and ex vivo cortical human slices (Sebollela et al. 2012; Umeda et al. 2015). It is believed that Aβ can impair synaptic integrity through receptor interaction and the reduction of markers of synaptic density, such as synaptophysin (Liu et al. 2010). Aβ oligomers have been described as synaptotoxins, leading to synapse elimination and reduced formation of new synapses (Vargas et al. 2014; Izzo et al. 2014). In addition, Aβ oligomers can be internalised into neurons and affect intracellular organelles, disrupting axonal transport and synaptic plasticity (LaFerla, Green, and Oddo 2007; Poon et al. 2011).

While these synthetic Aβ oligomers have provided significant insights into the mechanisms underlying AD, they also have several limitations. Firstly, natural oligomers exhibit heterogeneity in size and structure, and may significantly differ from synthetic oligomers (Varshavskaya et al. 2022). The administration, concentration and timing of synthetic oligomers in vitro can influence observed outcomes, as synthetic oligomers may not interact with other molecules in the same way as natural oligomers. Furthermore, most studies using synthetic oligomers in cell culture models

are short-term and neglect the complex interactions with other pathological features of AD, such as neuroinflammation (Akhtar et al. 2022).

It is clear that synthetic Aβ oligomers applied to in vitro models are suitable for expanding our understanding of the cellular mechanisms associated with the early stages of AD pathology, and may be useful for the development of therapeutics for earlier stages of disease.

1.4.2. Transgenic mouse models

Animal models of AD are crucial for unravelling molecular disease mechanisms and advancing preclinical research. In AD research, transgenic mouse models have been used extensively, and these models primarily overexpress mutated proteins associated with FAD, such as APP and presenilin (Sasaguri et al. 2017).

Several transgenic mouse models have been developed over the years, starting with the first generation AD mouse models, which overexpress human APP with or without FAD mutations, using different promoters, such as platelet-derived growth factor-β (PDGF-β), prion protein, and Thy1 (Hsiao et al. 1996; Games et al. 1995; Mucke et al. 2000). The first generation mouse models often feature multiple mutations in the APP gene, with the Swedish mutation being the most prevalent, leading to the increased production of total Aβ from APP (Citron et al. 1992). These mice develop extracellular Aβ deposits and exhibit cognitive dysfunction before amyloid plaques appear (Sasaguri et al. 2017). In some models APP mutations are overexpressed in combination with presenilin mutations to induce Aβ pathology (Holcomb et al. 1998; Oakley et al. 2006). Additionally, crossbreeding of transgenic tau mice with APP mutant mice can induce the formation of Aβ plaques and NFTs (Lewis et al. 2001), although, the mutations in the MAPT gene

are not related to AD pathogenesis but are more commonly associated with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Sasaguri et al. 2017).

The J20 mouse model, used in this thesis, overexpresses human APP with Indiana and Swedish mutations, and expression is driven by PDGF-β (Mucke et al. 2000). The Swedish mutation is immediately adjacent to the β-secretase site in APP and increases Aβ levels by facilitating APP cleavage by BACE-1 (Barman, Schürer, and Prabhakar 2011). The mutation also increases overall production of Aβ (Scheuner et al. 1996). The Indiana mutation at the γ-secretase cleavage site in APP increases Aβ42 production (Suzuki et al. 1994). The J20 transgene inserts within the genetic locus of the endogenous mouse gene Zbtb20 on chromosome 16, involved in hippocampal cell differentiation (Fisher et al. 2018). Transgene integration disrupts mRNA expression of Zbtb20 without altering protein levels, although further study is necessary (Fisher et al. 2018). J20 mice develop Aβ deposition and display AD-related phenotypes, such as synaptic loss, cognitive impairment, and gliosis (Wright et al. 2013).

The main limitation of first generation mice is that APP overexpression may contribute to potential artifacts due to the overproduction of APP fragments associated with enzymatic processing of APP to produce Aβ (Nhan, Chiang, and Koo 2015; Gunawardena and Goldstein 2001). The APP overexpression could potentially induce abnormalities before Aβ pathology is expressed (Sasaguri et al. 2017). In these models, Aβ pathology emerges significantly earlier than the onset of disease in humans, specifically in sporadic AD, suggesting that some phenotypes in AD mouse models may not reflect normal disease pathology (Foley et al. 2015).

The second generation of mouse models feature an APP knock-in strategy to produce Aβ without the need for APP overexpression (Saito et al. 2014). In these models, the native mouse Aβ sequence was humanised and two FAD mutations were introduced into the endogenous mouse

APP gene, the Swedish and Beyreuther/Iberian mutations, which significantly increases the Aβ42/Aβ40 ratio by a factor of 30 (Guerreiro et al. 2010; Lichtenthaler et al. 1999). APP knock-in mice with a third mutation, the Arctic mutation, were created to enhance the oligomerisation of Aβ (Gessel et al. 2012). These mice develop AD pathology and cognitive deficits faster and to a greater extent compared with previous APP knock-in models (Sasaguri et al. 2017). Several AD-associated features are observed in these mice, including amyloid plaques, synaptic loss, gliosis, and cognitive impairment (Saito et al. 2014). Studies comparing first generation mouse models with the knock-in mice have reported various inconsistencies, which may not accurately represent AD pathology and could be due to overexpression artifacts (Saito et al. 2016). A limitation of these models is the absence of tau pathology, which prevents the model from recapitulating all features of AD pathology. Furthermore, the multiple mutations in the APP gene may interact and produce an aggressive form of AD, which does not accurately reflect clinical AD (Sasaguri et al. 2017).

Mouse models of AD have greatly contributed to our understanding of AD pathology and the preclinical development of treatments. Although, it is important to acknowledge inherent differences between rodents and humans, which may restrict the potential of these models for AD (Molnár and Clowry 2012). The central proteins in AD, such as Aβ and tau, exhibit differences in terms of their sequences, pathogenic properties, and expressed isoforms (Kobro-Flatmoen, Hormann, and Gouras 2023; Hernández et al. 2020). Additionally, variations in the immune systems of the brain, such as microglial phenotypes and genetic expression patterns of genes related to inflammation, are also different in rodents and humans (Franco Bocanegra, Nicoll, and Boche 2017). The development of induced pluripotent stem cells from AD patients has been identified as a potential solution to tackle species differences, by introducing stem cells into AD mouse models (Sullivan and Young-Pearse 2017; Espuny-Camacho et al. 2017).

1.5. The hippocampus as a model system

1.5.1. Organisation of the hippocampus

The hippocampus is a brain structure located in the temporal lobe and forms part of the limbic system in the brain (Tatu and Vuillier 2014). Studies of patient HM in 1955 significantly advanced our understanding of hippocampal functions related to memory (Squire 2009). Patient HM underwent a medial temporal lobectomy, including the removal of parts of the hippocampus, to alleviate severe epilepsy (Penfield and Milner 1958). Following the surgery, the patient experienced anterograde amnesia and was unable to form new memories (Penfield and Milner 1958). This case highlighted the role of the hippocampus in the formation of new declarative memories, and encouraged further research into the functions of the hippocampus (Squire 2009). Studies using animal models have corroborated the importance of the hippocampus in the formation of episodic, declarative, and spatial memory (Neves, Cooke, and Bliss 2008). In addition, hippocampus-dependent memory is thought to depend on hippocampal synaptic plasticity (Malenka 1994).

The hippocampus has three distinct subregions: the dentate gyrus, the hippocampus proper, which includes the cornu ammonis regions (CA1, CA2, and CA3), and the subiculum (Figure 1.5) (Chauhan et al. 2021). The trisynaptic circuit consists of three main interconnected regions, including the dentate gyrus, CA3, and CA1 regions (Mercer and Thomson 2017). The hippocampus receives its main input from layer II neurons of the entorhinal cortex, specifically the perforant pathway (Neves, Cooke, and Bliss 2008). This pathway carries sensory information, including spatial cues, and synapses onto granule cells of the dentate gyrus, which in turn project mossy fibres to CA3 pyramidal neurons (Knierim 2015). CA3 neurons project Schaffer collaterals to CA1 pyramidal neurons, essential for certain forms of synaptic plasticity, such as LTP (Szirmai, Buzsáki, and Kamondi 2012). CA1 pyramidal neurons form the primary output from the

hippocampus, and project to various regions of the brain, including the entorhinal cortex, prefrontal cortex and amygdala (Orsini et al. 2011; Van Strien, Cappaert, and Witter 2009). Outside of the trisynaptic pathway, there are additional connections that are important for hippocampal function. Associational fibres connect different regions within the hippocampus and run longitudinally along the hippocampus to facilitate communication between different subfields (Zeineh et al. 2017). These fibres are thought to play a role in the integration and processing of information within the hippocampus (Dalton, McCormick, and Maguire 2019). CA3 neurons also have local recurrent collaterals, which transmit excitation between pyramidal neurons (Le Duigou et al. 2014). Back projections from CA1 to CA3 have also been observed, and may serve to provide feedback and regulate the activity of CA3 neurons during the memory retrieval and consolidation process (Ji and Maren 2008). The dentate gyrus and hippocampus proper are also composed of different layers, with neuronal projections connecting with other neurons in specific layers (Neves, Cooke, and Bliss 2008). For example, mossy fibres sent from the dentate gyrus synapse to CA3 neurons in the stratum lucidum, while Schaffer collateral fibres projecting onto CA1 neurons are found in the stratum radiatum (Van Strien, Cappaert, and Witter 2009).

Although the hippocampus contains a variety of cell types, pyramidal neurons in the CA1 and CA3 regions have been the most extensively studied (Zeisel et al. 2015). The pyramidal neurons form the primary glutamatergic connections within the hippocampus (Slomianka et al. 2011). Interneurons, which are inhibitory neurons, modulate the activity of other neurons and contribute to the regulation of hippocampal network dynamics (Zeisel et al. 2015). GABAergic interneurons form local circuits within the subfields of the hippocampus and control the synchronisation of neuronal activity (Pelkey et al. 2017). Interneurons also participate in mediating hippocampal oscillations, such as gamma and theta rhythms, and function in the generation of spatial and temporal patterns of activity (Colgin 2016). The hippocampus also contains glial cells, which

function to support and maintain the neuronal environment within the hippocampus (Jäkel and Dimou 2017; Keyser and Pellmar 1994).

The Schaffer collateral is the most studied pathway in the hippocampus, often used in electrophysiology experiments (Bashir and Collingridge 1994; Lin et al. 2008; Meighan et al. 2007). The pathway formed by Schaffer collaterals is highly ordered, easily identifiable, and relatively straightforward, which makes it easy to stimulate and record from specific regions along the pathway (Kumar 2011). The Schaffer collaterals are fairly consistent across preparations and experimental conditions, making them reliable targets for studying synaptic function and plasticity (Neves, Cooke, and Bliss 2008). Studying these synapses provides insights into the neuronal mechanisms underlying memory processes (Malenka and Nicoll 1993).

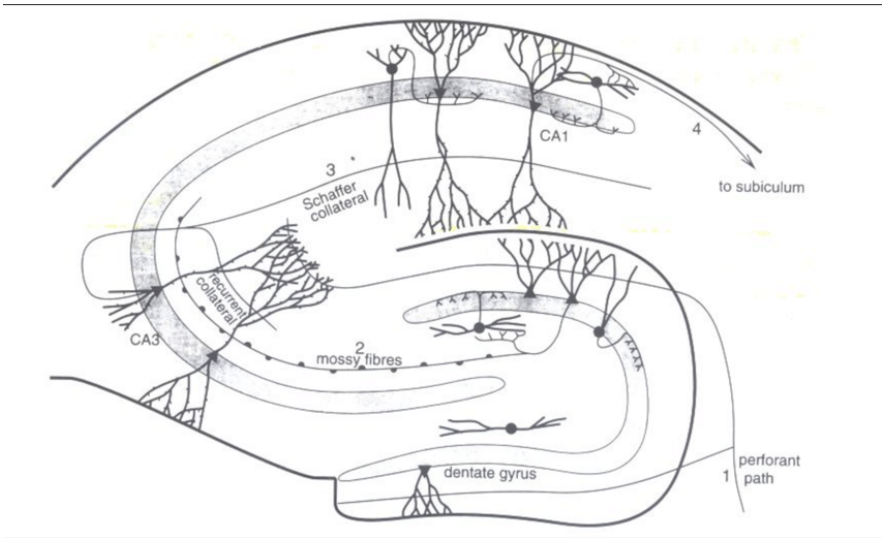

Figure 1.6: Representation of hippocampal connections. The entorhinal cortex projects to the dentate gyrus via the perforant path. Mossy fibres from the dentate gyrus project to CA3 pyramidal neurons. Schaffer collaterals from CA3 neurons project onto the dendrites of CA1 pyramidal neurons. CA1 neurons can send outputs to the subiculum. Reproduced from (Rolls and Treves 2012).

1.5.2. Dissociated primary hippocampal neurons

Dissociated hippocampal neurons are derived from rat or mouse embryos of very young postnatal animals (P1) (Peng, Xiong, and Mei 2013). This cell culture system offers a valuable approach to studying individual neurons and synapses (Mains and Patterson 1973). Dissociated hippocampal neurons can be cultured for extended time periods, allowing for long-term studies of neuronal development, synaptic function, and plasticity (Kaech and Banker 2007). These neurons are also ideal for experimental manipulation, as they are cultured under control conditions and various substances may be added to the basal culture media (Peng, Xiong, and Mei 2013). The main limitation of these cultures is that dissociated neurons do not fully recapitulate the physiological conditions and functions of neurons in vivo, as these neurons lack the organisation and connectivity observed in hippocampal slices, which are crucial for in vivo functions (Croft and Noble 2018). Dissociated neurons have altered properties leading to changes in gene expression, synaptic function, and cell survival compared to neurons in vivo (Marder and Goaillard 2006). For example, dissociated hippocampal neurons exhibit reduced spine density and differences in the composition and expression of ion channels (Brewer et al. 1993; Boyer, Schikorski, and Stevens 1998).

1.5.3. Organotypic hippocampal slice cultures

Organotypic slices are prepared from young animals (P7) and can be maintained for extended time periods, allowing for chronic manipulations (Gähwiler 1981). Organotypic slices preserve synaptic organisation and cytoarchitecture of the hippocampus (Collin, Miyaguchi, and Segal 1997). After 1 week in culture, the debris of dead cells is removed, providing a highly suitable surface for electrophysiological recordings, and appropriate optical access for imaging experiments (Lein, Barnhart, and Pessah 2011). Organotypic slices continue to develop connectivity, which is initially similar to neurons in vivo, however, after two weeks in culture, neurons form excessive

connections, leading to increased synaptic activity (Gähwiler 1981; De Simoni, Griesinger, and Edwards 2003). Due to the lack of external connections and the environment of a constrained neuronal network, connections might become reorganised, neurons display increased dendritic branching and enhanced spontaneous activity (Debanne et al. 1995; De Simoni, Griesinger, and Edwards 2003). However, these changes are often minor and organotypic slices remain an important model for investigating hippocampal function.

1.5.4. Acute hippocampal slices

Acute hippocampal slices are prepared from adult rodents and are used on the day of slicing, therefore, these slices retain much of the in vivo brain characteristics and functionality (Lein, Barnhart, and Pessah 2011). The synaptic circuits of the hippocampus and network activity are preserved, however, imaging quality worsens over time in acute slices compared with organotypic slices (Reid et al. 1988). Nevertheless, acute hippocampal slices provide a suitable experimental model for studying different forms of synaptic plasticity and the underlying mechanisms involved (Mathis, Furman, and Norris 2011).

1.6. Metabolomics

Metabolomics refers to the comprehensive analysis of metabolites in biological samples and systems-level understanding of biological processes (Johnson, Ivanisevic, and Siuzdak 2016). By integrating metabolomics information with other omics data, researchers can gain a better understanding of cellular physiology, metabolic regulation, and disease mechanisms (Chen et al. 2023). Metabolomic studies offer high sensitivity for detecting subtle changes in metabolic pathways, associated with biological processes or early disease pathogenesis (Wilkins and Trushina 2018). Furthermore, metabolomics offers diverse applications in clinical research, including disease diagnosis and monitoring of treatment responses (Gonzalez-Covarrubias, Martínez-Martínez, and

Bosque-Plata 2022). Profiling metabolites in patient samples can aid in the development of biomarkers and guide treatment decisions by identifying individuals at risk for disease development or progression (Zhang et al. 2015).

1.6.1. Analytical platforms

Metabolomics includes various techniques, including untargeted and targeted metabolomics, lipidomics, and fluxomics (Triebl et al. 2017; Patti, Yanes, and Siuzdak 2012). Untargeted metabolomics analyses hundreds of metabolites to identify metabolic signatures associated with specific disease states, providing relative changes in metabolites and the discovery of metabolic pathways (Schrimpe-Rutledge et al. 2016). Targeted metabolomics quantitively measures a distinct set of metabolites within a specific pathway (Roberts et al. 2012). Lipidomics examines changes in lipid profiles, including the detection of water-insoluble metabolites (Yang and Han 2016). Fluxomics is the study of the rates of metabolic reactions within biological systems, providing an insight into the dynamic behaviour of metabolic networks (Emwas et al. 2022). Usually, heavy carbon precursors (^{13}C) are introduced into a system, which enables tracing through a metabolic pathway of interest by measuring various mass isotopomers (Wilkins and Trushina 2018). Metabolites are defined as small molecules (<1,500 Da) involved in most biological pathways (Wishart 2007). Multiple analytical platforms have been developed for the accurate detection of metabolites, including mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Patti, Yanes, and Siuzdak 2012).

MS provides high sensitivity, detecting metabolites at femtomole levels, and can cover an extensive range of metabolites (Urban 2016). MS involves ionising molecules to create ions, and subsequently, separating these ions based on their mass-to-charge ratio, and detecting and measuring the resulting signals (Alseekh et al. 2021). Prior to MS analysis, biological samples are

often separated using different chromatography methods, such as gas chromatography (GC), liquid chromatography (LC), ultraperformance LC, and high pressure LC (Trushina 2015; Nassar et al. 2017; Garcia and Barbas 2011).

NMR is another analytical technique used for the detection of metabolites. NMR spectroscopy uses the magnetic properties of atomic nuclei with non-zero spin, such as ^{1}H (Emwas et al. 2019). When placed in a strong magnetic field, the nuclei absorb energy and encounter nuclear magnetic resonance, resulting in the emission of particular electromagnetic signals (Kwan et al. 2011). NMR spectra displays resonance peaks that are representative of the chemical environment surrounding each nucleus (Marion 2013). The chemical shift, which is measured in parts per million (ppm), reflects the magnetic environment of the nucleus and offers insights about its molecular structure and surrounding electron environment (Tognarelli et al. 2015). The spectra enables the identification of metabolites based on their chemical shifts (Marion 2013). Additionally, by comparing the intensity of NMR signals in the spectra, the relative concentrations of metabolites can be quantitively measured (Crook and Powers 2020). NMR spectroscopy allows for metabolic profiling, comprising the detection and quantification of numerous metabolites, which reflect the metabolic state of biological samples under specific conditions (Larive, Barding, and Dinges 2015).

1.6.2. Clinical applications of metabolomics in AD

Brain hypometabolism in AD emerges approximately two decades before clinical symptoms appear, indicating that metabolic dysfunction plays a role in the development of AD (Small et al. 2000). A range of metabolic networks are disrupted in early AD, producing a unique metabolic signature associated with the disease (Trushina et al. 2013; Kaddurah-Daouk et al. 2013). Recently, metabolomic analysis has emerged as an important tool in biomedical research, enabling the identification of new biomarkers and the investigation of disease mechanisms by expansively

analysing the entire biological system of metabolites (Wilkins and Trushina 2018). Metabolomics also provides crucial biochemical information for evaluating drug candidates throughout the process of drug discovery and development (Xia et al. 2017; Van Gijsel-Bonnello et al. 2017).

Metabolomic studies in AD patients and mouse models have identified alterations in various metabolic pathways related to AD pathology, such as lipid and energy metabolism (Trushina et al. 2013; Han et al. 2011). These changes can be used as potential biomarkers for detection and monitoring disease progression, however, the development of accessible and precise tests using biological fluids must also be researched for the application of metabolomics in clinical practice (Wilkins and Trushina 2018).

Initial studies investigated metabolic changes in post-mortem brains from AD patients using untargeted metabolomics approaches (Snowden et al. 2017). Brain region-specific metabolic alterations were examined using LC-MS and GC-MS, which identified several unsaturated fatty acids that correlated with AD pathology, as well as metabolites involved in mitochondrial dysfunction (Snowden et al. 2017; Paglia et al. 2016). Studies assessing CSF levels from AD patients also discovered metabolites that were significantly increased with AD, including sphingomyelins and glycerophospholipids (Koal et al. 2015). Sphingomyelins are prominent components of myelin sheaths, and the breakdown of myelin and sphingomyelin has been demonstrated to offer an alternative energy supply, in the form of ketones, associated with AD and aging (Klosinski et al. 2015).

Plasma is a highly suitable biofluid for metabolomics analysis. Several studies have analysed plasma samples from AD patients to identify metabolic alterations (Graham et al. 2015; Trushina and Mielke 2014). Research has also shown that metabolic changes related to disrupted energy balance, detected in the CSF of patients, could be reliably corroborated in plasma samples (Trushina et al.

2013). Longitudinal studies to examine metabolite changes in plasma samples from AD patients have also been able to determine alterations associated with different stages of AD (Graham et al. 2015). Studies have found changes in membrane lipids in the early stages of AD associated with abnormal Aβ42 levels, and alteration in lipid metabolism and mitochondrial bioenergetics associated with hyperphosphorylated tau (Toledo et al. 2017). Metabolic transitions that occur during AD pathogenesis provide insights into disease mechanisms at specific stages of AD (Toledo et al. 2017; Fiandaca et al. 2015).

Biomarkers in the blood metabolome reflect the states of brain tissue and other organs, and therefore, hold promise as a minimally invasive and cost-effective method for AD diagnosis (Lin et al. 2019). Further research and more detailed analyses are needed to develop blood-based metabolomic biomarkers (Varma et al. 2018), although, more recently, an immunoassay for plasma p-tau217 has shown similar accuracies to CSF biomarkers in identifying AD pathology (Ashton et al. 2024). Additionally, standardising protocols related to sample preparation and analytical methods is important to reduce inconsistent results between studies (O'Bryant et al. 2015). As blood-based biomarkers become more sensitive and analytical methods improve, their clinical applications become increasingly feasible.

1.7. Thesis aims

The primary aim of this thesis was to investigate intracellular Ca^{2+} signalling and altered metabolic pathways in models of AD pathology. The specific aims of the thesis are:

1. To examine the effect of $Ca_v2.1$ heterozygous knockout at the presynaptic terminal on synaptic neurotransmission and Aβ-induced LTP impairment.

Aβ oligomers, in hippocampal neurons, produce a sustained enhancement in evoked neurotransmitter release by increasing presynaptic Ca^{2+} influx through $Ca_v2.1$ calcium channels (Jeans et al., Unpublished). In Chapter 3, I will investigate the impact of a heterozygous knockout of $Ca_v2.1$ on basal synaptic function, Aβ-mediated inhibition of LTP, and neuroinflammation in J20 mice. I hypothesise that $Ca_v2.1$ heterozygous knockout presynaptic terminals, which rely less on these VGCCs for functioning, should exhibit reduced sensitivity to the effects of Aβ oligomers. Consequently, the impairment of LTP should be rescued at Aβ oligomer-treated synapses in hippocampal slices.

2. To characterise specific features of lysosomal activity in response to Aβ oligomer treatment.

In Chapter 4, I will identify changes in lysosome biology in response to treatment with Aβ oligomers in vitro and in the J20 mouse model. This work will include an assessment of lysosome number, Ca^{2+} signalling, fusion with the plasma membrane, and the release of proteases required for the maintenance of long-term structural plasticity at excitatory synapses in hippocampal slices. This project may yield novel insight into the pathological mechanisms of AD by examining the mechanisms underlying lysosome dysfunction in the disease and identifying molecular pathways or targets that are potentially amenable to therapeutic intervention.

3. To investigate the effect of Aβ oligomers on activity-dependent aspects of ER Ca^{2+} signalling and the impact on structural plasticity of dendritic spines in hippocampal neurons.

ER Ca^{2+} signalling is dysregulated in AD, however, the mechanisms underlying this dysfunction are still unclear. Since neuronal Ca^{2+} signalling is tightly correlated to structural plasticity

mechanisms, I will investigate activity-dependent aspects of Ca^{2+} signalling from the ER and the impact on structural plasticity of dendritic spines in hippocampal neurons in organotypic slices treated with Aβ oligomers and acute hippocampal slices from J20 mice. I hypothesise that dysregulated ER Ca^{2+} signalling may play a role in the impairment of structural plasticity in dendritic spines, which is characteristic of AD. Furthermore, I hope to identify targets implicated in the disruption of ER Ca^{2+} signalling in AD that may be pharmacologically altered to rescue the effects of Aβ oligomers.

4. To assess metabolic changes in the CNS and peripheral tissues in response to aging and AD pathogenesis in mice.

^{1}H NMR analysis will be used to evaluate metabolite changes in the prefrontal cortex, hippocampus, liver, spleen, and serum during regular aging and the early and late stages of AD in wild type and J20 mice. I hypothesise that numerous metabolites will be altered in the tissues of J20 mice, which may allow for the identification of novel metabolic pathways associated with AD.

2. Materials and Methods

2.1. Transgenic mice and preparation of hippocampal neurons and slices

2.1.1. Transgenic mice

All mouse work was carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, under project licence PP8326000 approved by the Home Office (UK). The J20 hAPP and *Cacna1a* knockout transgenic mouse lines were on a C57BL/6 background. Animals were housed in a pathogen-free facility with *ad libitum* access to food and water. Genotyping of mice was carried out by Transnetyx Inc.

2.1.2. Dissociated primary hippocampal neurons

Dissociated hippocampal cultures were prepared from male P1 Wistar rats (Envigo RMS, UK). Rats were sacrificed by cervical dislocation and decapitation, subsequently, the brain was extracted and placed into ice-cold Hanks' Balanced Salt Solution (HBSS) and the hippocampus of each hemisphere was dissected and the meninges removed. Hippocampi were placed into a trypsin solution at 37°C for 15 minutes. The hippocampi were washed with a solution of 2% Fetal Bovine Serum (FBS) in HBSS and then with HBSS alone. They were then transferred to a solution of Neurobasal medium A (NBA) and triturated. Once dissociated, cells were counted with a hemacytometer and diluted with more NBA to result in approximately 30,000 cells in 100 μl. The cells were then seeded onto poly-D-lysine coated 18 mm coverslips and placed into wells containing NBA supplemented with 2% FBS, 2% B27 Plus (Gibco), 1% GlutaMAX (Gibco), and 1% penicillin/streptomycin. The next day, half the medium was exchanged for a different NBA solution, supplemented with 2% B27 Plus and 1% GlutaMAX only, which was replaced every 3 days.

2.1.3. Organotypic hippocampal slices

Transverse 350 μm organotypic hippocampal slices were prepared from male P7 Wistar rats (Envigo RMS, UK). Rats were sacrificed by cervical dislocation and decapitation. The brain was dissected out and placed into ice-cold Earle's Balanced Salt Solution (EBSS)-based dissection buffer with additional 21 mM HEPES, 27.8 mM D-glucose (pH adjusted to 7.2-7.4 with 5 mM NaOH). The hippocampus of both hemispheres was dissected and transferred to a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd. And Cavey Laboratory Engineering Co. Ltd.) to cut hippocampi into slices. Slices were placed back into dissection buffer and those exhibiting damage or incomplete hippocampal anatomy were discarded. Slices were cultured on Millicell CM culture plate inserts (pore size 0.4 μm, diameter 12 mm, Merck Millipore) in a six-well plate with 1 ml culture medium and maintained at 37°C and 5% CO_2 for 10 days prior to use. Culture medium was replaced every 3-4 days and contained 78.8% Minimum Essential Medium, GlutaMAX, 20% heat-inactivated horse serum, 1% B27 plus, 30 mM HEPES, 26 mM D-glucose, 5.8 mM $NaHCO_3$, 1 mM $CaCl_2$ and 2mM $MgSO_4$. During experiments, slices were constantly perfused with artificial cerebrospinal fluid (ACSF) (145 mM NaCl, 16 $NaHCO_3$, 11 mM D-glucose, 2.5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 1.2 NaH_2PO_4).

2.1.4. Acute hippocampal slices

Acute hippocampal slices were prepared from transgenic or wild-type (C57BL/6) mice of varying ages. Mice were sacrificed by cervical dislocation and decapitation. The brain was dissected and submerged in ice-cold dissection medium (65 mM sucrose, 85 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10mM D-glucose, 7mM $MgCl_2 \cdot 6H_2O$, and 0.5 mM $CaCl_2 \cdot 6H_2O$), which was bubbled with 95% O_2 and 5% CO_2 for 30 minutes prior to use. The cerebellum was detached with a scalpel and then the brain was glued onto the vibratome plate together with a 2% agar block by the dorsal side of the brain, to stabilise it during slicing. The vibratome slicing stage was then filled with ice-cold dissection medium. 350 μm coronal slices were cut using a Leica

VT1000S vibrating blade microtome and the hemispheres of the brain were separated using a scalpel. Slices were transferred to a recovery chamber containing 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 constantly bubbled with 95% O_2 and 5% CO_2. Slices were first placed in a water heating bath at 37°C for 10 minutes before being kept at room temperature for 1 hour to recover. Slices were maintained for up to 5 hours.

2.2. Electrophysiology

2.2.1. Field excitatory postsynaptic potential (fEPSP) recordings in acute hippocampal slices

Acute hippocampal slices were transferred to a custom-built interface chamber constantly 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 $CaCl_2 \cdot 6H_2O$) bubbled with 95% O_2 and 5% CO_2 and heated to a near-physiological temperature of 35°C. For drug treatments, Aβ oligomers (10 nM) were diluted in ACSF and slices were incubated for >1 hour during which recordings were performed. Field excitatory postsynaptic potentials (fEPSPs) in the CA1 region of the hippocampus were recorded. A bipolar stimulating electrode was positioned in the Schaffer collaterals of CA3 neurons to provide stimuli and a borosilicate glass recording electrode filled with ACSF was placed in the stratum radiatum of CA1 neurons. Recordings were amplified using a Digitimer NeuroLog amplifier, filtered below 3 Hz and above 3 KHz, and digitised with a BNC-2090A converter (National Instruments). Data was acquired using WinWCP software (Strathclyde Electrophysiology Software). Neurons were stimulated with an appropriate stimulating intensity, which could produce fEPSPs of approximately 0.5 mV, every 14.5 seconds with a paired pulse stimulation delivered every four stimuli. Once a stable response was observed, a baseline recording was made for 15-20 minutes.

2.2.2. LTP induction and fEPSP analysis

After a stable baseline was recorded, LTP was induced using a theta burst stimulus (TBS) consisting of 4 bursts of 100 Hz repeated 20 times over 20 seconds, each burst being 200ms apart. Recordings were then resumed for 1 hour.

fEPSP traces were analysed in Clampfit 9 (Version 10.6.2.2, Molecular Devices). To remove any confounds associated with population spiking, the initial slope of the fEPSP was analysed. For each experiment, the size of the response before and after LTP induction was measured by determining the slope (mV/ms) of the initial phase of the fEPSP. The magnitude of LTP was expressed as percent changes of fEPSP slope normalised to baseline values, the average value of 15-20 minutes of baseline recording before LTP induction. Blinding was used in data analysis.

2.2.3. Paired-pulse ratio (PPR) analysis

A paired pulse stimulation, with an interstimulus interval of 50 ms, was delivered every four stimuli. PPR was obtained by calculating fEPSP2 slope/fEPSP1 slope. Blinding was used in data analysis.

2.2.4. Whole-cell patch clamp electrophysiological recordings in organotypic and acute hippocampal slices

Organotypic hippocampal slices were transferred to a recording chamber and secured onto the stage with vacuum grease (Glisseal HV, Borer), while acute hippocampal slices were stabilised with a 'harp' (Warner Instruments). During experiments, slices were constantly perfused with ACSF heated to 32-34°C and bubbled with 95% O_2 and 5% CO_2. Ascorbic acid (0.2 mM) and Trolox (1 mM, Sigma Aldrich) were added to ACSF to minimize photodynamic damage during imaging. Patch pipettes (3-5 MΩ) were made with a horizontal micropipette puller (Sutter Instrument Co.) and filled with standard internal solution (135 mM K-Gluconate, 10 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 2 mM Na_2ATP, and 0.4 mM Na_3GTP, pH 7.2-7.4). Electrophysiology data was recorded in whole-cell patch clamp mode with WinWCP (Strathclyde Electrophysiology Software)

using an Axoclamp 2B amplifier. Cells were clamped at -70 mV and a minimum access conductance of Ga ≥ 25 nS was used for all experiments. For acute slices, neurons found in deeper cell layers were chosen for experiments to ensure only healthy neurons were recorded from.

2.3. Fluorescence confocal imaging

2.3.1. Lysosomal labelling and imaging

Dissociated neuronal cells were used 21 days after plating and incubated with Lysotracker yellow (50 nM; Thermo Fisher) for 1-2 hours at 37°C and 5% CO_2 before imaging. Coverslips were mounted on a Chamlide EC-B18 stimulation chamber (Live Cell Instrument), which was filled with standard Tyrode's solution (128 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 4.2 mM $NaHCO_3$, 20 mM D-glucose and 15 mM HEPES buffer; pH=7.2-7.4), on the stage of an Olympus IX-71 inverted microscope equipped with a 100X, NA 1.40 UPlanSApo objective and an Andor iXon EM CCD camera. A 100W mercury lamp with appropriate neutral density filters was used for fluorescence illumination. Aβ oligomers were applied at 200 nM for 2 hours prior to imaging two fields of view per coverslip. GPN (200 μM; Sigma) was used to disrupt lysosomal labelling. Fluorescence was calculated in arbitrary units in ImageJ using the following calculation:
Integrated density - (selected area × mean fluorescence of background readings). Blinding was used in data collection and analysis.

2.3.2. Total Internal Reflection Fluorescence Microscopy (TIRFM)

Dissociated hippocampal neurons (DIV 21) were labelled with 50 nM Lysotracker Yellow, as previously described, and imaged using TIRFM (Olympus IX83 TIRF microscope) with a 60X water immersion objective (Olympus; NA=0.90), Photometrics Evolve Delta EMCCD camera and software application (CellSens) at the Wolfson Imaging Centre (Weatherall Institute of Molecular Medicine). TIRFM images were taken before and after a 45 mM K^+ Tyrode's solution

(88 mM NaCl, 45 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 4.2 mM $NaHCO_3$, 20 mM D-glucose and 15 mM HEPES buffer; pH=7.2-7.4) was applied. Images were analysed using ImageJ and changes in fluorescence were reported in arbitrary units using the previous calculation. Ned-19 (50 μM; Tocris) was used as a NAADP antagonist to prevent lysosomal Ca^{2+} release. Blinding was used in data collection and data analysis.

2.3.3. Induction of structural plasticity using glutamate uncaging at dendritic spines and imaging

CA1 pyramidal neurons from hippocampal slices were patched and loaded with Alexa Fluor 488 (0.1-0.2 mM; Thermo Fisher), diluted in internal solution, and the dye was able to diffuse throughout the neuron for 15-20 minutes. After identification of a dendritic spine well separated from the dendritic branch, a pipette filled with 4-Methoxy-7-nitroindolinyl-caged-L-glutamate (MNI glutamate; 10 mM; Tocris) diluted in Tyrode's was placed just above the slice in the recording chamber and connected to a picospritzer to provide focal delivery of MNI-glutamate, which was uncaged in a ~ 1 μm diameter spot above the dendritic spine head using a 405 nm UV laser. The location of the uncaging spot was marked on the computer screen and the movable stage of the recording chamber was used to place the dendritic spine under the photolysis spot. An external shutter and shutter control box was used to regulate photolysis. Laser intensity was adjusted to trigger a ~ 1 mV EPSP. MNI-glutamate was uncaged using a train of 4-6 ms pulses, 30 times at 0.5 Hz, paired with postsynaptic depolarisation, which was controlled by TTL pulses programmed in a WinWCP stimulus protocol. Cells were patched-off after induction of the protocol and a maximum of two spines were stimulated per cell, and 2-3 cells were used for experiments per slice.

Images were taken on an Olympus BX50WI microscope with a 60x water immersion objection (NA = 0.90) and a BioRad Radiance 2000 confocal scanhead (BioRad/Zeiss) using a 488 nm argon laser. Images of the length of dendrite containing the target dendritic spine were acquired using

Lasersharp 2000 software as z-stacks (0.5 μm steps) with a 512 x 512 resolution. To establish a baseline, images were taken once per minute for three minutes, and then at 0, 1, 30 and 60 mins. Dendritic spine size was analysed in ImageJ and the changes in fluorescence were used to estimate fractional changes in spine size and calculated as fold Δ volume = $(F - F_{background}) \div (F_{initial} - F_{background})$. Blinding was used in data analysis.

2.3.4. Ca^{2+} imaging

CA1 neurons were patched with patch pipettes containing internal solution with the Ca^{2+}-sensitive dye, Oregon Green 488 BAPTA-1 (OGB-1; 0.5 mM; Thermo Fisher) and the dye was able to diffuse throughout the neuron for 15-20 minutes.. The line scan axis was aligned to bisect a secondary dendrite and line-scans were acquired and synchronised to action potentials triggered by current injection (1-2 nA, 10ms) to produce Ca^{2+} transients. The timing and external trigger to start the line scans was controlled by WinWCP stimulus protocol. 10 line scans evoked at 20 second intervals were acquired and merged into a single line scan for every condition. Experiments were analysed in ImageJ to acquire the plot profile of the line scan and then Excel to calculate fractional changes in fluorescence with: $\Delta F/F = (F_{total\ F\ over\ ROI} - F_{background}) \div (F_{baseline\ average} - F_{background})$. The baseline average was calculated as the average value of the signal of the first 50 ms of the line scan before stimulus. Peak Ca^{2+} transients were determined by taking the average of a 20 ms time window following the action potential stimulus. Blinding was used in data analysis.

2.3.5. Induction of structural plasticity with Theta Burst Stimulation (TBS) in organotypic slices

CA1 pyramidal neurons from organotypic hippocampal slices were patched and loaded with Alexa Fluor 488 (0.1-0.2 mM; Thermo Fisher), diluted in internal solution, and the dye was able to diffuse throughout the neuron for 15-20 minutes. After identification of a secondary dendrite, a TBS protocol (4 stimuli at 100 Hz repeated 20 times with an interval of 200 ms) was triggered with WinWCP stimulus protocol. Imaging and analysis of dendritic spine size was conducted as before

to measure fold Δ volume; 4-5 spines were analysed within 20 μm of dendrite per neuron. Blinding was used in data analysis. AP5 was used as a negative control (Supplementary Figure 2).

2.3.6. Viral-based transfection of organotypic hippocampal slices to monitor ER dynamics

At DIV 3, organotypic hippocampal slices were microinjected at $3.37x10^{11}$ GC/ml in the CA1 region with a custom-made recombinant AAV2 virus made by Vector Builder: pAAV[Exp]-SYN1-EGFP(ns):rErlec1[NM_001106023.1] (Vector ID: VB230608-1474djg) and ViralEntry Transduction Enhancer (1:100; NBS Biologicals Ltd). Images were taken 10-12 days later. CA1 neurons were patch-filled with Alexa 546 (0.2 mM; Thermo Fisher) and the dye was able to diffuse throughout the neuron for 15-20 minutes to visualise the structure of the neuron. To visualise ER fluorescence, the laser settings used were: excitation/emission 488/515-530 nm; to visualise neurons patched with Alexa 546, the laser settings used were: excitation/emission 514/570 nm. Bleed-through of ER-GFP fluorescence into the red channel, and Alexa 546 into the green channel was quantified and subtracted from the fluorescence calculations. To determine the effects of structural plasticity induction at dendritic spines on ER dynamics, using the red-pass filter, a dendritic spine was identified on a secondary dendrite. MNI-glutamate uncaging was performed and z-stack images of the dendritic spine were taken at -5, 0, 1, 5 and 30 mins. Images were analysed in ImageJ to measure ER fluorescence with: integrated density - (area of selected cell × mean fluorescence of background readings), and dendritic spine fold Δ volume: $(F - F_{background}) \div (F_{initial} - F_{background})$. Blinding was used in data analysis.

In all imaging experiments, if the neuron showed signs of photodynamic damage or lost responsiveness, the experiments were excluded from analysis.

2.4. Histology

2.4.1. Immunohistochemistry with DAB

Fixed tissue was sectioned (10 μm thick) using the Leica Cryostat, mounted onto gelatin-coated slides, and stored at -20°C until staining. Sections were first dried at 37°C and briefly washed in PBS, before being placed in citrate buffer (1.92 g citric acid in 1 L distilled water) at 45°C for 10 minutes. Sections were washed in PBS again and placed into a 1% H_2O_2 in methanol solution for 10 minutes to quench endogenous peroxidase activity. Sections were blocked for non-specific binding with 10% goat serum in PBS-T for 1 hour at room temperature. PBS-T is a mixture of PBS with added 0.1% Tween-20, which causes cell permeabilization and helps antibodies bind to antigens. Sections were then incubated in primary antibody LAMP-2A (1:100; Invitrogen; PA1-655) diluted in 1% goat serum in PBS-T, overnight at 4°C. The following day, sections were washed in PBS and incubated in goat α-rabbit biotinylated secondary antibody (1:200; Vector Labs) diluted in 1% goat serum in PBS-T for 2 hours at room temperature. Sections were washed again in PBS and incubated in Avidin-Biotin Complex (ABC; 1:100 in PBS; Thermo Fisher) for 1 hour at room temperature. Subsequently, sections were incubated in the chromogenic reporter 3,3'-Diaminobenzidine (DAB) until staining was visualised. Cresyl violet counterstaining was used and then sections were dehydrated through graded ethanol (80%, 90%, 100%) for a few minutes each. Sections were cleared with xylene and coverslipped with DPX.

For quantification, slides were imaged using a Nikon light microscope with Basler camera and slide scanning software (Manual WSIScanner) at 10X magnification. Images were analysed with ImageJ by applying a suitable threshold and quantifying the % area that was stained of the hippocampus. A negative control using secondary antibody only without the addition of primary antibody was performed. Blinding was used in data collection and analysis.

2.4.2. Golgi-Cox staining

Mice were sacrificed by cervical dislocation and decapitation. Brains were rinsed in distilled water, sagittally bisected, and treated with the FD Rapid Golgi Stain Kit (FD Neurotechnologies Inc) to label neurons. Following treatment, brains were embedded in agarose and sectioned coronally (100 μm thick) on the Leica VT1000S vibrating blade microtome. Sections were transferred to gelatin-coated slides and air dried in the dark at room temperature. Slides were briefly rinsed in distilled water, stained in the developing solution provided in the kit, and dehydrated through graded ethanol (50%, 75%, 95% and 100%). Sections were cleared with xylene and coverslipped with Permount Mounting Medium (Fisher Scientific). Images were taken on an Olympus Fluoview FV1000 confocal software with an Olympus IX-81 inverted microscope under 100X, NA 1.40 UPlanSApo oil immersion objective. Differential Interference Contrast (DIC) images were acquired as a z-stack of secondary and tertiary dendrites of pyramidal neurons in the CA1 region of the hippocampus. Images were analysed using ImageJ to measure spine density by counting the number of dendritic spines per μm of dendrite. Blinding was used in data collection and analysis.

2.4.3. Immunofluorescence

Mice were sacrificed by cervical dislocation and decapitation. The brain was dissected and stored at 4% PFA. To prepare sections, brains were submerged in PBS in the vibratome slicing chamber, and 100 μm thick coronal slices were cut using a Leica VT1000S vibrating blade microtome. Sections were transferred with a glass pipette dropper into a 12 well plate for the staining protocol. Sections were rinsed in PBS (2x 10 minutes), washed with sodium borohydrate solution (1 mg/ml in PBS) while kept on ice (3x 10 minutes), washed in Tris-Triton (3x 10 minutes), then incubated in citrate buffer (1.92 g citric acid in 1 L distilled water, pH 6 with added 0.5 ml Tween-20) at 60°C for 30 minutes. Sections were briefly rinsed in Tris-Triton solution and then incubated in a blocking solution (10% goat serum in Tris-Triton) for one hour at room temperature. Primary antibody (rabbit α-mouse Iba-1; 1:2000; Abcam; or rabbit α-mouse GFAP; 1:1000; Dako) diluted

in Tris-Triton containing 2% goat serum and 0.2% Triton X-100 was placed in each well (300 µl per well). Sections were incubated overnight with continuous agitation at 4°C. Sections were then washed in Tris-Triton (3x 10 minutes). The secondary antibody, goat anti-rabbit IgG Alexa Fluor 488 (1:1000; Thermo Fisher) was diluted in Tris-Triton and 2% goat serum. Sections were incubated in the secondary antibody for 30 minutes at room temperature. Sections were then washed again in Tris-Triton (3x 10 minutes) and then mounted on gelatin-coated slides and left to air dry. Slides were coverslipped with ProLong Antifade Mountant with DNA stain DAPI (Thermo Fisher) and stored in the dark at 4°C. Images were taken on an Olympus Fluoview FV1000 confocal software with an Olympus IX-81 inverted microscope under 10X and 20X objectives. The hippocampus was located by visualising the DAPI stain and z-stack images were taken of the CA1 region. Images were analysed in ImageJ to count the number of astrocytes and microglia within the CA1 area of the hippocampus. Blinding was used in data collection and analysis.

2.5. Molecular biology

2.5.1. mRNA extraction

Mice were sacrificed by cervical dislocation and decapitation. The brain was dissected, the region containing the hippocampus was isolated and immediately snap-frozen in isopentane. Tissue was then stored in Eppendorf tubes at -80°C until use. RNA was extracted from approximately 30 mg of tissue, using the Qiagen QIAShredder and RNeasy Mini Kit, as per manufacturer's instructions. Briefly, the tissue was kept on dry ice until 300 µl RLT lysis buffer with 1% ß-mercaptoethanol was added to the tube and the tissue homogenised using a pestle homogeniser and then moved to ice. A further 300 µl of lysis buffer was added and then vortexed for 10 seconds. 300 µl of tissue lysate was then transferred to a QIAshredder spin column, which was centrifuged for 3 minutes

at 13,300 rpm at 20°C. This step was repeated with the remaining 300 μl of tissue lysate. The column was then discarded and the filtrate was mixed with 600 μl of 70% ethanol. 600 μl was transferred to an RNeasy spin column and centrifuged for 30 seconds at 10,000 rpm. The filtrate was discarded and the remaining 600 μl was added to the column and the step was repeated. 700 μl of RW1 wash buffer was added to the column and then centrifuged at 10,000 rpm for 30 seconds. Next, two washes of 500 μl of RPE buffer were added to the column and centrifuged at 10,000 rpm for 30 seconds the first time, and 10,000 rpm for 2 minutes the second time. During each wash step, the filtrate was discarded. The spin column was dried by transferring the column to a new collecting tube and centrifuging at 13,300 rpm for 1 minute. Columns were then transferred into a clean tube and RNA was eluted by adding 30 μl of nuclease free water to the membrane and centrifuging at 10,000 rpm for 1 minute. A NanoDrop 1000 spectrophotometer (Thermo Fisher) was used to assess RNA quantity and purity. An acceptable ratio of sample absorbance at 260 nm and 280 nm was approximately 2, and the ratio of sample absorbance at 260 nm and 230 nm was 1.8-2.2. RNA samples were stored at -80°C.

2.5.2. cDNA conversion and quantitative real-time polymerase chain reaction (qPCR)

To convert RNA to cDNA, the Applied Systems High Capacity cDNA conversion kit was used. 1000 ng of RNA was used with the reverse transcription master mix provided. Samples were briefly centrifuged before being placed in the thermocycler (Bio-Rad) for incubations of 25°C for 10 minutes, 37°C for 2 hours, and 85°C for 5 minutes. cDNA samples were then stored at -20°C until qPCR.

cDNA was diluted with nuclease-free water and 10 ng of cDNA was used per reaction. qPCR was performed on samples in duplicate with SYBR green master mix (Primerdesign or Bio-Rad) using a LightCycler 480 machine (Roche Diagnostics). Primers were purchased from Primerdesign or Sigma Aldrich and used at a concentration of 300 nM (Table 2.1). A melt curve was generated

after each run to ensure only a single PCR product was generated for each reaction. Relative gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as the housekeeping gene, and relative gene expression of the samples was normalised to expression of GAPDH.

Gene	Forward primer	Reverse primer
GAPDH	5'-AACGACCCCTTCATTGAC-3'	5'-TCCACGACATACTCAGCAC-3'
MMP-9	5'-GCTGACTACGATAAGGACGGCA-3'	5'-TAGTGGTGCAGGCAGAGTAGGA-3'
PSD-95	5'-TCTGTGCGAGAGGTAGCAGA -3'	5'-AAGCACTCCGTGAACTCCTG-3'

Table 2.1: qPCR primers with primer sequences.

2.5.3. MMP-9 activity assay

MMP-9 activity was assessed using an MMP-9 specific fluorogenic substrate (PEPDAB052; Biozyme) with excitation at 485 nm and emission at 530 nm. Organotypic hippocampal slices were first briefly washed in Tyrode's solution, then individually placed in wells of a 12 well plate, and incubated with 10 μM of the fluorogenic substrate in 200 μl of either a normal or a 45 mM K^+ Tyrode's solution, to augment neuron activity, for two hours at 37°C and 5% CO_2. The fluorescence of 100 μl of solution was measured using a plate reader (CLARIOstar Plus) and final measurements were expressed in arbitrary fluorescent units (AFU) after the subtraction of blank controls. In some experiments, additional drugs were added to the incubation solution.

2.6. Metabolomics

2.6.1. Sample preparation

Animals were sacrificed by cardiac puncture under terminal anaesthesia (3% isoflurane in oxygen) and blood was collected into Eppendorf tubes. Blood was left to stand at room temperature for 30 minutes, followed by centrifugation 1500 x g for 10 minutes at 4°C for serum collection. Serum

samples were then stored in -80°C. For the collection of brain, liver, and spleen: animals were transcardially perfused with cold saline (0.9% m/v) containing heparin (5000 USP/L) and fresh tissue was immediately snap frozen in -80°C.

Samples were prepared in batches to minimize variation, using an optimised protocol based on previously published methods (Waters et al. 2001).

2.6.1.1. Liver

Frozen liver (100-125 mg) was homogenised with a pestle and mortar kept on dry ice. Ice-cold 50% acetonitrile in distilled water was added to the samples (8 μl/mg of tissue) and then vortexed. Samples were centrifuged at 5070 x g for 5 minutes at 4°C. 750 μl of supernatant was transferred into a fresh tube and then lyophilised. Samples were then stored at -80°C until NMR analysis.

Lyophilised samples were resuspended in 600 μl of 75 mM NMR buffer (5:1 disodium phosphate [Na_2HPO_4] and monosodium phosphate [NaH_2PO_4] in 100% D_2O, pH = 7.4), then centrifuged at 2500 x g for 5 minutes at 4°C to remove any particulate matter. Samples were transferred to 5 mm NMR tubes (Norell) using a glass pipette dropper, being careful to avoid the pellet.

2.6.1.2. Spleen

Frozen spleen (20-40 mg) was prepared as previously described in the liver protocol. After the addition of 50% acetonitrile, the samples were homogenised using an electric pestle homogeniser. Samples were centrifuged at 5070 x g for 5 minutes at 4°C. 200 μl of supernatant was transferred into a new tube. A double extraction was performed for these samples and after the first extraction, acetonitrile solution was added again to the pellet, the sample was vortexed, centrifugation was repeated, and a total of 400 μl of supernatant was collected. The samples were then lyophilised and stored at -80°C until NMR analysis. The samples were then processed as previously described and transferred to NMR tubes.

2.6.1.3. Brain

Frozen brain samples were cut on the cryostat (Leica) at a thickness of 25 μm for prefrontal cortex collection (18-35 mg). The hippocampal region (40-65 mg) was isolated using a scalpel. Brain samples underwent double extraction. A 1:1 solution of acetonitrile and double distilled water was added to samples (10 μl/mg). The prefrontal cortex (PFC) samples were homogenised by being vortexed, while the hippocampal samples were homogenised by electric pestle homogeniser. The homogenised samples were centrifuged at 5070 x g for 5 minutes at 4°C. 170 μl of supernatant was collected for PFC samples and 350 μl for hippocampal samples. The extraction process was repeated, yielding a total of 340 μl of supernatant for PFC samples and 700 μl for hippocampal samples. The samples were then lyophilised and stored at -80°C until NMR analysis. The samples were then processed as previously described and transferred to NMR tubes.

2.6.1.4. Serum

Serum samples were processed with a collaborator in Numares AG (Regensburg, Germany). Briefly, 100 μl of serum was thawed at room temperature and 550 μl of 75 mM NMR buffer was added to the sample. The samples were triturated, vortexed, centrifuged at 2000 x g for 30 seconds, and then transferred to NMR tubes.

2.6.2. ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy

All samples were measured using a 700 MHz Bruker AVII spectrometer operating at 16.4 Tesla fitted with a ^{1}H (^{13}C/^{15}N) TCI cryoprobe, as previously described (Jurynczyk et al. 2017). Sample temperature remained stable at 310 K. The ^{1}H NMR spectra were obtained by using a one-dimensional nuclear Overhauser effect spectroscopy (NOESY) presaturation scheme. The NOESY scheme reduces water signal to produce less noise and easier metabolite compound recognition.

The serum samples were measured using a 600-MHz Bruker AVIII ^{1}H NMR spectrometer with the zgpr30 pulse sequence for spectra acquisition as previously described (Stratmann et al. 2016).

2.6.3. NMR data pre-processing

Spectra were processed using TopSpin 2.1 (Bruker, Germany), zero filled by a factor of 2 and multiplied by an exponential function corresponding to 0.3 Hz line broadening. All spectra were manually phased, the baseline was corrected, and chemical shifts referenced to the lactate-CH_3 doublet resonance at δ = 1.33 ppm. Processed spectra were exported to ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc.). Regions of interest were set and other regions were discarded from analysis. Spectra belonging to the same tissue were overlayed and the region of interest was binned manually by adding bins around each resonance signal. Bins were 0.02 ppm wide and the absolute value of integral of each spectral bin was unit variance scaled. These values were then exported to excel and labelled with their corresponding animal ID and treatment groups.

2.6.4. Multivariate analysis (PCA and OPLS-DA)

Analysis was performed in R software 3.4.3 (R foundation for statistical computing, Vienna, Austria). Principal component analysis (PCA) was initially used to visualise differences between groups for each tissue in an unbiased way and to identify and remove outliers that may be present. To investigate metabolic differences between groups in each tissue and to examine which metabolites contributed most to the group differences, orthogonal partial least square discriminant analysis (OPLS-DA) was used. The process is outlined in Figure 2.1. OPLS-DA is a supervised multivariate analysis, which allows for identification of significant metabolite changes between groups. The analysis was performed in R using the *ropls* package and in-house scripts. Briefly, unequal group sizes were adjusted and separated into a training set (90%) and test set (10%). The training set was employed to build a model on which the test set was applied using an external 10-fold cross-validation with 100 repetitions, to build a total of 1000 models. The mean accuracy,

sensitivity and specificity of the model ± standard deviation was compared to that of a separate model set created by modifying the class assignments to create a random null distribution. Accuracy of the model relates to the degree to which it accurately categorizes all samples, sensitivity reflects the capability of the model to correctly detect true positives, and specificity refers to the correct detection of true negatives. Discriminatory metabolites were identified by calculating the average of the variable importance in projection (VIP) scores of the models, which signify the key metabolites driving variability between groups. An inflection point (cut-off) was chosen in the line plot of VIP scores for choosing the discriminatory metabolites.

The correlation matrix of VIP metabolites from all tissues was created in R software 3.4.3 using the Pearson correlation method and FDR correction for multiple testing above the diagonal. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

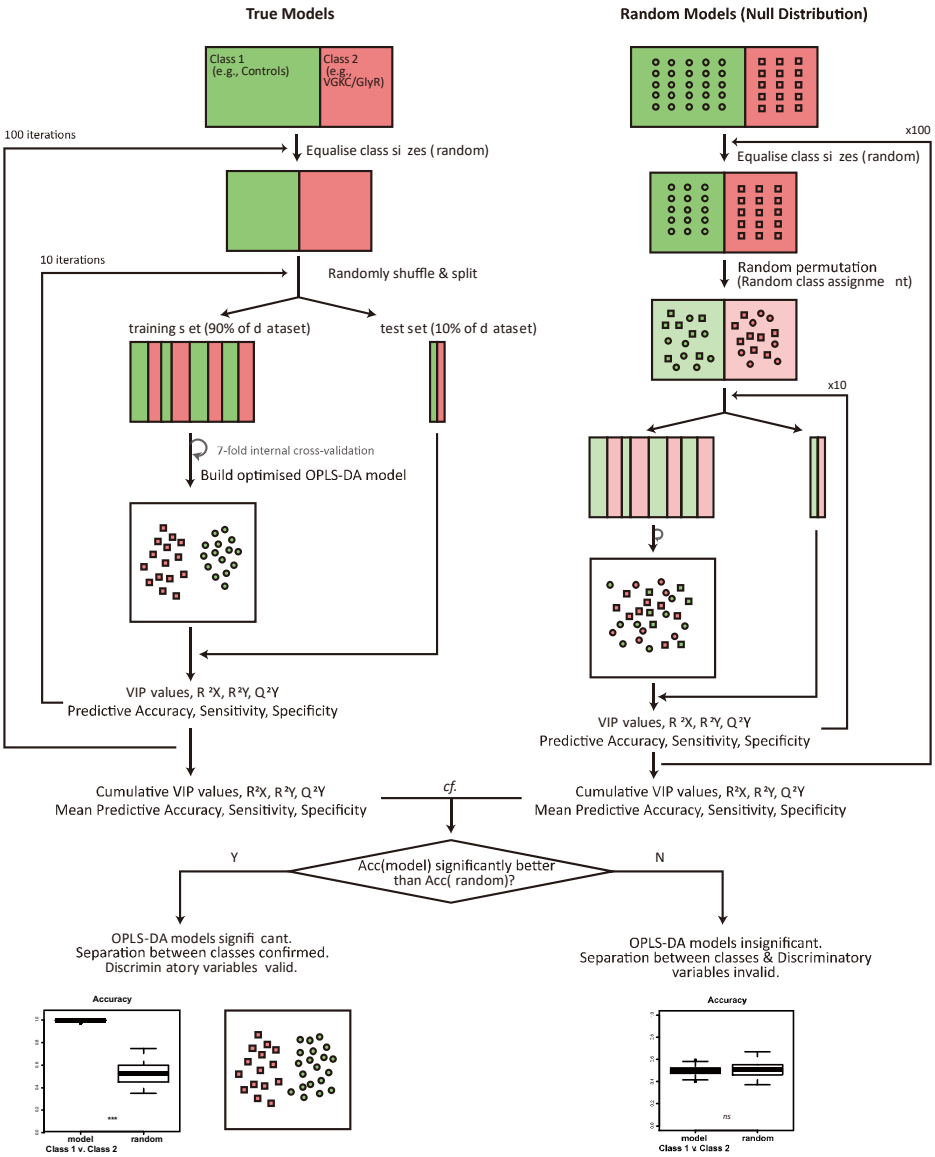

Figure 2.1. Schematic of OPLS-DA analysis. OPLS-DA is a supervised multivariate method to investigate group variation. Class sizes are equalised, shuffled, and split into a training set (90%) and test set (10%). The training set is employed to build models on which the test set is applied using an external 10-fold cross-validation with 100 repetitions, to build a total of 1000 models. The mean accuracy, sensitivity and specificity of the model is then compared to that of a random model (null distribution). Figure reproduced from (Lennox et al. 2022).

2.6.5. Statistical analysis and metabolite assignment

Statistical tests were performed in Prism 10 (Graph Pad). The values corresponding to the bins, identified by VIP scores, were analysed by two-way analysis of variance (ANOVA) with post-hoc Tukey's test. Significance was set at $p < 0.05$ with 95% confidence intervals. All quantitative data

were expressed as integral values (AU) and presented as boxplots including the median, interquartile range, minimum and maximum data points.

Metabolite assignment was achieved by referring to the Human Metabolome Database (HMDB), literature values, and inspection of the 2D spectra in TopSpin.

2.7. Soluble Aβ oligomer synthesis

Oligomers were synthesised as previously described (Klein, 2002). Briefly, solid $A\beta_{1-42}$ peptide (Sigma Aldrich) was dissolved in ice-cold hexafluoro-2-propanol (HFIP; Sigma). The peptide was left at room temperature for at least one hour to establish monomerization and randomisation of structure. The HFIP solution was aliquoted and left to evaporate overnight, following by 10 minutes in a Savant Speed Vac. The peptide was stored as a film at -80°C. The film was then dissolved in anhydrous dimethylsulfoxide (Sigma) to 5 mM, then diluted to 100 μM in Ham's F12 (without phenol red, with glutamine; Caisson Laboratories) and vortexed. This solution was incubated at 4°C for 24 hours and soluble oligomers were acquired by centrifugation at 14,000 x g for 10 minutes at 4°C. Supernatant was aliquoted and oligomers were stored at -80°C. A single batch of $A\beta_{1-42}$ peptide was used for all experiments.

2.8. Statistics

Statistical tests were performed using Prism 10 (Graph Pad) and are specifically stated in figure legends where used. The Shapiro-Wilk test was used to assess normality of data and the appropriate parametric or non-parametric statistical tests were used subsequently. The unpaired Student's t-test or Mann-Whitney test was used to compare the means between two groups. One- and two-

way ANOVA or Kruskal-Wallis test, with appropriate post-hoc tests stated, were used to compare means of three or more groups, and post-hoc tests were used to correct for multiple comparisons. Significance is represented as: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$). In all statistical tests, alpha = 0.05, and all quantitative data is presented as mean ± standard error of mean (SEM).

3. Altering presynaptic Ca^{2+} channel function normalises Aβ oligomer-induced LTP deficits

3.1. Introduction

The preclinical stage of AD, before irreversible changes have taken place in later stages of neurodegeneration in the disease, has become a prominent focus of the research community. Imaging studies in AD patients have shown evidence of amyloid deposition or network-level abnormalities that can occur years or even decades in advance of clinical symptoms (Sperling, Mormino, and Johnson 2014), so there is a long latent period, sometimes referred to as "preclinical AD", in which therapeutic intervention may be possible with the aim of preventing disease development.

It is currently widely accepted that smaller, soluble Aβ species, most likely oligomers or protofibrils rather than amyloid plaques are the neurotoxic species that drive the disease, and these have been more directly implicated in neurotoxic effects (Mucke and Selkoe 2012). Aβ deposition begins decades before the onset of clinical symptoms and disease (Hardy and Higgins 1992), therefore, it is important to uncover the mechanisms by which upstream pathogenic agents, such as soluble Aβ oligomers, disrupt neuronal function at the level of synapses. The dysfunction of synaptic plasticity has been linked to cognitive and memory impairment, characteristic hallmarks of AD (Colom-Cadena et al. 2020).

It was first shown in 1998 that fibril-free soluble Aβ oligomers lead to rapid inhibition of LTP, the main form of synaptic plasticity and the principal candidate for a cellular mechanism of learning and memory (Lambert et al. 1998). Aβ oligomers exert neurotoxic effects through binding at

synapses, preferentially targeting excitatory synapses (Wilcox et al. 2011). Prior to synapse loss, oligomers are able to induce changes in synaptic membranes, including loss of surface glutamate NMDA and AMPA receptors, which is likely to underlie the loss of LTP (Wang et al. 2004). LTP impairment and a delay in LTD reversal in AD transgenic mice correlate well with the accumulation of soluble Aβ oligomers (Chang et al. 2003). Interestingly, blocking specific signalling pathways related to LTD can prevent the inhibition of LTP by Aβ oligomers, implying that induction of LTD pathways by Aβ could underlie, at least in part, the deficits seen in LTP (Wang et al. 2004).

Additionally, Aβ oligomers induce microglia and astrocyte activation, initiating chronic neuroinflammation in AD (Heneka et al. 2015). The reactive glial cells and resultant pro-inflammatory environment have been linked to synaptic degeneration and consequential cognitive decline (Rajendran and Paolicelli 2018), however, it is still unclear whether glial activation alone is necessary and/or sufficient to induce synaptic dysfunction and loss.

The primary mechanisms by which Aβ oligomers mediate early synaptic dysfunction are still incompletely understood. It has long been presumed that synaptic toxicity is likely to be initiated following Aβ oligomer binding to certain sites on the surface of particular neurons, and numerous candidate receptors for Aβ oligomers on the postsynaptic terminal have been proposed (Magdesian et al. 2008; Yaar et al. 1997; Knowles et al. 2009). However, none of the candidates comprise all the necessary characteristics of a true oligomer receptor, and none can account for all aspects of AD pathophysiology (Wilcox et al. 2011). In part due to these issues, the focus of research is beginning to shift towards events at the presynaptic terminal.

There is already a body of evidence implicating Aβ in presynaptic function. It has been reported that endogenous, physiological Aβ acts as a positive regulator of neurotransmitter release at the

presynaptic level. Increased extracellular Aβ levels are able to enhance probability of synaptic vesicle release and increase neuronal activity in the hippocampal network, albeit in a manner that is dependent on the history of activity at that synapse (Abramov et al. 2009). Pathological (mainly oligomeric) forms of Aβ have also been associated with alterations in presynaptic function, although the effects reported have been diverse and often contradictory (Li et al. 2009; Abramov et al. 2009; Nimmrich et al. 2008). This may reflect the fact that these studies have largely been carried out in less intact in vitro systems, and using a variety of assembly states of exogenously applied Aβ. Nonetheless, these findings raise the possibility that changes in presynaptic function driven by Aβ oligomers could play an early, upstream role in the pathological cascade. Interestingly, it has been shown that an Aβ oligomer-driven increase in extracellular glutamate is able to bring about both the loss of LTP (Li et al. 2011) and the induction of LTD at hippocampal synapses via activation of perisynaptic NR2B-containing NMDARs, which are normally required for LTD induction (Li et al. 2009; Liu et al. 2004). Although one of these studies found evidence for a failure of glutamate reuptake underlying this glutamate rise (Li et al. 2009), it could be hypothesised that increased synaptic release may have a similar effect.

Recent work in our lab has attempted to clarify the effects of Aβ oligomers on presynaptic terminals using optical assays, allowing for Aβ oligomer-induced changes in neurotransmitter release to be observed at individual presynaptic terminals without the confounds that accompany indirect postsynaptic measures such as electrophysiology (Jeans et al., Unpublished). Experiments have revealed that Aβ oligomers are able to produce an increase in action potential-evoked neurotransmitter release, which is dependent on increased Ca^{2+} entry through $Ca_v2.1$ presynaptic voltage-gated Ca^{2+} channels (VGCC). Upstream of this lies a novel signal transduction pathway in which Aβ elicits presynaptic Ca^{2+} influx through binding α7-nicotinic acetylcholine receptors (nAChR), stimulating insertion of epithelial sodium channels (ENaC) into the presynaptic membrane from a pre-existing intracellular pool. The increased influx of Na^+ causes a change in

the resting membrane potential and leads to increased $Ca_v2.3$ VGCC opening, to allow Ca^{2+} entry. The increased intracellular concentration of Ca^{2+} activates protein kinase C (PKC), which phosphorylates glycogen synthase kinase-3β (GSK-3β), thereby inactivating it. Since GSK-3β is a negative regulator of $Ca_v2.1$ channel function, the result is increased action potential-evoked Ca^{2+} entry through $Ca_v2.1$ channels and enhanced neurotransmitter release.

In the mammalian hippocampus, both P/Q-type ($Ca_v2.1$) and N-type ($Ca_v2.2$) VGCC mediate the presynaptic sources of Ca^{2+} influx (Wheeler, Randall, and Tsien 1994). The functional properties of the VGCCs differ at individual synapses (Ariel, Hoppa, and Ryan 2013), and it has been determined that homeostatic changes in evoked presynaptic Ca^{2+} currents and neurotransmitter release efficacy are regulated specifically through P/Q-type VGCCs (Jeans et al. 2017). It has been shown that Aβ oligomers directly increase P/Q-type Ca^{2+} channel current in an in vitro model, and such modulation can lead to excitotoxic neurodegeneration in AD (Mezler et al. 2012). In contrast, another study has demonstrated that Aβ oligomers inhibit presynaptic P/Q-type VGCC-mediated currents (Nimmrich et al. 2008). The effects of Aβ oligomers on presynaptic terminal activity are therefore currently still unclear.

Our laboratory has further shown that the enhancement of P/Q-type function and neurotransmitter release seems to be an early and influential event in Aβ oligomer toxicity that is required for many key synaptic and cognitive phenotypes associated with AD. This work has therefore identified $Ca_v2.1$ P/Q-type VGCC as a potential target for therapeutic intervention in AD. Since both the loss of LTP and the induction of LTD at excitatory synapses caused by Aβ oligomers, important and related elements of synaptic toxicity, have been linked to increased extracellular glutamate levels, we hypothesised that increased neurotransmitter release mediated by P/Q-type VGCC may be at least partly responsible. While it is not readily possible to completely remove P/Q-type VGCC in order to test this hypothesis, due to the profound effects on

neurotransmission, it has previously been shown in our laboratory that heterozygous knockout (*Cacna1a*$^{+/-}$) of the channel greatly decreases its contribution to neurotransmission whilst retaining normal synaptic function. We therefore hypothesised that P/Q-type heterozygous knockout in presynaptic terminals, being less dependent on these channels for their functioning, should be less sensitive to the effects of Aβ oligomers, and these experiments will therefore attempt a rescue of LTP via this manipulation at Aβ oligomer-treated synapses.

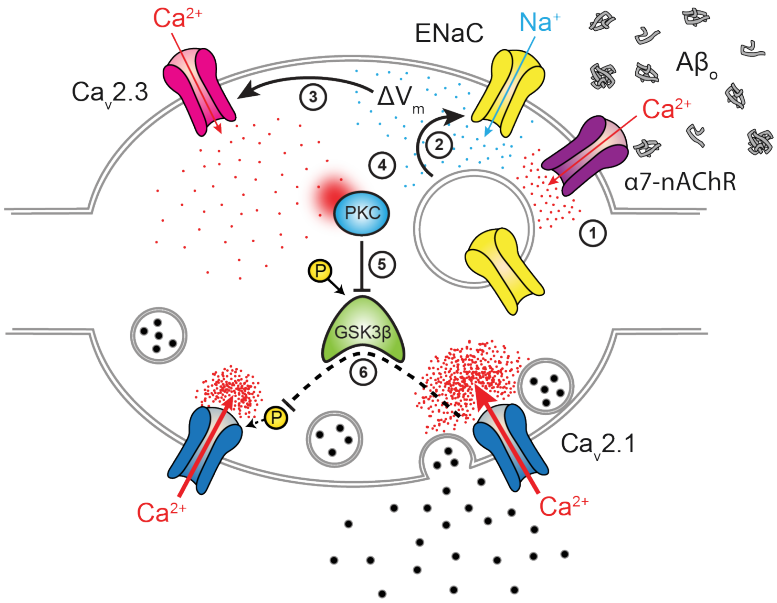

Figure 3.1: Mechanistic model for enhancement of neurotransmitter release by Aβ. Arrows and blunted lines indicate activating and inhibitory processes respectively, and a dashed line indicates inhibition by Aβ. Our data support a model in which pathological Aβ elicits presynaptic Ca^{2+} entry via α7-nAChR (1) to drive insertion of an intracellular pool of ENaC channels into the presynaptic membrane (2). This causes an increase in Na^+ influx and a resulting change in presynaptic resting membrane potential ΔVm, which enhances $Ca_V2.3$ VGCC opening to elevate resting [Ca^{2+}] at the presynaptic terminal (3). This [Ca^{2+}] increase activates protein kinase C (PKC) (4), a negative regulator of GSK-3β, increasing the fraction of phosphorylated, inactive GSK-3β (5) and thereby inhibiting GSK-3β-mediated negative regulation of $Ca_V2.1$ function (6). The result is increased Ca^{2+} influx via $Ca_V2.1$ channels, with enhancement of activity-evoked neurotransmitter release. Reproduced from (Jeans et al., Unpublished).

3.2. Results

3.2.1. $Ca_v2.1$ heterozygous knockout causes no differences in intrinsic presynaptic function

For these experiments, I performed extracellular field potential recordings from Schaffer collateral-CA1 synapses in acute hippocampal slices from 7-8 week old C57BL/6 mice. Firstly, it was necessary to determine if the heterozygous P/Q-type VGCC knockout (*Cacna1a*$^{+/-}$) caused any differences in intrinsic presynaptic function in comparison to wild-type presynaptic terminals. Paired-pulse facilitation (PPF) is a form of short-term synaptic plasticity, which can be used as an index of probability of neurotransmitter release (Pr) at the presynaptic terminal. PPF occurs when two presynaptic action potentials are evoked in close succession and the postsynaptic response to the second action potential is increased in comparison to the first. It is widely accepted that this facilitation is due to residual Ca^{2+} at the presynaptic terminal from the first action potential, which contributes to enhanced release and a consequently larger postsynaptic response following the second action potential (Catterall, Leal, and Nanou 2013). Facilitation is quantified by calculating the paired pulse ratio (PPR), the ratio of the slope of the second field excitatory postsynaptic potential (fEPSP) divided by the slope of the first (fEPSP2/fEPSP1). No significant difference was observed between the PPRs of wild-type and *Cacna1a*$^{+/-}$ hippocampal slices (Figure 3.2A), suggesting that the heterozygous knockout of the channel does not impact on basal Pr.

The overall strength of basal synaptic transmission was measured with an input-output analysis of fEPSP slope versus increasing stimulus strength. Both wild-type and *Cacna1a*$^{+/-}$ slices demonstrated similar basal synaptic transmission, with the slope of fEPSPs increasing as the stimulus intensity was increased (Figure 3.2B). This analysis further validated that a heterozygous knockout of the channel does not alter intrinsic synaptic function.

To determine if the induction of LTP caused changes in presynaptic release probability, PPR was measured before and after LTP induction. It was previously shown that PPR does not change with the LTP protocol used, and this is supported by the results obtained. In both wild-type and *Cacna1a*$^{+/-}$ experiments, PPR did not change significantly after LTP induction, representing no change in presynaptic release probability (Figure 3.2C).

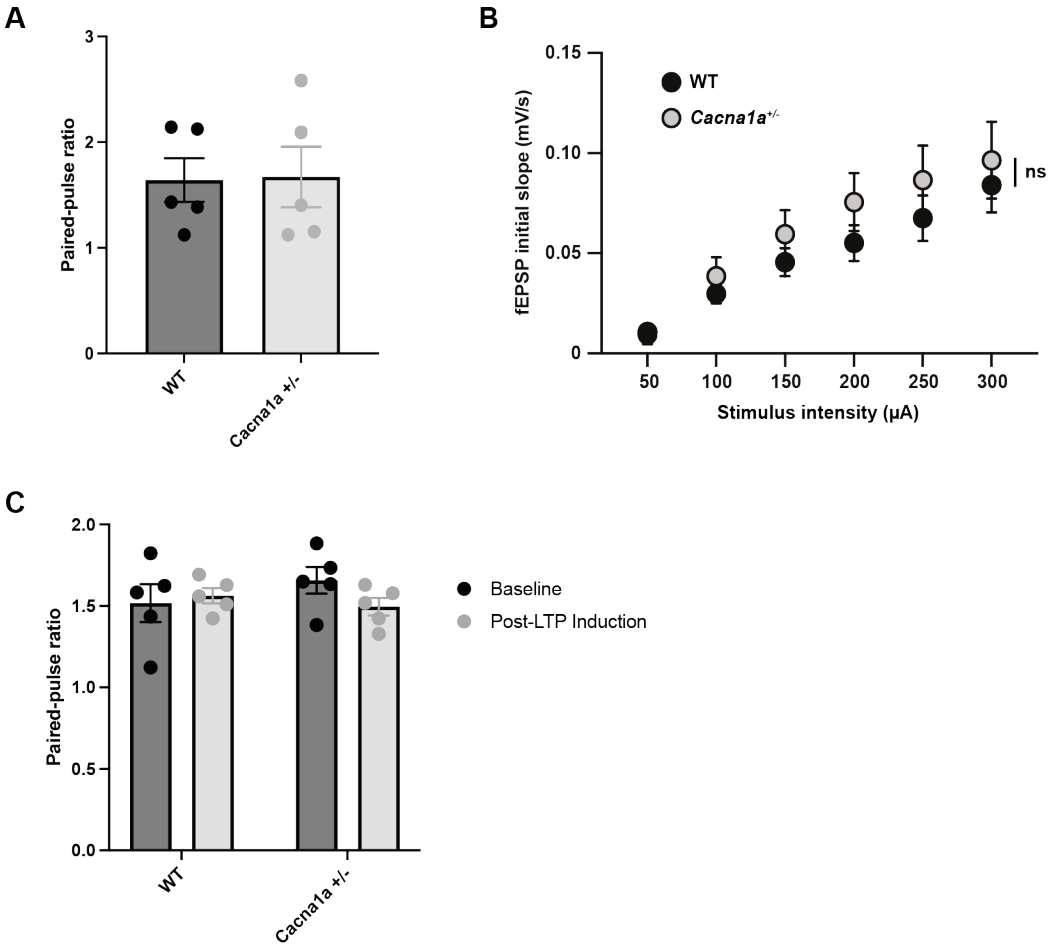

Figure 3.2: *Cacna1a*$^{+/-}$ CA1 neurons in acute hippocampal slices demonstrate normal synaptic neurotransmission. **a)** Paired-pulse ratio (PPR) assessed in hippocampal slices from wild-type and *Cacna1a*$^{+/-}$ mice (both n = 5 slices from 5 mice). Unpaired Student's t-test. **b)** Input-output relationship at increasing stimulus intensities in hippocampal slices from wild-type and *Cacna1a*$^{+/-}$ mice (both n = 5 slices from 5 mice). Repeated measures two-way ANOVA. **c)** PPR assessed in hippocampal slices from wild-type and *Cacna1a*$^{+/-}$ mice (both n = 5 slices from 5 mice) before and after LTP induction. Repeated measures two-way ANOVA.

3.2.2. $Ca_v2.1$ heterozygous knockout rescues LTP impairment caused by soluble Aβ oligomers

LTP was induced by theta burst stimulation, which is considered to be a physiologically-relevant protocol (Larson and Munkácsy 2015) (Figure 3.3A). Wild-type slices showed strong potentiation that remained stable for 1 hour of recording (160.3 ± 4.852 % of baseline fEPSP, n=6). However, LTP was strongly suppressed by exogenous application of Aβ oligomers (10 nM) (117.3 ± 1.951 % of baseline fEPSP, n=7), and this suppression was stable throughout the 1 hour of LTP recording. This is entirely in keeping with previous reports (Shankar et al. 2008; Li et al. 2011). The difference between the mean fEPSP slopes of wild-type and Aβ-incubated slices 60 minutes after LTP induction was highly significant ($p<0.0001$).

Careful consideration was given to the concentration of Aβ oligomers used in experiments. While CSF samples from AD patients exhibit total $A\beta_{1-42}$ values in the a range 0.1-0.3 nM (Andreasen et al. 1999; Gustafson et al. 2007), Aβ oligomers have been measured at 11 pM in the interstitial fluid of aged APP transgenic mice (Takeda et al. 2013). However, these measurements may not accurately reflect local concentrations in regions of synaptic pathology, where Aβ concentrations are significantly higher near amyloid plaques that act as a reservoir, releasing soluble oligomeric Aβ into the surrounding space (Koffie et al. 2009). Consequently, we reasoned that 10 nM of Aβ represented an appropriate choice for our experiments, as even higher concentrations show neither acute nor sub-chronic toxicity in neuronal cultures (data not shown), nor do they alter cellular ATP levels (Berman et al. 2008).

Cacna1a$^{+/-}$ hippocampal slices showed strong potentiation of the fEPSP in response to the theta burst induction protocol, with no significant difference in the magnitude of LTP between *Cacna1a*$^{+/-}$ and wild-type experiments. Both wild-type and *Cacna1a*$^{+/-}$ experiments showed a significant difference ($p<0.0001$) in mean fEPSP slopes compared with wild-type slices incubated

with Aβ (Figure 3.3B). The application of Aβ oligomers (10 nM) produced no significant difference in LTP response in the *Cacna1a*$^{+/-}$ hippocampal slices, in comparison with either wild-type or *Cacna1a*$^{+/-}$ hippocampal slices alone. Soluble Aβ-induced attenuation of LTP was therefore rescued by the heterozygous knockout of $Ca_v2.1$ with fEPSP slopes showing a return to control levels (175.4 ± 3.918 % of baseline fEPSP, n=5), and a significant difference ($p<0.0001$) detected between mean fEPSP slopes of the hippocampal slices from *Cacna1a*$^{+/-}$ mice with Aβ oligomers, and wild-type slices incubated with Aβ oligomers.

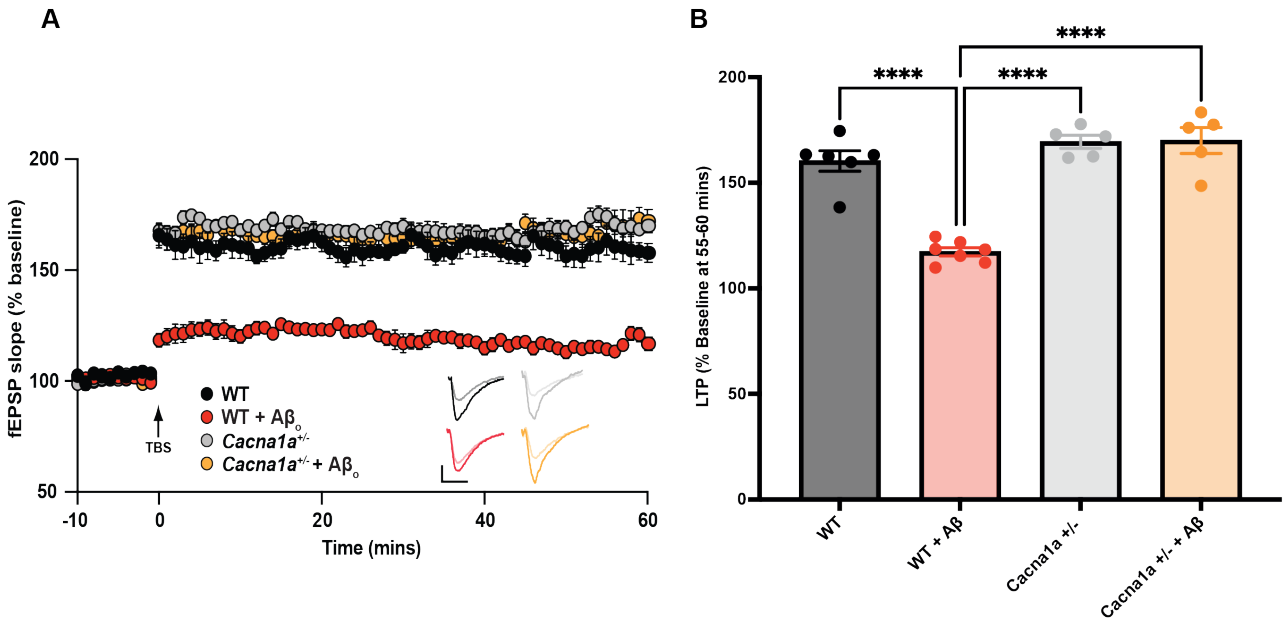

Figure 3.3: *Cacna1a*$^{+/-}$ hippocampal slices are resistant to Aβ oligomer-induced inhibition of LTP. **a)** Traces showing LTP following theta burst stimulation (TBS) in wild-type and *Cacna1a*$^{+/-}$ mice treated as indicated. Representative traces show fEPSPs before (faint) and after (bold) LTP induction. Scale bars: 0.5 mV and 5 ms. **b)** Mean fEPSP slopes 55-60 minutes after LTP induction (% of baseline). WT (n = 6 slices from 6 mice), WT + Aβ (n = 7 slices from 7 mice), *Cacna1a*$^{+/-}$ (n = 5 slices from 5 mice), *Cacna1a*$^{+/-}$+ Aβ (n = 5 slices from 5 mice). One-way ANOVA with post-hoc Tukey's multiple comparison test. ****$p<0.0001$.

3.2.3. $Ca_v2.1$ heterozygous knockout in J20 mice prevents cognitive impairment independent of glial proliferation

To investigate the role of $Ca_v2.1$ activity and its contribution to synaptic function in AD pathogenesis in a more intact model, the extensively used and well-established 'J20' AD mouse model was used. J20 mice overexpress human APP with two mutations linked to familial AD (Swedish and Indiana mutations) (Mucke et al. 2000). Previous results corroborated the effects of Aβ oligomers, demonstrating enhanced neurotransmitter release in J20 mice (Jeans et al., Unpublished). The J20 mice also presented with decreased dendritic spine density (Supplementary Figure 1) and learning and memory impairment (Jeans et al., Unpublished). To determine whether the *Cacna1a*$^{+/-}$ knockout is able to normalise neurotransmitter release and prevent the development of disease-relevant phenotypes, the hAPP J20/*Cacna1a*$^{+/-}$ mouse line was created. The performance of these mice was unimpaired in tests of hippocampus-dependent learning and memory, dendritic spine density was similar to that of wild-type mice, and *Cacna1a*$^{+/-}$ knockout prevented premature mortality of J20 mice (Jeans et al., Unpublished).

Current evidence presents AD as a complex series of events with numerous components, and interactions between different cell types, such as neurons and glial cells (Ibrahim et al. 2020). The sequence of events in AD pathogenesis is still unclear, and it is not completely known how changes that occur during the early stages of AD impact further downstream changes, which drive cognitive decline (Selkoe and Hardy 2016). A prominent characteristic of AD, which is also exhibited in J20 mice, is a significant increase in the number of reactive astrocytes and microglia, which are able to degrade synapses, in the hippocampus (Wright et al. 2013). Although, it is not entirely understood how Aβ triggers glial activation, and whether activated astrocytes and microglia are essential for synaptic degeneration (Ibrahim et al. 2020). Studying neuroinflammation in the hAPP J20/*Cacna1a*$^{+/-}$ mouse line, which is synaptically and cognitively preserved whilst presumably maintaining pathological levels of Aβ with accompanying tissue pathology, offers potential insight

into this question. Accordingly, I labelled brain sections with the astrocytic marker glial fibrillary acidic protein (GFAP) and microglial marker ionized calcium-binding adapter molecule 1 (Iba1) to study neuroinflammatory changes in the brains of these mice.

As expected, 6-8 month old J20 mice showed a significantly increased number of astrocytes (543.9 ± 11.77; n=4; **p<0.01) and microglia (271.6 ± 10.11; n=4; *p<0.05) in the CA1 region of the hippocampus compared to wild-type mice (astrocytes 432.9 ± 22.94; n=5; microglia 247.5 ± 6.38; n=5) (Figure 3.4B,D). Interestingly, compared with wild-type mice, the J20/*Cacna1a*$^{+/-}$ mice also exhibited an increase in the number of astrocytes (520.5 ± 7.22; n=5; **p<0.01) and microglia (280.1 ± 6.54; n=5; *p<0.05), similar to the J20 mice. Additionally, the astrocytes and microglia in both J20 and J20/*Cacna1a*$^{+/-}$ mice showed morphological changes including the development of thickened processes, which is typical of activated glial cells. These results demonstrate that neuroinflammatory pathology is retained despite the rescue of synapse loss in J20/*Cacna1a*$^{+/-}$ mice, indicating that synaptic decline and neuroinflammation, both critical processes in AD progression, can be dissociated.

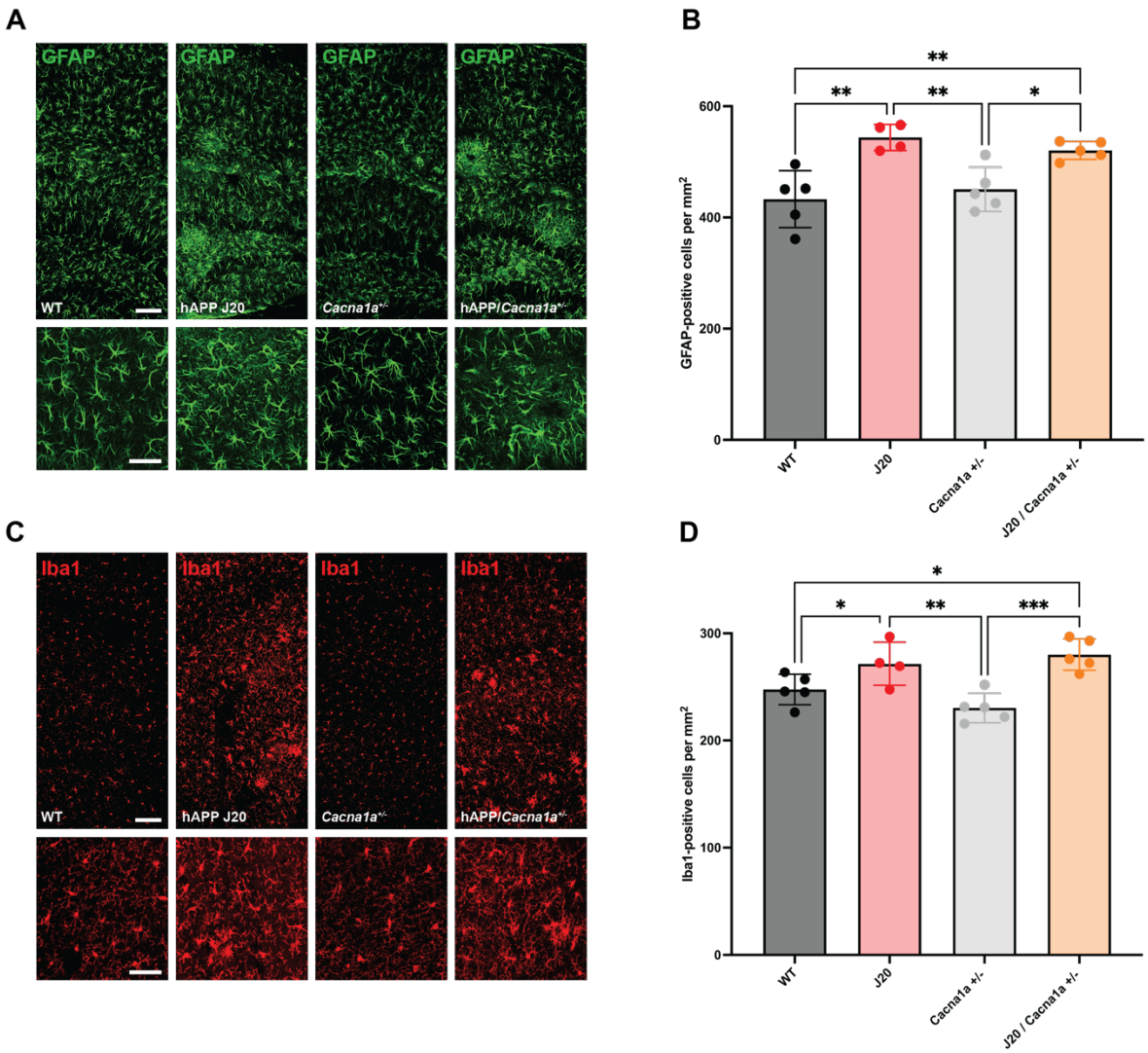

Figure 3.4: Partial block of $Ca_v2.1$ has no effects on glial proliferation in hAPP J20 mice. **a)** Representative images of the CA1 region of 6-8 month old wild-type, hAPP J20, *Cacna1a*$^{+/-}$, and hAPP/ *Cacna1a*$^{+/-}$ mice labelled for the astrocytic marker GFAP. Scale bar = 100 μm. **b)** Quantification of GFAP-positive cells in a 1 mm^2 area. Cells were quantified across two sections per mouse, in two fields of view per section. WT (n = 5 mice), J20 (n = 4 mice), *Cacna1a*$^{+/-}$ (n = 5 mice), J20/*Cacna1a*$^{+/-}$ (n = 5 mice). One-way ANOVA with Tukey's multiple comparison test. **c)** Representative images of the CA1 region of 6-8 month old wild-type, hAPP J20, *Cacna1a*$^{+/-}$, and hAPP/ *Cacna1a*$^{+/-}$ mice labelled for the microglial marker Iba1. Scale bar = 100 μm. **d)** Quantification of Iba1-positive cells in a 1 mm^2 area. Cells were quantified across two sections per mouse, in two fields of view per section. WT (n = 5 mice), J20 (n = 4 mice), *Cacna1a*$^{+/-}$ (n = 5 mice), J20/*Cacna1a*$^{+/-}$ (n = 5 mice). One-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

3.3. Discussion

The results presented in this Chapter contribute to a larger research paper, which uncovers a new presynaptic signalling pathway, which converges on the $Ca_v2.1$ channel (Jeans et al., Unpublished). This new pathway sheds light on the mechanisms behind Aβ oligomer-induced synaptic dysfunction and the connection with synapse loss and cognitive decline. A heterozygous knockout of the gene coding for the $Ca_v2.1$ channel (*Cacna1a*$^{+/-}$) was able to prevent the effects of Aβ oligomers by rescuing the enhanced presynaptic Ca^{2+} influx and resulting potentiation of neurotransmitter release, which resulted in the normalisation of synaptic function and mitigation of numerous AD phenotypes, such as synapse loss, cognitive decline, and premature mortality (Jeans et al., Unpublished). It is clear that the enhanced function of $Ca_v2.1$ channels plays a crucial role in the development of AD pathology.

Since it has previously been shown that P/Q-type VGCCs are critical mediators of basal fast neurotransmission at glutamatergic terminals, as well as being necessary for presynaptic homeostatic changes in Ca^{2+} currents and neurotransmitter release (Jeans et al. 2017), complete removal of these channels would result in very severe impairments in synaptic transmission which are incompatible with an unbiased experiment. However, the heterozygous knockout of $Ca_v2.1$ significantly decreased the contribution of these channels to neurotransmission whilst allowing for preservation of normal basal neurotransmission.

Experiments had to be performed to assess any changes in basal synaptic function that may introduce confounding factors. PPR analysis was used to evaluate changes in short-term presynaptic plasticity and intrinsic presynaptic function between wild-type and *Cacna1a*$^{+/-}$ presynaptic terminals. Most synapses exhibit multiple types of presynaptic plasticity, including depression and facilitation, and net synaptic potency reflects the interaction between these

different forms of plasticity (Dittman, Kreitzer, and Regehr 2000). The action potential-evoked influx of Ca^{2+} plays a prominent role in many forms of synaptic plasticity, and presynaptic plasticity is controlled by initial neurotransmitter release probability and presynaptic activity pattern (Dittman, Kreitzer, and Regehr 2000). It is perhaps surprising, then, that no significant difference was observed in PPRs between *Cacna1a*$^{+/-}$ and wild-type presynaptic terminals, providing evidence that there is no apparent difference in presynaptic function and release efficacy. Since both P/Q-type and N-type VGCCs mediate presynaptic Ca^{2+} influx (Ishikawa et al. 2005), N-type VGCCs may compensate for the heterozygous knockout of P/Q-type channels. Alternatively, functional up-regulation of a variety of other presynaptic and active zone proteins could account for the preservation of presynaptic function, and indeed this has been found by transcriptomic and functional analysis of homozygous P/Q-type knockout neurons in culture (Piedras-Renteria et al. 2004).

PPR analysis was also performed before and after LTP induction and represents a relative measure of presynaptic release probability. Our results showed no significant difference in PPR before and after LTP induction, suggesting no change in presynaptic release probability. This is a rather controversial topic in the field, relating to the question of whether LTP is mediated by enhanced presynaptic release or enhanced postsynaptic function, with various studies reporting opposite results. Previous experiments have shown that average PPF is not altered by the induction of LTP (Zalutsky and Nicoll 1990; Manabe et al. 1993; Schulz, Cook, and Johnston 1995). However, another study used a fluorescent marker of presynaptic activity, FM 1-43, to image changes in presynaptic activity during LTP induction, and found direct evidence for altered presynaptic function (Zakharenko et al. 2003). Since PPF is an indirect, postsynaptic measure of presynaptic release probability, it may be more reliable to use direct measures of presynaptic function such as FM 1-43 dye. The dye is loaded into the presynaptic terminals of CA3 neurons, which are then electrically stimulated to unload the dye and determine the average fluorescent decay constant,

with faster unloading confirming enhancement of release (Kavalali and Jorgensen 2014). Changes in PPR are not exclusively mediated by alterations in presynaptic release probability. PPR may be influenced by modifications of short-term plasticity, which impact PPR but are not associated with a change in presynaptic release probability (Geppert et al. 1997). Furthermore, PPR changes may be masked by AMPAR desensitisation, since it has been shown that changes in PPR induced by Aβ are only evident in the presence of cyclothiazide, a positive allosteric modulator of AMPARs that prevents desensitisation (Jeans et al., Unpublished).

Additionally, overall basal synaptic neurotransmission was studied in wild-type and *Cacna1a*$^{+/-}$ neurons by progressively increasing the stimulus intensity and measuring the changes induced in fEPSP slope. Synaptic neurotransmission was preserved, and *Cacna1a*$^{+/-}$ synapses were indistinguishable from wild-type.

To determine if a heterozygous knockout of $Ca_v2.1$ is able to rescue the LTP impairment observed in the presence of Aβ oligomers, fEPSP recordings were obtained from acute hippocampal slices. Aβ oligomer-induced inhibition of LTP has been clearly demonstrated in numerous previous studies, and this loss of LTP has been associated with an increase in extracellular glutamate concentration, which can perturb LTP induction and facilitate LTD by activating extra-synaptic NR2B-containing NMDARs (Li et al. 2009; Li et al. 2011). Furthermore, the overall expression level of NMDARs is reduced in the context of AD, suggesting an adaptive change as a long-term consequence of excess glutamate neurotransmission and excitotoxity (Hynd, Scott, and Dodd 2001). Since previous results demonstrated that Aβ oligomers cause an increase in action potential-dependent neurotransmitter release, this led to a hypothesis that the $Ca_v2.1$-mediated increase in synaptic neurotransmitter release may contribute towards the impairments in LTP seen in models of AD. Since a heterozygous knockout of P/Q-type VGCCs significantly decreases their

contribution to neurotransmission (Jeans et al., Unpublished), I predicted that LTP deficits could be rescued to some degree by this manipulation.

The persistent increase in presynaptic efficacy induced by pathological levels of soluble Aβ oligomers is not subject to homeostatic regulation, and consequently, oligomer-enhanced release is sustained at a high level (Jang and Chung 2016). Excessive release eventually results in the gradual weakening of synapses, and then progressing to synapse loss (Jeans et al., Unpublished), possibly via mechanisms of chronic long-term depression (LTD), which may lead to the cognitive phenotypes seen in AD. Another consequence of the sustained increase in presynaptic activity would be an increase in Aβ production, due to the activity-dependent release of Aβ (Cirrito et al. 2005). The increase in Aβ production would facilitate Aβ oligomer assembly and create a positive feedback loop to maintain and worsen the hyperactivity, suggesting a specific enhancement of glutamatergic neurons by Aβ oligomers.

Cacna1a$^{+/-}$ hippocampal slices treated with Aβ oligomers were hypothesized to be less sensitive to the effects of Aβ oligomers owing to their reduced dependence on $Ca_v2.1$, and although a rescue of LTP was predicted, it was considered likely to be partial due to the incomplete nature of the manipulation. However, a full rescue was seen with LTP magnitude returning to control levels, suggesting that a heterozygous knockout of $Ca_v2.1$ is sufficient to rescue the impairment in LTP. This may be because the effects of soluble Aβ oligomers on the remaining channels produce a much more modest increase in Ca^{2+} influx, so that presynaptic Ca^{2+} concentrations do not reach the threshold for recruiting the mechanisms of enhancement.

Aβ oligomers induce a neuroinflammatory response, with the recruitment and activation of astrocytes and microglia (Mun, Park, and Choi 2024; Meda et al. 1995). My results demonstrated that in the J20/*Cacna1a*$^{+/-}$ mice, synaptic dysfunction can be rescued even in the presence of glial

activation. This suggests that the neuroinflammation present in J20 mice is not sufficient for Aβ oligomer-induced synaptic impairment. Previous studies have shown that activated microglia can degrade synapses in AD pathology (Rajendran and Paolicelli 2018), however, it is unknown whether activated microglia in AD are sufficient to cause a significant loss of synapses. By introducing the *Cacna1a*$^{+/-}$ genotype in the J20 mice, evoked synaptic vesicle exocytosis was normalised and several AD-related phenotypes were rescued despite the increased number of microglia and astrocytes (Jeans et al., Unpublished). The interaction of Aβ oligomers with the signalling pathway described previously is necessary to cause a decrease in dendritic spine density, however, microglia and astrocytes may play a subsequent role in downstream pathophysiological processes. Furthermore, a previous study has demonstrated that Aβ oligomers bound to synapses may anchor C1q, the initiating protein of the classical complement cascade, to synapses, or expose a C1q receptor, allowing microglia to eliminate synapses through the complement pathway (Hong et al. 2016). This suggests that microglia are downstream effectors of synapse removal, however, this process is dependent on the actions of Aβ at the synapse, and if this is prevented, the inflammation is not significant for synapse loss. In this Chapter, I have not looked further into the neuroinflammatory response, however, future studies should identify the cellular state of activated microglia and astrocytes by measuring gene expression of disease-associated microglial and astrocytic genes. Furthermore, future research should determine if these results are translatable to other mouse models of AD, including models which feature both pathogenic tau and Aβ oligomers.

The P/Q-type VGCC $Ca_v2.1$ is an appealing target for pharmacological manipulation of presynaptic function since it is specific to presynaptic terminals, while the other mechanistic elements identified serve multiple roles in other processes. A heterozygous knockout of the channel suppresses its involvement in neurotransmission, while leaving synaptic input-output relationships and probability of release unchanged. This supports the idea that it might be a valid

therapeutic target, although, with the caveat that presynaptic mechanisms of compensation for the missing $Ca_v2.1$ channel may be more readily recruited developmentally in a constitutive knockout animal than in a more mature system in which the channels have been acutely blocked pharmacologically. The P/Q-type channel is characterised by the α_{1A} subunit, which functions as the conducting channel and voltage sensor (Nimmrich and Gross 2012). The *Cacna1a* gene, which encodes the pore-forming subunit of the channel, has multiple splice variants that are differentially distributed throughout the CNS (Nimmrich and Gross, 2012). Targeting a particular splice variant expressed in the brain region of interest, such as the hippocampus, may represent a further improved approach for drug development to bypass off-target effects. Currently, the expression pattern of the various isoforms is incomplete, however, it has been reported that variant $\alpha_{1A\text{-}b}$ shows predominant expression in the hippocampus and could represent a potential target in the treatment of AD (Bourinet et al. 1999). Additionally, state-dependent P/Q-type VGCC blockers, which bind to the inactivated state of the channel at overactive synapses, may be useful in the pathological context of AD with increased presynaptic activity (Nimmrich and Gross, 2012).

In the study by Jeans et. al, the identification of a role for ENaC in the pathway engaged by Aβ is also noteworthy, as it is the target of potassium-sparing diuretics, such as amiloride, which is typically used to treat hypertension and heart failure (Teiwes and Toto 2007). Studies have discovered an inverse association between the use of diuretics in hypertensive patients and the incidence of AD (Chuang et al. 2014). There have been relatively few studies dedicated to investigating ENaC expression and function in the CNS, therefore, more studies are needed to develop our understanding of the role of ENaC in the pathogenesis of AD.

In conclusion, my results show that alterations in presynaptic Ca^{2+} channel function are a key event in early AD pathogenesis, and specific manipulations of $Ca_v2.1$ may protect from Aβ oligomer-induced toxicity, including synaptic and cognitive decline.

4. Activity-dependent lysosomal Ca^{2+} release and the regulation of structural plasticity of hippocampal neurons in AD

4.1. Introduction

Lysosomes are membrane-bound organelles containing an array of enzymes in an acidic lumen environment, and act as the digestive system of the cell by digesting macromolecules (Settembre et al., 2013). Lysosomes are critically involved in protecting neurons from the build-up of malfunctioning or misfolded proteins and damaged organelles (Saftig & Klumperman, 2009). Lysosomes also function as intracellular stores for Ca^{2+}, and membrane proteins, which function as Ca^{2+}-conducting channels are responsible for the release of Ca^{2+} from lysosomes, required for lysosomal fusion and exocytosis (Gómez et al., 2018). Lysosomal Ca^{2+} release is reported to be mediated by mucolipin 1 (MCOLN1) (Xu & Ren, 2015) and Two-Pore Channels (TPCs) (Ruas et al., 2015). Ca^{2+} release from TPCs is regulated by the second messenger NAADP, which is synthesised by ADP-ribosyl cyclases in response to specific physiological stimuli (Galione, 2015). Lysosomal Ca^{2+} signalling is likely to play an important role in neuron function as it has been shown to promote neuronal differentiation (Brailoiu et al., 2006), drive spontaneous neurotransmitter release (Brailoiu et al., 2003) and aid membrane repair processes via lysosomal Ca^{2+}-dependent exocytosis (Reddy et al., 2001).

Neurons are highly dependent on optimal lysosome function due to their demands of metabolic activity and neurotransmission (Ferguson, 2019). This dependence is highlighted by lysosome storage diseases (LSDs), which link lysosomal dysfunction with progressive neurodegeneration (Platt et al., 2018). Furthermore, Niemann-Pick disease type C, an LSD characterised by the accumulation of substrates in lysosomes, is sometimes referred to as 'childhood Alzheimer's' since

the condition involves progressive dementia (Nixon 2004). Lysosomes are most prominently found in neuronal cell bodies and proximal dendrites, and relatively rarely in axons (Ferguson, 2019). Lysosomes also play a central role in the degradation of integral membrane proteins, such as glutamate receptors (Ehlers, 2000). The endosomal trafficking of AMPARs to lysosomes for degradation is vital for AMPAR downregulation in the context of synaptic plasticity, such as LTD (Fernández-Monreal et al., 2012). The stimulation of NMDA and AMPA receptors increases the abundance of lysosomes within dendritic spines, this provides further evidence for lysosome trafficking to sites of activity and local degradation involved with receptor turnover (Goo et al. 2017). In addition to the degradative function of lysosomes, these organelles participate in exocytosis and subsequent remodelling of the extracellular matrix and regulating long-term structural plasticity (Padamsey et al., 2017). The contribution of lysosomes to the architecture of dendrites has also been demonstrated by abnormal dendritic arborization in *Drosophila* mutants for a lysosome membrane transporter (Lin et al., 2015). These studies highlight novel functions of lysosomes as important drivers of localised synaptic remodelling which is associated with synaptic plasticity.

Research has provided abundant evidence implicating lysosome dysfunction in a number of neurological diseases including Alzheimer's Disease (AD) (Wang et al., 2018), however, the molecular mechanisms underlying the contribution of lysosomes to the cellular pathology seen in these diseases is still being elucidated. Autophagy has been reported to be impaired in AD, resulting in an increase in the number and size of lysosomes in AD neurons, with lysosomes accumulating within the distal ends of swollen axons (Nixon et al., 2005). The significant enlargement of endosomal compartments, containing low levels of multiple lysosomal proteases, precedes the appearance of amyloid plaques (Morena et al., 2017; Nixon & Yang, 2011). Evidence has linked the failure of the lysosome autophagy system to the increase in amyloidogenesis, neuritic dystrophy and apoptosis (Yu et al., 2005; Yang et al., 2008). However, manipulating the autophagy

system may become counterproductive during these late stages in the face of lysosome clearance deficits, by overburdening the already failing system and exacerbating autophagic accumulation (Nixon & Yang, 2012). Reports have shown that autophagy induction may activate the amyloid pathway and increase Aβ secretion in more advanced stages of the disease (Yu et al., 2005; Nilsson & Saido, 2014), further providing evidence to support the claim that inducing autophagy may be ineffective. Furthermore, AD-related mutations in Presenilin 1 (PS1) have been shown to result in failure of V-ATPase specific subunit maturation and assembly of the complex, necessary for lysosomal acidification and protease activity (Lee et al., 2015). This impairment also contributes to the alterations in Ca^{2+} homeostasis, specifically the increased efflux of Ca^{2+} from lysosomes resulting in higher cytosolic levels of Ca^{2+} (Bezprozvanny, 2012). Defective lysosome Ca^{2+} storage and release may likely have a multifactorial origin, including contributions from Aβ neurotoxicity, and may contribute to impaired lysosome fusion and disrupted normal neuronal function (LaFerla, 2002).

Previous work in our lab (Padamsey et al., 2017) has shown that back-propagating action potentials trigger Ca^{2+} release from lysosomes in hippocampal pyramidal neurons. This Ca^{2+} release is a critical trigger for the fusion of lysosomes with the plasma membrane, resulting in the release of Cathepsin B, which increases the activity of matrix metalloproteinase 9 (MMP-9), an enzyme involved in extracellular matrix remodelling and synaptic plasticity (Figure 4.1). These findings suggest that activity-dependent exocytosis of Cathepsin B from lysosomes regulates the long-term structural plasticity of dendritic spines. Persistent dendritic spine enlargement is associated with stable LTP, and MMP-9, which is activated after LTP induction, is both necessary and sufficient to drive spine enlargement and synaptic potentiation (Wang et al., 2008). As dendritic spine dysfunction, LTP deficits, and lysosome impairment are well-documented features of AD pathology, we hypothesised that lysosome-mediated structural remodelling may also be dysregulated in AD models.

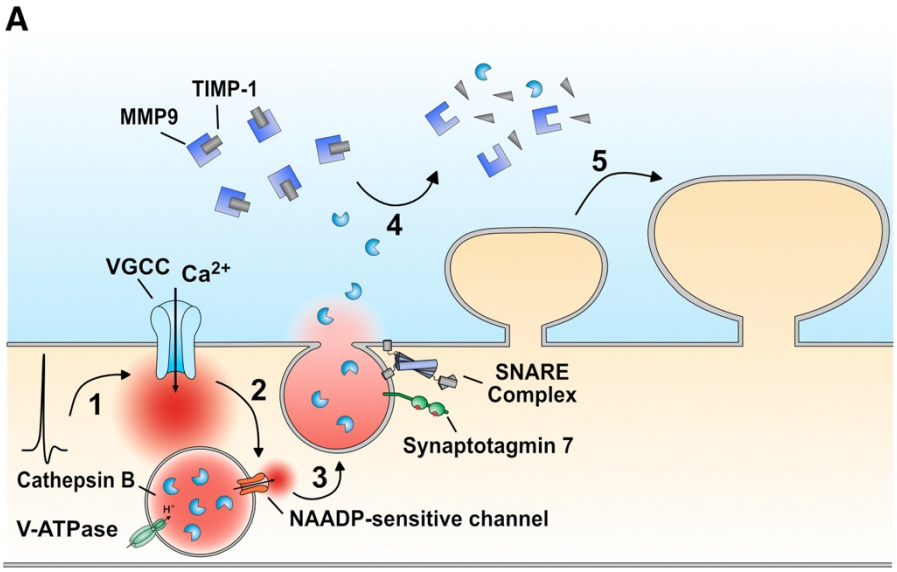

Figure 4.1: Schematic diagram of proposed model of MMP-9-mediated long-term structural plasticity. 1. Back-propagating action potentials activate dendritic voltage-gated calcium channels VGCCs. 2: VGCC-mediated Ca^{2+} influx triggers Ca^{2+} release from the lysosome via an NAADP-sensitive channel. 3: lysosomal Ca^{2+} release triggers fusion of the lysosome with the plasma membrane, resulting in the release of Cathepsin B. 4: Cathepsin B cleaves tissue inhibitor of metalloproteinase 1 (TIMP-1), releasing MMP-9 from inhibition. 5: MMP-9 activity maintains the long-lasting structural plasticity of dendritic spines. Reproduced from (Padamsey et al., 2017).

4.2. Results

4.2.1. The effect of Aβ oligomers on lysosome dynamics

Due to the lysosomal dysfunction observed in AD, we might expect to see differences in their number and dynamics. To investigate the acute effects of Aβ oligomers on lysosome accumulation, dissociated hippocampal neurons were incubated with Aβ oligomers (200 nM) for two hours and then with LysoTracker yellow (50 nM) for one hour. LysoTracker fluorescence was measured in neurons on coverslips and there was no significant difference between control (82.52 ± 3.1 AFU, n = 5) and Aβ-treated coverslips (84.26 ± 3.0 AFU, n = 5) (Figure 4.2B). Glycyl-L-phenylalanine 2-naphthylamide (GPN) was used to disrupt lysosome labelling to confirm the specificity of

LysoTracker. GPN is degraded within lysosomes by Cathepsin C (Berg et al., 1994). The cleavage product becomes membrane impermeable, generating an osmotic effect and rupturing the lysosomal membrane (Berg et al., 1994). GPN treatment (200 μM for 10 mins) abolished the majority of LysoTracker labelling in neurons (12.78 ± 1.6 AFU, n = 5) with a significant difference compared with control and Aβ-treated coverslips ($p<0.0001$), suggesting the specificity of LysoTracker for lysosomes.

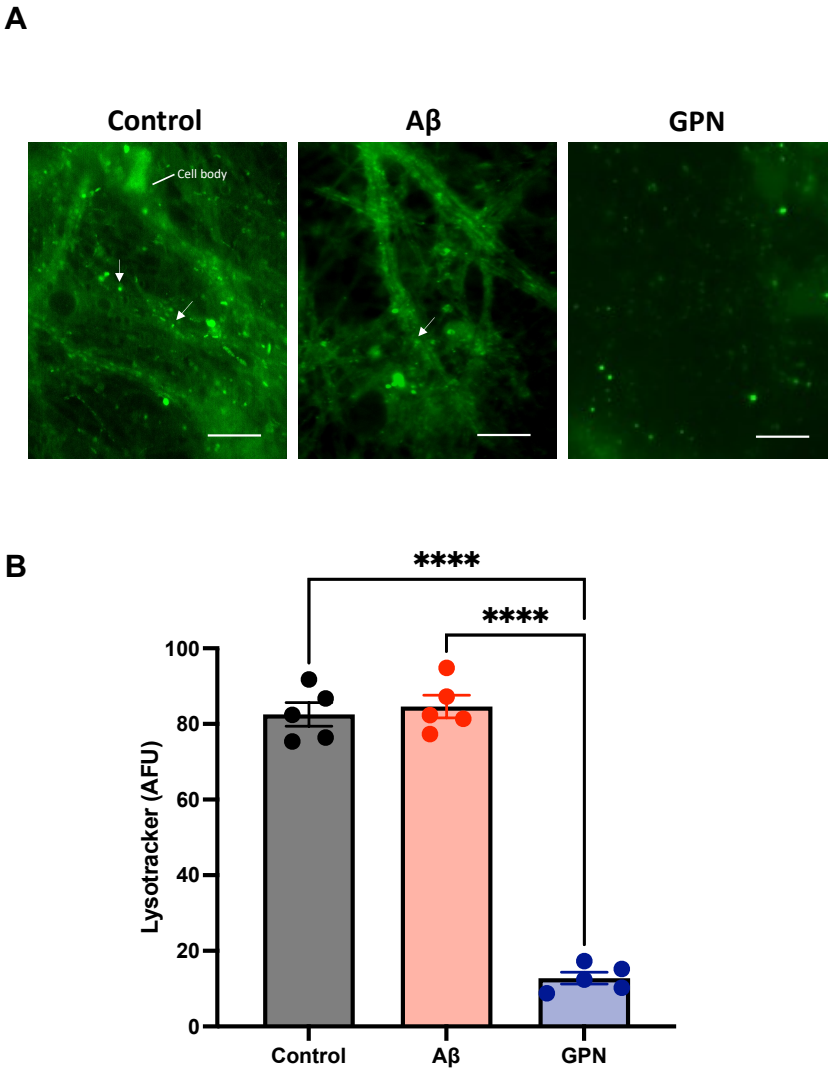

Figure 4.2: Treatment with Aβ oligomers produced no change in lysosome number. **a)** Representative fields of dissociated hippocampal neurons incubated with LysoTracker under various conditions (scale bar = 5 μm). Arrows highlight lysosome puncta. **b)** Average LysoTracker staining (AFU) (n = 10 fields from 5 coverslips per condition). One-way ANOVA with post-hoc Tukey's test. ****$p<0.0001$.

Lysosomes are able to undergo Ca^{2+}-dependent fusion with the plasma membrane (Luzio et al., 2007). Lysosomal fusion can release enzymes responsible for extracellular matrix remodelling and maintenance of long-term dendritic spine growth (Padamsey et al., 2017). To investigate activity-dependent fusion of lysosomes with the plasma membrane in neuronal dendrites, total internal reflection fluorescence microscopy (TIRFM) was used to observe the dynamics of lysosomes close to the plasma membrane in dissociated hippocampal neurons under various conditions and fluorescence was quantified. TIRFM provides an exponentially-decaying fluorescence excitation field approximately 100 nm above the coverslip/liquid interface (Steyer & Almers, 1999). This depth of illumination is well-suited to observe fluorescently-labelled lysosomes close to the plasma membrane at the surface of a cell adherent to the cover slip. When the concentration of K^+ in Tyrode's solution was elevated to 45 mM to stimulate depolarisation and voltage-gated calcium channel activity, LysoTracker fluorescence increased significantly (control: 48.93 ± 2.8 AFU, n = 3, K^+: 64.21 ± 4.1 AFU, n = 3; $p<0.05$) (Figure 4.3B). The pre-incubation of neurons with Aβ oligomers (200 nM for two hours) or Ned-19 (100 μM for one hour), used to disrupt Ca^{2+} release from lysosomes by inhibiting NAADP, produced no increase in LysoTracker fluorescence after depolarisation with the application of 45 mM K^+ Tyrode's solution, suggesting that Aβ oligomers may prevent lysosomal fusion with the plasma membrane in response to neuronal depolarisation.

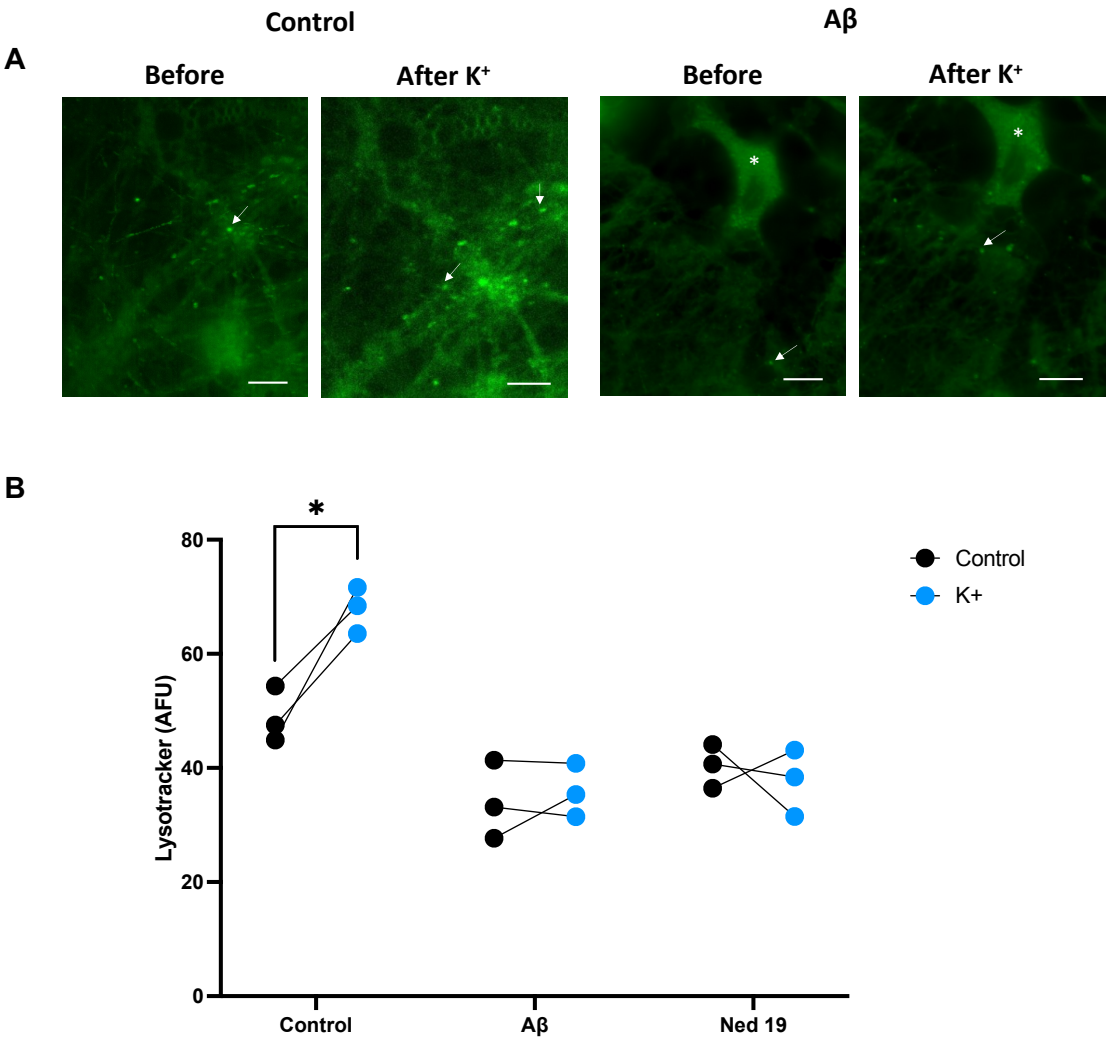

Figure 4.3: Aβ oligomers may prevent lysosome fusion with the plasma membrane in neurons. **a)** Representative fields of dissociated hippocampal neurons labelled with LysoTracker and imaged with TIRFM before and after the application of 45 mM K^+ Tyrode's solution (scale bar = 10 μm). Arrows highlight lysosome puncta and asterisk for cell body. **b)** Average LysoTracker fluorescence (AFU) under various conditions (n = 6 fields from 3 coverslips per condition). Paired Student's t-test (single paired comparisons). *$p<0.05$.

4.2.2. LAMP-2 expression is increased in the hippocampus of J20 mice

Following the experiments investigating the effect of acute Aβ oligomers treatment, the accumulation of lysosomes was explored in a more intact model of AD, the J20 mouse, to explore the effects of chronic Aβ accumulation on lysosomes. The expression of LAMP-2, a lysosomal

membrane glycoprotein, was examined in brain sections of 9 month old wild-type and J20 mice with immunohistochemistry (Figure 4.4A). The average percentage of total LAMP-2 stained area in the hippocampus was significantly increased in the sections from J20 mice (71.13 ± 1.4 %, n = 5) in comparison to the sections from wild-type mice (43.78 ± 1.5 %, n = 5) ($p<0.0001$) (Figure 4.4D).

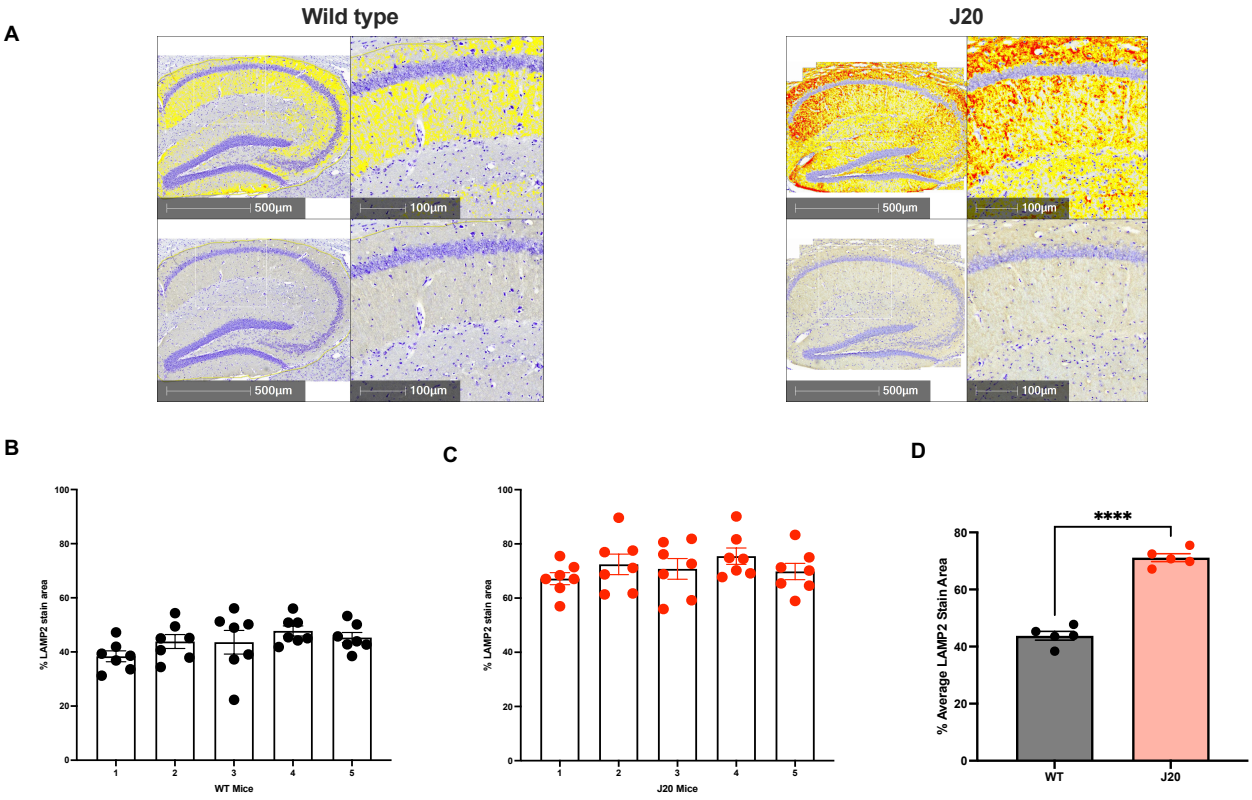

Figure 4.4: LAMP-2 expression is increased in J20 mice. **a)** Representative images of LAMP-2 staining in the hippocampus of wild type and J20 mice. Yellow – weak staining; red – strong staining assigned by HALO Image Analysis software; brown DAB staining represents LAMP-2 signal. **b,c)** Percentage of total LAMP-2 positive stained area of the hippocampus (n = 7 sections per mouse, for both wild type and J20 mice). **d**) Average percentage of total LAMP-2 positive stained area of the hippocampus of wild type (n = 5) and J20 (n = 5) mice. Unpaired Student's t-test. ****$p<0.0001$.

4.2.3. Lysosomes do not contribute to the Aβ-induced increased peak Ca^{2+} signal following back-propagating action potentials

Lysosomal Ca^{2+} release is influenced by VGCC-mediated Ca^{2+} influx evoked by back-propagating action potentials (Padamsey et al., 2017). Disruptions to this process may affect lysosomal fusion capacity, and therefore, prevent the release of lysosomal contents and impair the maintenance of LTP. Ca^{2+} transients were investigated in response to the exogenous application of Aβ oligomers. Hippocampal CA1 neurons from organotypic slices were patch-clamped and loaded with the Ca^{2+}-sensitive dye OGB-1, and dendritic Ca^{2+} was imaged in response to back-propagating action potentials (Figure 4.5A). Line scan imaging was coupled to action potential stimulation, and this protocol was repeated 10 times per experiment. Peak Ca^{2+} transients were averaged across the 10 trials and determined by taking the average of a 20 ms time window following the action potential stimulus. After incubating hippocampal slices with 200 nM Aβ oligomers for two hours, there was a significant rise in peak Ca^{2+} signal evoked after an action potential (2.424 ± 0.071 fold Δ fluorescence, n = 6) compared to control (1.892 ± 0.076 fold Δ fluorescence, n = 6) ($p<0.001$) (Figure 4.5B). Both GPN (200 μM for 10 mins) and Ned-19 (100 μM for one hour) reduced the Ca^{2+} rise evoked by action potentials, previously observed in (Padamsey et al., 2017). However, the application of Aβ in addition to the lysosome inhibitors produced no difference compared to the peak Ca^{2+} signal in response to Aβ alone (Figure 4.5B, C). Ca^{2+} imaging experiments were then replicated in acute hippocampal slices from 6-9 month old wild type and J20 mice, which recapitulates Aβ accumulation over time and exhibits pathological hallmarks of amyloid. Similarly as with the application of synthetic Aβ oligomers, J20 mice showed significantly increased peak Ca^{2+} levels in response to action potentials (2.157 ± 0.092 fold Δ fluorescence, n = 4) compared with wild type mice (1.670 ± 0.101 fold Δ fluorescence, n = 4) ($p<0.05$) (Figure 4.5D). Once again, the application of GPN diminished the peak Ca^{2+} signal in wild type mice, however, in J20 mice, the peak Ca^{2+} signal remained elevated after GPN incubation (Figure 4.5D).

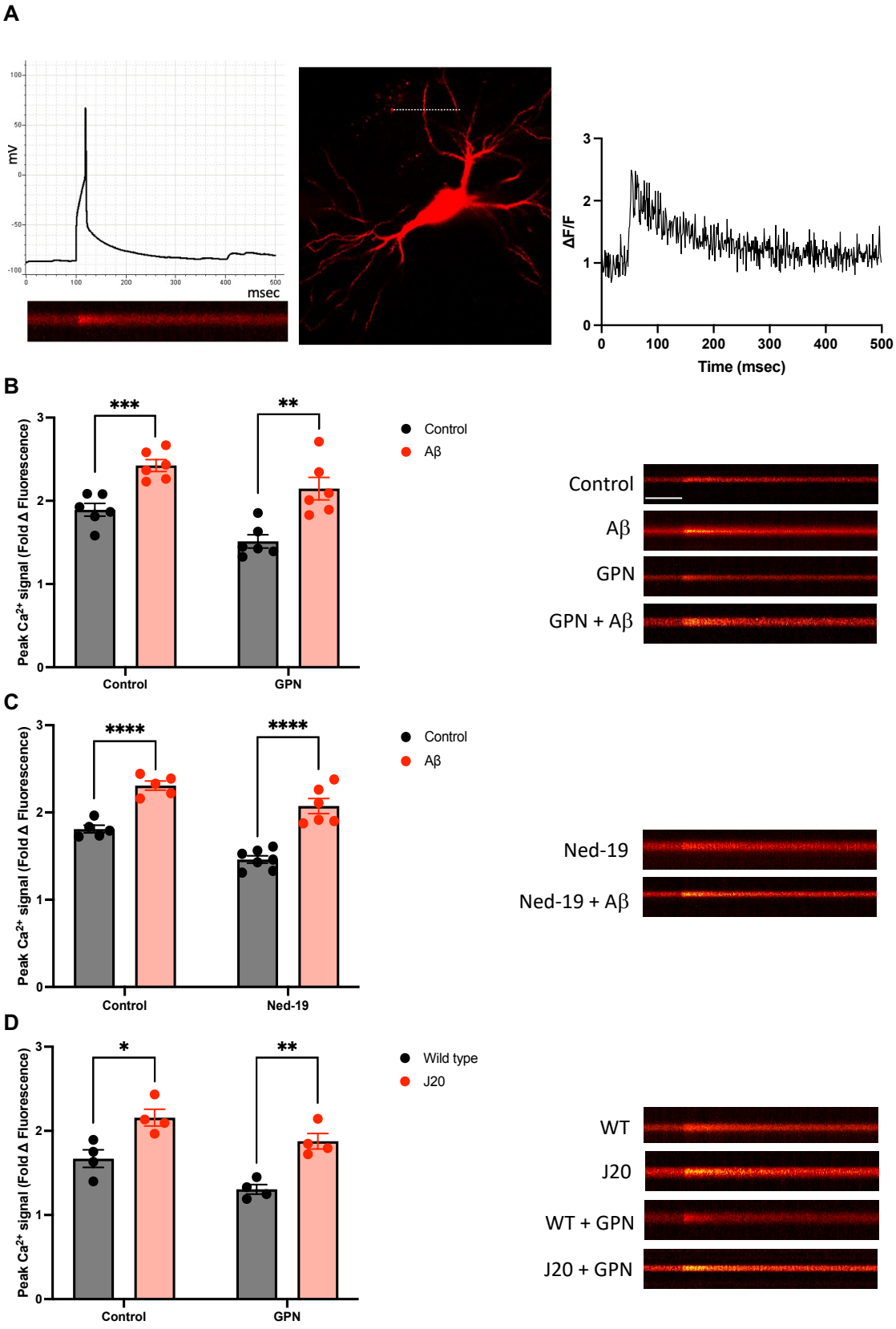

Figure 4.5: Aβ-induced increased peak Ca^{2+} signal following back-propagating action remains unchanged after the application of lysosome inhibitors. **a)** Experimental set-up: Ca^{2+} line scan images were coupled to action potential stimulation following patch-clamping CA1 hippocampal neurons in organotypic slices filled with OGB-1. Dashed line indicates line scanning region

through the dendrite. The ΔF/F graph indicates the quantification of the signal, the changes in fluorescence compared to the average fluorescence of the baseline (first 50 ms of recording). **b-d)** Average peak Ca^{2+} signals (fold Δ fluorescence) measured across different conditions indicated (n = 4-6 neurons per condition, n = the average of 10 line scans per experiment) with examples of line scan images (scale bar = 50 ms). Unpaired Student's t-test (single comparisons). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.2.4. Alterations in MMP-9 activity following Aβ incubation and in J20 mice

Following lysosomal Ca^{2+} release and the fusion of lysosomes with the plasma membrane, exocytosis of Cathepsin B releases MMP-9 from its inhibition by TIMP-1, causing an increase in extracellular MMP-9 activity (Padamsey et al., 2017). MMP-9 is critical in LTP maintenance, and MMP-9 knock-out mice show deficits in spatial learning (Nagy et al., 2006). Additionally, MMP-9 has been shown to contribute to the pathophysiological mechanisms of AD (Wang et al. 2014; Lorenzl et al. 2003; Kamat et al. 2014). To examine MMP-9 activity, hippocampal slices were incubated with Tyrode's solution and MMP-9 fluorogenic substrate (PEPDAB0502), which fluoresces upon MMP-9 mediated cleavage and is selective for MMP-9 (Miller et al. 2011). Fluorescence of the Tyrode's solution after 2 hours of incubation was assessed using a plate reader. MMP-9 activity was significantly increased after the application of a 45 mM K^+ Tyrode's solution to drive neuronal depolarisation (39027 ± 3068 AFU, n = 4) compared with control Tyrode's solution (29549 ± 2157 AFU, n = 4) ($p<0.05$). The addition of GPN (200 μM), Ned-19 (100 μM) and Cathepsin B inhibitor (1 μM) with K^+ Tyrode's solution all significantly decreased the levels of MMP-9 activity (Figure 4.6A) Incubating organotypic hippocampal slices with Aβ oligomers (200 nM) produced a significant decrease in MMP-9 activity levels (27037 ± 2463 AFU, n = 4) compared with control conditions (36504 ± 1653 AFU, n = 4) ($p<0.01$), both in K^+ Tyrode's solution (Figure 4.6B). To investigate the levels of MMP-9 activity in 6-9 month old J20 mice, acute hippocampal slices were used under the same protocol. In physiological Tyrode's solution, slices from J20 mice showed elevated MMP-9 activity (8698 ± 641 AFU, n = 4) compared with

wild type mice (5999 ± 543 AFU, n = 4) ($p<0.01$). Similarly, in K^+ Tyrode's solution, slices from J20 mice also displayed increased MMP-9 activity levels (12355 ± 903 AFU, n = 4) compared with wild type mice (9833 ± 488 AFU, n = 4) ($p<0.01$) (Figure 4.6C). To determine if higher levels of MMP-9 activity in J20 mice were due to an increase in enzyme expression, quantitative polymerase chain reaction (qPCR) was used to examine relative MMP-9 expression in the hippocampus of 6-9 month old wild type and J20 mice. Since MMP-9 activity is associated with the maintenance of LTP and dendritic spine growth (Wang et al. 2008), postsynaptic density protein 95 (PSD-95) expression was also investigated. PSD-95, one of the most abundant proteins in the PSD, functions as a scaffolding protein that regulates the localisation of many synaptic receptors and signalling proteins (Chen et al. 2015). The mechanisms preceding synapse degeneration in AD suggest that loss of PSD-95 is an early event, and the depletion of PSD-95 has been observed in the brains of AD mouse models and patients (Kivisäkk et al. 2022; Shao et al. 2011; Gylys et al. 2004). Interestingly, MMP-9 expression is increased in J20 mice (2.092 ± 0.232 relative MMP-9 expression, n = 6) compared with wild type mice (1.062 ± 0.239 relative MMP-9 expression, n = 6) ($p<0.05$) (Figure 4.6D). However, PSD-95 expression was decreased in J20 mice (1.290 ± 0.256 relative PSD-95 expression, n = 8) compared with wild type mice (2.339 ± 0.299 relative PSD-95 expression, n = 8) ($p<0.05$) (Figure 4.6E).

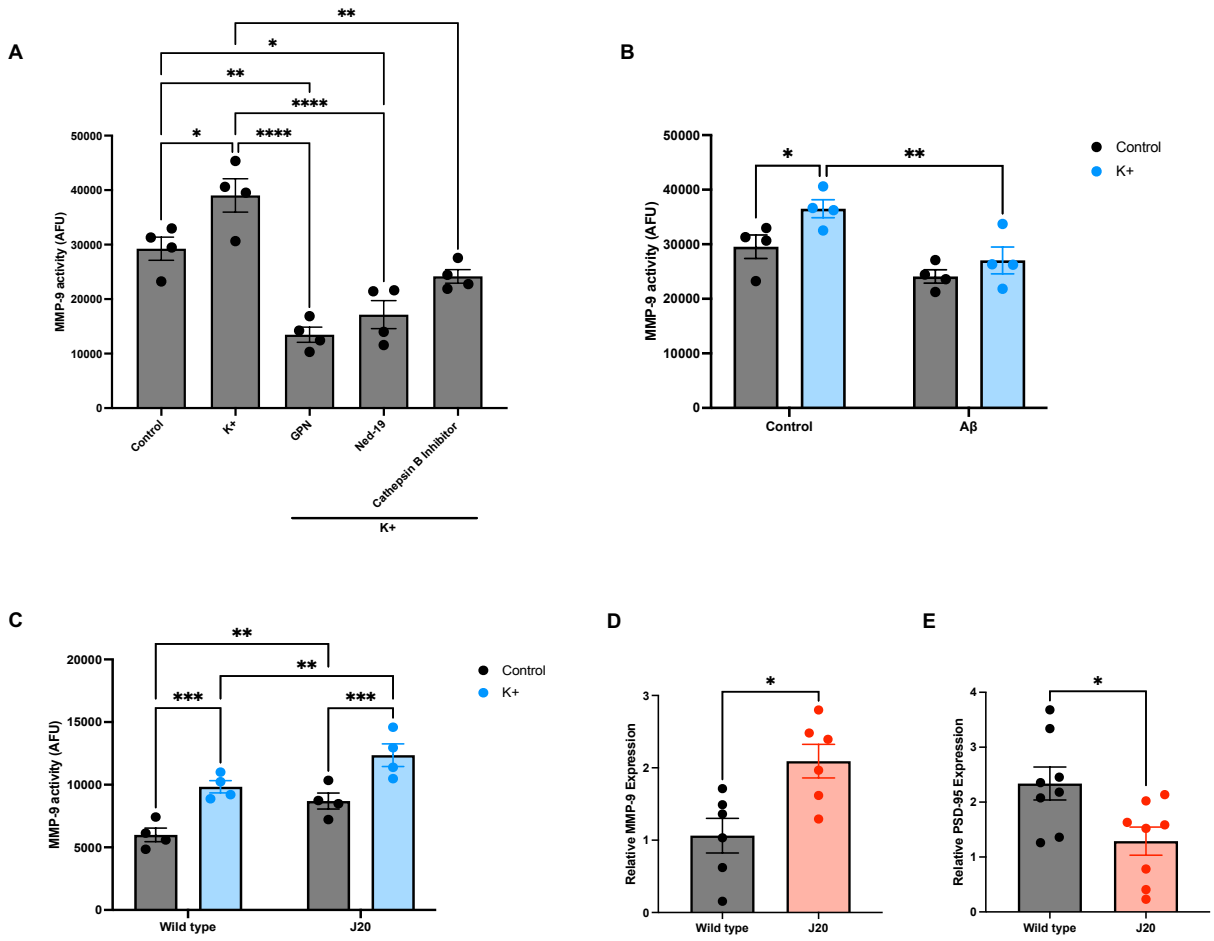

Figure 4.6: MMP-9 activity is decreased following Aβ incubation and increased in J20 mice. **a,b)** Average MMP-9 activity (AFU) in organotypic hippocampal slices under various conditions (n = 4 slices per condition). One-way ANOVA (a) and two-way ANOVA (b) with post-hoc Tukey's test. **c**) Average MMP-9 activity (AFU) in acute hippocampal slices from wild type and J20 mice (n = 4 slices per condition). Two-way ANOVA with post-hoc Tukey's test. **d,e)** Relative MMP-9 and PSD-95 expression following qPCR analysis of the hippocampus in wild type (n = 6-8) and J20 (n = 6-8) mice. Unpaired Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.2.5. MMP-9 does not rescue Aβ oligomer-induced impairment of long-lasting changes in the structure of dendritic spines in response to glutamate uncaging

Since MMP-9 is crucial for the maintenance of LTP (Padamsey et al., 2017), dendritic spine growth in response to an LTP protocol was examined under various conditions. Firstly, it was important to determine the effects of Aβ oligomers on dendritic spine growth in response to LTP. Dendritic spines of CA1 hippocampal neurons in organotypic slices were subjected to an established

structural plasticity protocol using MNI-glutamate photolysis (Matsuzaki et al. 2004), which produced an increase in dendritic spine size, restricted to a region within 1 μm of the uncaging laser beam, and remained stable for at least 30-60 minutes (Figure 4.7A, B). Under control conditions, the glutamate uncaging protocol induced a robust increase in spine size by 60 minutes (1.69 ± 0.034 fold Δ volume at 60 mins, n = 5) and the application of 200 nM Aβ oligomers for 2 hours (one hour incubation pre stimulation and one hour during the duration of experiment) significantly diminished growth of spines (1.351 ± 0.070 fold Δ volume at 60 mins, n = 5) ($p<0.001$). The application of GPN (200 μM, applied prior to baseline recordings and present for entire experiment) and GPN + Aβ also exhibited a significantly lower change in spine size compared to the control. In the previous experiment, MMP-9 activity levels were reduced by Aβ oligomers, which could explain the impairment in dendritic spine growth. Organotypic slices incubated with active MMP-9 (5 μg/mL for 10 mins post-stimulation) displayed an increase in spine size similar in magnitude to control conditions (1.586 ± 0.050 fold Δ volume at 60 mins, n = 5), however, the addition of MMP-9 with Aβ was not able to rescue the impairment in spine growth (Figure 4.7D). The glutamate photolysis experiments were repeated in CA1 hippocampal neurons in acute slices from 6 month old wild type and J20 mice to investigate the effects of chronic Aβ accumulation on the growth of dendritic spines. Once again, dendritic spines from wild type mice displayed an increase in size following glutamate photolysis (1.614 ± 0.056 fold Δ volume at 30 mins, n = 3) and spines from J20 mice showed significantly reduced growth (1.343 ± 0.022 fold Δ volume at 30 mins, n = 3) ($p<0.01$). The effects of GPN in wild type and J20 mice were also comparable to the reduced growth of spines in J20 mice alone. Once again, incubating wild type slices with active MMP-9 produced a normal increase in spine volume (1.636 ± 0.031 fold Δ volume at 30 mins, n = 3), and in J20 slices, MMP-9 caused a similar reduction in spine growth compared to control conditions in J20 slices (Figure 4.7G).

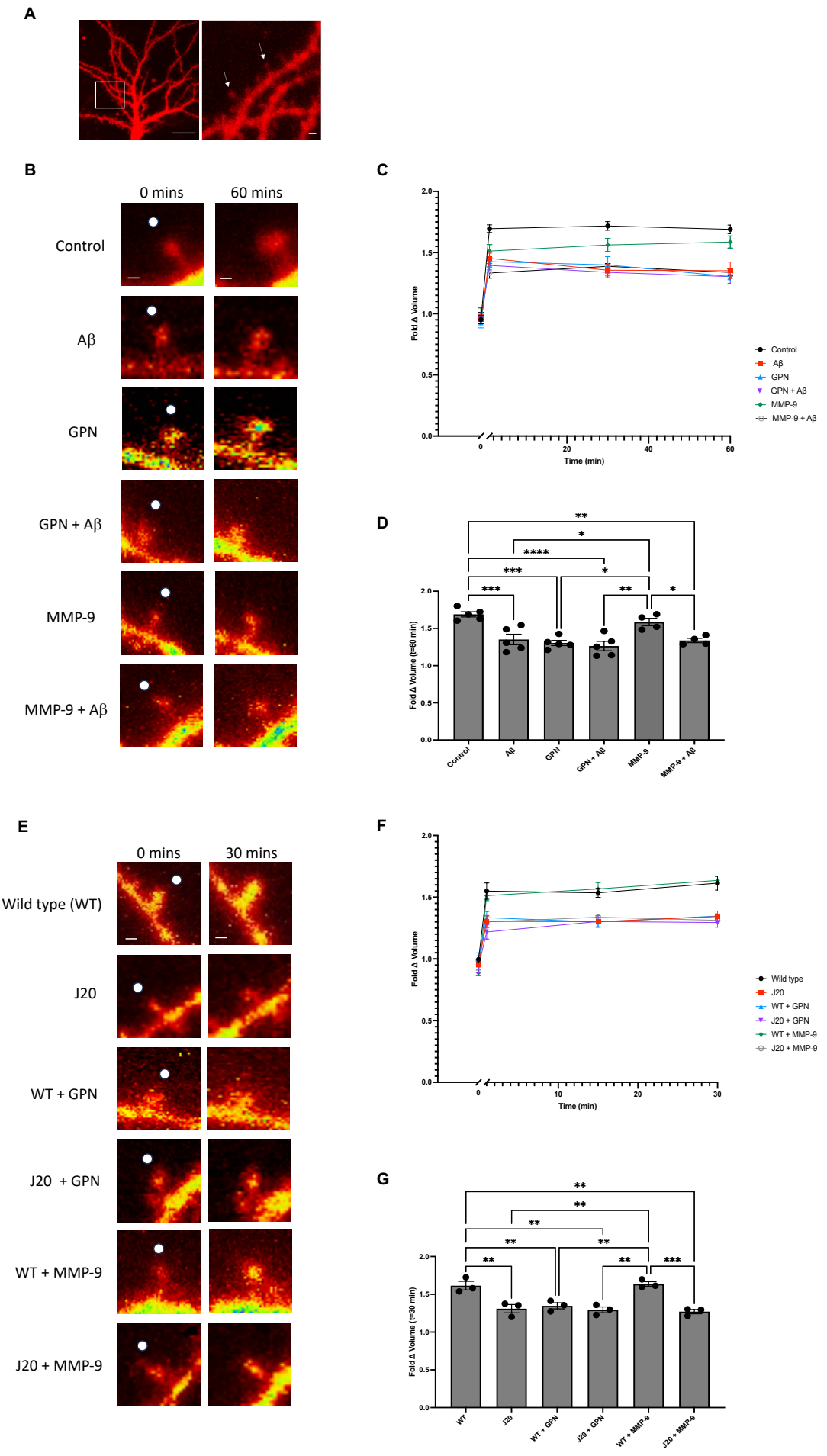

Figure 4.7: Aβ oligomer-induced impairment of dendritic spine growth is not rescued by

MMP-9. **a)** Representative image of dye-filled neuron with highlighted secondary dendrite and examples of typical spines selected for measurements; scale bar = 10 μm in the first panel and 1 μm in the second panel. **b,e)** Representative images of dendritic spines from the hippocampus of organotypic (a) or acute (d) slices at 0 and 60/30 mins after glutamate photolysis. White dot represents photolysis spot; scale bar = 1 μm. **c,f)** Fold Δ volume of dendritic spines over time (mins) under various conditions. **d)** Average fold Δ volume of dendritic spines at 60 minutes after photolysis (n = 5 spines per condition). One-way ANOVA with post-hoc Tukey's test. **g)** Average fold Δ volume of dendritic spines at 30 minutes after photolysis (n = 3 spines per condition). One-way ANOVA with post-hoc Tukey's test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.3. Discussion

It is well accepted that the lysosomal autophagy system is disrupted in AD (Nixon & Yang, 2011). Following the identification in our laboratory of an additional role for lysosomes as critical drivers of synaptic plasticity (Padamsey et al. 2017), I set out to examine whether this important aspect of lysosomal function might also be disrupted in AD models, which could help explain known deficits in plasticity in AD. For each experiment, cultured organotypic hippocampal slices incubated with synthetic Aβ oligomers were used as an acute model of Aβ build-up in early AD, as well as acute hippocampal slices from J20 mice, to represent the chronic accumulation of Aβ in later disease stages.

Firstly, I examined the expression of lysosomes in dissociated hippocampal neuron cultures. The addition of Aβ oligomers had no effect on the number of lysosomes observed, which may be due to the short incubation time. However, LAMP-2 expression was significantly increased in the hippocampus of 9 month old J20 mice when compared with wild type mice. Studies have previously shown that Aβ can be degraded by lysosomal enzymes, however, with the accumulation of oligomers, which then build-up into amyloid plaques, lysosomes become dysfunctional (Torres et al. 2012; Gowrishankar et al. 2015). Multiple studies have reported an increased abundance of lysosomes in neurons surrounding amyloid plaques (Gowrishankar et al. 2015; Nixon et al. 2005).

These lysosomes contain significantly lower levels of several proteases, display impaired axonal transport and maturation, and exhibit impaired acidification (Gowrishankar et al. 2015; Avrahami et al. 2013). Autophagic upregulation in later stages of AD may represent a compensatory mechanism to boost autophagic flux, although, the dysfunctional lysosomes are unable to efficiently degrade proteins (Orr and Oddo 2013). The reduced competence of lysosomal degradation coupled with additional effects of oxidative damage lead to the impairment of autophagy, which accelerates AD pathogenesis (Terman and Brunk 2006; Nixon and Yang 2011).

Ca^{2+} release from lysosomes is necessary for lysosomal fusion with the plasma membrane and the exocytosis of its contents into the extracellular space. The strong coupling between VGCCs activation and lysosomal Ca^{2+} release indicates that lysosome fusion and exocytosis is activity-dependent (Padamsey et al. 2017). The 45 mM K^+ Tyrode's solution was used to drive neuronal depolarisation and lysosome fusion with the plasma membrane, as evidenced by the experiments using TIRFM. Since lysosomal Ca^{2+} stores are disrupted in response to Aβ (McBrayer and Nixon 2013), this could lead to the impairment in lysosomal fusion with the plasma membrane as observed in Figure 4.3B. One limitation of this experiment is that TIRFM only visualises lysosomal mobilisation to the surface of the plasma membrane and does not confirm membrane fusion. The use of a fluorescently tagged antibody targeting the luminal domain of a lysosomal protein would be required to confirm lysosomal fusion with the plasma membrane upon the observation of fluorescent labelling.

Next, I performed Ca^{2+} line scan imaging experiments to gain a better understanding of the effect of Aβ on Ca^{2+} signalling in response to back-propagating action potentials. Aβ oligomers were able to significantly increase the peak Ca^{2+} signal and the application of GPN and Ned-19 with Aβ was not able to return the peak Ca^{2+} signal to control levels. The same result was observed in acute hippocampal slices from J20 mice. One possible explanation is that Aβ oligomers triggers

unregulated flux of Ca^{2+} through VGCCs, contributing to the elevated cytosolic concentration of Ca^{2+} (Demuro et al., 2005). Additionally, Aβ neurotoxicity may initiate increased Ca^{2+} efflux from other intracellular Ca^{2+} stores, contributing to a higher peak Ca^{2+} signal (Bezprozvanny, 2012). Studies have also shown abundant evidence that neuronal Ca^{2+} regulation is weakened in AD, resulting in an increase in intracellular Ca^{2+} concentration and overloaded intracellular stores (Kuchibhotla et al. 2008; Ge et al. 2022).

The exocytosis of lysosomal cathepsin B cleaves TIMP-1 and recruits MMP-9 activity, which plays a crucial role in remodelling the extracellular matrix, allowing for the expansion of the postsynaptic density and long-lasting enlargement of dendritic spines (Padamsey et al. 2017). Treatment of hippocampal organotypic slices with 45 mM K^+ Tyrode's solution, to drive depolarisation, and Aβ produced a significant decrease in MMP-9 activity levels, which may reflect the inability of lysosomes to fuse with the plasma membrane following Aβ incubation. The reduced MMP-9 activity may also be a result of disrupted protease activity, as decreased levels of several proteases, including cathepsin B, have been observed in models of early AD (Gowrishankar et al. 2015). Another explanation for reduced MMP-9 activity may be due to Aβ competing as a substrate for MMP-9, causing a 'pseudo' reduction in fluorescent signal produced by the fluorogenic substrate. Interestingly, in the slices from J20 mice, MMP-9 activity was increased in both regular and K^+ Tyrode's solution compared with wild type mice, suggesting that the basal MMP-9 activity is increased and further augmented after neuronal depolarisation in AD pathogenesis. Several studies have corroborated these results and observed that MMP-9 expression is increased in AD and contributes to the inflammatory pathways associated with the disease (Lorenzl et al., 2003). However, in early stages of AD, MMP-9 overexpression is a compensatory mechanism and exhibits a neuroprotective function with its role in the formation of new synaptic connections and its ability to degrade Aβ (Kaminari et al., 2017). The compensatory mechanism, such as was the case with the accumulation of lysosomes in AD, eventually becomes dysfunctional and exacerbates

neuronal degeneration. MMP-9 is released from astrocytes following brain injury, which increases the risk of neurodegenerative disease (Kamat et al. 2014). High levels of MMP-9 are implicated in aberrant synaptic plasticity, neuronal loss and dendritic degeneration (Stawarski, Stefaniuk, and Wlodarczyk 2014). In this Chapter, MMP-9 expression levels measured in the hippocampus of J20 mice using qPCR were elevated compared with wild type mice. In contrast, PSD-95 expression was decreased in J20 mice, reflective of postsynaptic degeneration and neuronal loss, potentially owing to the elevated MMP-9 expression and activity levels. Previous studies have confirmed these results, detecting a decrease in hippocampal PSD-95 in J20 mice and AD patients (Gylys et al. 2004; Shao et al. 2011).

Finally, I investigated the structural plasticity of dendritic spines on CA1 pyramidal neurons of organotypic and acute hippocampal slices. I used an established protocol (Matsuzaki et al. 2004), which utilised MNI-glutamate photolysis within a small focal volume to produce marked increases in dendritic spine size. In accordance with previous reports (Ortiz-Sanz et al. 2020), Aβ prevented the growth of spines to control levels, similar to the results obtained following GPN treatment since lysosomes play an important role in the long-term maintenance of structural plasticity (Padamsey et al. 2017). The application of GPN + Aβ also reduced the change in spine growth, which may be due to the large rise in Ca^{2+} observed following Aβ treatment that may disturb the mechanisms underlying physiological structural plasticity. The addition of MMP-9 in control experiments did not further augment spine size, suggesting that there might be a limit to spine growth. MMP-9 included with Aβ treatment did not restore persistent expansion of the dendritic spine to control levels, suggesting that Aβ might act at different sites to disturb structural plasticity mechanisms. The application of active MMP-9 might also overwhelm the homeostasis of the extracellular matrix, and further impair the expansion of spines, as evidenced by the effect of increased levels of MMP-9 in AD pathology contributing to synaptic degeneration (Mizoguchi et al. 2009). The same results were observed in acute slices from wild type and J20 mice under various

treatments, which imply that acute and chronic Aβ accumulation has a similar effect on the expansion of dendritic spines, further validating the notion that oligomeric Aβ is the synaptotoxic species in AD (Lambert et al. 1998).

This Chapter has uncovered several significant changes in lysosomal biology in response to Aβ oligomers, which may contribute to the pathophysiological mechanisms of AD. Since excessive levels of cathepsin B and MMP-9 have been reported in a pathological context (Gan et al. 2004; Sundelöf et al. 2010; Lorenzl et al. 2003), it might be constructive for future studies to explore whether inhibiting lysosome fusion with the plasma membrane may provide a novel way to prevent excessive protease release. The use of Ned-19 to prevent lysosomal Ca^{2+} release may reduce the levels of cathepsin B and MMP-9 in the extracellular space and rescue certain features of AD pathology. In addition, the effects of a cathepsin B or MMP-9 inhibitor on structural plasticity of dendritic spines in an AD context could be studied to further elucidate the link between cathepsin B/MMP-9 and LTP mechanisms in a pathological context. Transcription Factor EB (TFEB) promotes lysosome biogenesis and has been shown to ameliorate several AD-related phenotypes in mice and reduce plaque burden (Li et al. 2022). Future research could also explore the use of upregulating TFEB in the early stages of AD in J20 mice, before lysosome accumulation becomes deleterious, and monitoring the effects on lysosome biology and structural plasticity. The data in this Chapter supports the notion that there is a great deal to learn before the relationship between lysosome biology and AD is adequately understood, not least, whether lysosomes are damaged by the pathogenic process or whether changes in their biology occur in direct response to the disease.

5. Activity-dependent ER Ca^{2+} signalling and the effect on structural plasticity of hippocampal neurons in AD

5.1. Introduction

The endoplasmic reticulum (ER) is comprised of an intricate network of microtubules and cisternae, present in both neurons and glia (Alberts et al. 2002). The ER consists of the ribosome-rich rough ER, which is mainly located in the somatodendritic area and is the site of protein synthesis, and the smooth ER in axons and synapses, which is the site of lipid synthesis (Alberts et al. 2002). In neurons, the ER serves numerous important functions – it acts as a primary transport route for proteins, it forms a particular environment for post-translation protein processing, and functions as an intracellular signalling system with the dynamic release of its internal store of Ca^{2+} (Chanaday and Kavalali 2022). The ER maintains cytosolic Ca^{2+} homeostasis by either removing or releasing Ca^{2+} via specialised Ca^{2+} channels located on the ER membrane (Chanaday and Kavalali 2022). The sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a Ca^{2+} ATPase, which transports Ca^{2+} from the cytosol into the ER, utilising the energy from ATP hydrolysis (Lytton et al. 1992). Inositol triphosphate receptors (IP_3Rs) and ryanodine receptors (RyR) mediate ER Ca^{2+} release (Furuichi et al. 1989; Takeshima et al. 1989). These receptors use calcium-induced calcium release (CICR) and are activated by low Ca^{2+} concentrations and inhibited by high Ca^{2+} concentrations; additionally, IP_3 regulates the sensitivity of IP_3Rs to Ca^{2+} (Woll and Van Petegem 2022). Stromal interaction molecule (STIM) proteins, specifically STIM1 and STIM2, are located on the ER membrane and function as sensors for ER Ca^{2+} concentration (Roos et al. 2005). When ER Ca^{2+} levels drop, the sensors stimulate Ca^{2+} influx from the extracellular space through Orai1 channels in the plasma membrane (Prakriya et al. 2006). Neurons contain the largest ER that connects the whole cell, while also regulating restricted signals

in specific neuronal domains (Berridge 2002). Previous studies have demonstrated that the release of Ca^{2+} from internal stores, such as the ER and lysosomes, can augment neurotransmitter release at synapses of hippocampal pyramidal neurons (Emptage, Reid, and Fine 2001). Different forms of neurotransmission rely on specific Ca^{2+} sources and signalling proteins; for example, the spontaneous release of GABA from Purkinje cells is facilitated by Ca^{2+} release via RyRs (Llano et al. 2000), while both RyRs and IP_3Rs are responsible for triggering dopamine release from nigrostriatal neurons without the influence of extracellular Ca^{2+} influx (Patel et al. 2009). At glutamatergic synapses, STIM proteins are negative regulators of NMDAR-induced Ca^{2+} elevations and they also control AMPAR trafficking, influencing the processes associated with LTP and LTD (Gruszczynska-Biegala et al. 2020; Yap et al. 2017). RyRs contribute to the generation of fast Ca^{2+} sparks in dendrites, whereas CICR facilitated by IP_3Rs results in a more sustained Ca^{2+} signal that propagates as waves (Miyazaki and Ross 2013). The Ca^{2+} waves achieve high Ca^{2+} concentrations and spread over long distances, however, their function in neuronal physiology remains largely unknown (Ross 2012). Ca^{2+} release through IP_3Rs stimulates postsynaptic signalling and unsilences synapses through a CaMKII mechanism, although, in Purkinje cells, Ca^{2+} release from IP_3Rs leads to LTD expression (Kelly et al. 2005; Miyata et al. 2000). A recent study suggested that the interplay of various factors such as stimulation frequency, duration, activation of IP_3Rs and RyRs, and saturation level of SERCA, collectively influence whether a specific synapse will undergo LTP or LTD (Singh et al. 2021).

The activity levels of neurons can determine the dynamics of intracellular organelles (Chanaday and Kavalali 2022). Only a portion of dendritic spines (15-50%) include ER at a given moment, however, the dynamic nature of the ER indicates that it may transiently enter the majority of dendritic spines (Holbro, Grunditz, and Oertner 2009; Perez-Alvarez et al. 2020). In larger dendritic spines, the ER forms a structure known as the spine apparatus (Spacek and Harris 1997). Research has revealed that the ER enters spines during periods of synaptic activity and serves an

essential function in preventing excessive potentiation of spines, maintaining most of them at an intermediate strength from which both LTP and LTD remains achievable (Perez-Alvarez et al. 2020). In ER-containing spines, Ca^{2+} release of a large amplitude was associated with strengthened synapses and an enlarged ER contributing to the spine apparatus (Holbro, Grunditz, and Oertner 2009). The ER also facilitates local translation and delivery of synaptic proteins, enabling the dynamic regulation of the morphology and synaptic strength of dendritic spines in response to various forms of neuronal activity and pathological conditions (Chanaday and Kavalali 2022).

The ER stress response is recognised as a significant process in the development of AD (Hashimoto and Saido 2018). The build-up of pathogenic misfolded proteins and the disturbance of Ca^{2+} homeostasis are regarded as fundamental mechanisms leading to the initiation of heightened ER stress, ultimately resulting in neuronal death (Hashimoto and Saido 2018). The unfolded protein response (UPR) serves as a protective mechanism activated in response to ER stress induced by various biological factors (Li et al. 2015). These protective mechanisms include an increase in molecular chaperones, translational attenuation, and activation of endoplasmic reticulum-associated protein degradation (ERAD) (Walter and Ron 2011). Several reports have described elevated ER stress markers in post-mortem brains from AD patients and AD mouse models (Lai et al. 2022; Hashimoto et al. 2018). Presenilin 1 (PS1), a component of γ-secretase that is involved in cleaving APP, is a fundamental membrane protein of the ER (Area-Gomez et al. 2009). PS1 mutations associated with FAD induce ER stress by inhibiting the UPR as well as damaging ER organisation within neurons (Katayama et al. 1999). The ER plays an important role in the processing of APP (Plácido et al. 2014). Chronic ER stress, which persists throughout AD progression, disrupts the trafficking and processing of proteins, such as APP (Plácido et al. 2014). This disruption results in the elevated production of Aβ, which subsequently triggers various neurotoxic pathways leading to neuronal apoptosis (Takahashi et al. 2009).

Cultured hippocampal neurons from 3xTg AD neurons, PS1/PS2 FAD mutant fibroblasts, and primary lymphoblasts from FAD patients display enhanced intracellular Ca^{2+} release from the ER and diminished store-operated Ca^{2+} entry, as a result of ER Ca^{2+} overload (Popugaeva and Bezprozvanny 2013; Leissring et al. 2000). It has been reported that soluble Aβ oligomers can enhance the liberation of Ca^{2+} from the ER by stimulating the excessive production of IP_3 (Demuro and Parker 2013). Similarly, RyR activity is amplified in neurons of 3xTg-AD mice (Goussakov, Miller, and Stutzmann 2010), and increased expression of RyRs has been depicted in brains of AD patients (Bruno et al. 2012). PS1 mutations also disturb Ca^{2+} signalling in neurons (Popugaeva and Bezprozvanny 2013). Physiological presenilins function as Ca^{2+} leak channels to preserve ER Ca^{2+} homeostasis by leaking Ca^{2+} into the cytosol, thereby balancing the activity of SERCA (Tu et al. 2006). PS1 mutations disturb the Ca^{2+} leak function, resulting in overfilling of the ER with Ca^{2+} and the amplified release of ER Ca^{2+} (Tu et al. 2006; Nelson et al. 2007). Fibroblasts from PS1-M146V knock in mice also exhibit substantial impairments in store-operated Ca^{2+} entry (Leissring et al. 2000). Alterations in STIM1 and STIM2 expression were observed in FAD mutant cells, and these changes may lead to the destabilisation and elimination of dendritic spines (Sun et al. 2014). In another study, disrupted ER Ca^{2+} signalling resulted in synaptic plasticity impairments in presenilin knockout neurons (Zhang et al. 2010; Zhang et al. 2009). Dendritic spine deterioration due to ER Ca^{2+} signalling impairment may act synergistically with other mechanisms impacted by Aβ synaptotoxicity, terminating in the loss of dendritic spines.

Since the precise mechanisms of Aβ-mediated disruption of intracellular Ca^{2+} signalling in neurons is still not fully understood, I investigated the effect of acute and chronic Aβ exposure on activity-dependent aspects of ER Ca^{2+} signalling and the impact on structural plasticity of dendritic spines in hippocampal neurons.

5.2. Results

5.2.1. Aβ oligomers recruit Ca^{2+} release from the ER following back-propagating action potentials in hippocampal CA1 neurons

A number of studies have demonstrated that Aβ increases Ca^{2+} levels in neurons, causing intracellular Ca^{2+} overload, and ultimately, neuronal death (Kuchibhotla et al. 2008). Given the relationship between intracellular Ca^{2+} signalling and AD, I examined whether activity-dependent aspects of Ca^{2+} signalling, such as Ca^{2+} release from the ER, are altered by Aβ. As previously described in Chapter 4, section 4.2.3, hippocampal CA1 neurons from organotypic slices were patch-clamped and loaded with the Ca^{2+}-sensitive dye OGB-1, and dendritic Ca^{2+} transients were imaged in response to back-propagating action potentials. Once again, following the incubation of organotypic hippocampal slices with 200 nM of Aβ oligomers for two hours, there was a significant increase in the peak Ca^{2+} signal (2.4 ± 0.119 fold Δ fluorescence, n = 5) compared to the control peak Ca^{2+} signal (1.87 ± 0.082 fold Δ fluorescence, n = 5) ($p<0.01$) (Figure 5.1A). The application of thapsigargin (15 μM for 20 mins), which blocks SERCA and depletes ER Ca^{2+} stores, had no effect on the amplitude of Ca^{2+} signals, however, when applied with Aβ, it was able to return the Ca^{2+} signal to control levels. Similarly, incubating slices with ryanodine (20 μM for 20 mins), which blocks RyRs when used at a micromolar concentration and prevents ER Ca^{2+} release, produced no effect on peak Ca^{2+} signals, but prevented the elevated peak Ca^{2+} signal triggered by Aβ exposure (Figure 5.1A, B). Ca^{2+} line scan imaging was then performed in acute hippocampal slices from 6-9 month old wild type and J20 mice. Results were comparable to those observed in organotypic slices, with a significant increase in peak Ca^{2+} signal in J20 mice (2.268 ± 0.104 fold Δ fluorescence, n = 4) compared with the peak Ca^{2+} signal in wild type mice (1.795 ± 0.118 fold Δ fluorescence, n = 4) ($p<0.05$) (Figure 5.1C). Incubating the wild type slices with thapsigargin made no difference to peak Ca^{2+} signals compared to wild type control conditions, however, thapsigargin was able to prevent the large rise in peak Ca^{2+} signals in J20 mice (Figure 5.1C).

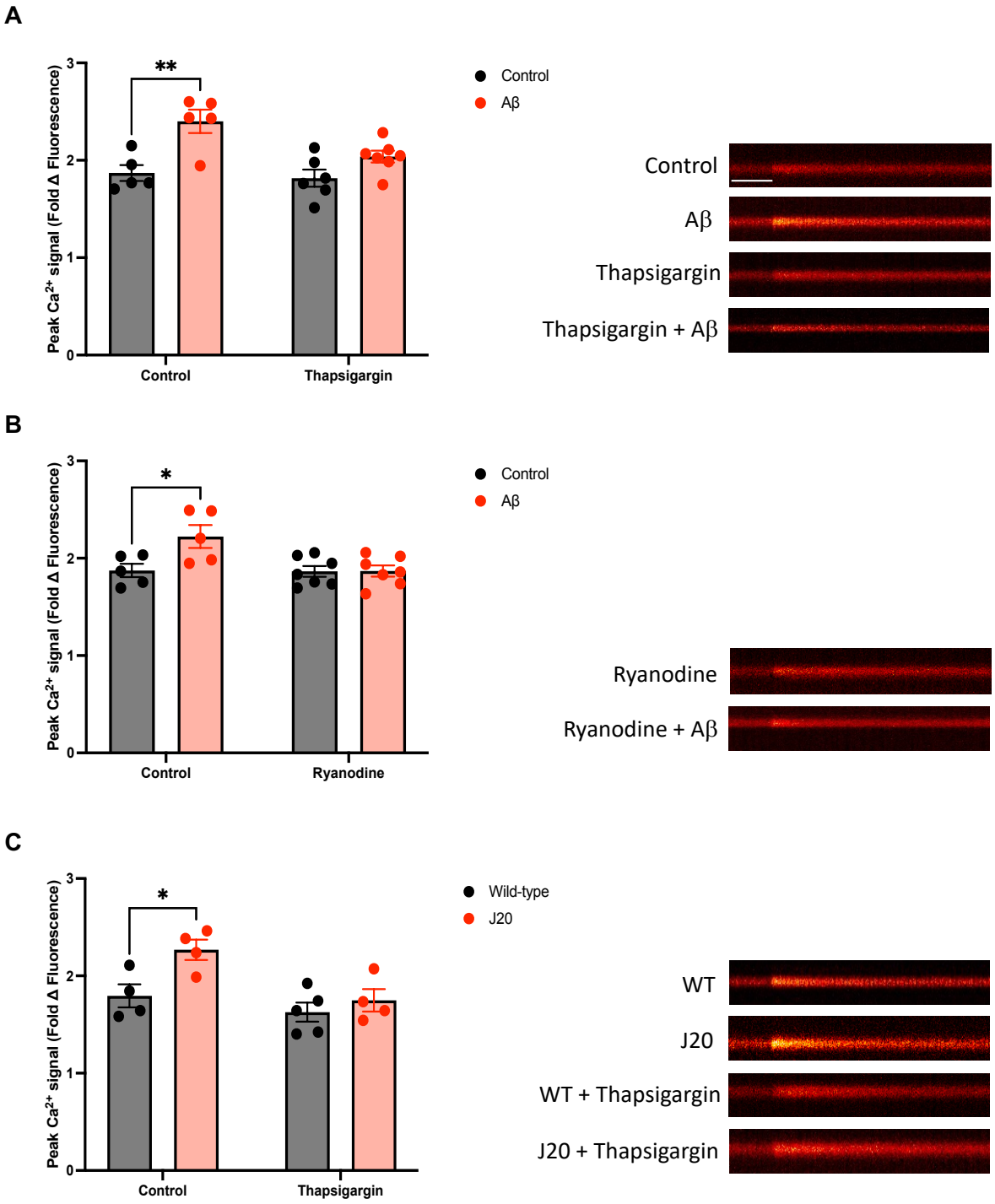

Figure 5.1: Aβ increases bpAP-triggered peak Ca^{2+} signal by recruitment of Ca^{2+} release from the ER. **a-c)** Average peak Ca^{2+} signals (fold Δ fluorescence) measured across different conditions indicated (n = 4-7 neurons per condition, n = the average of 10 line scans per experiment) with examples of line scan images (scale bar = 50 ms). Unpaired Student's t-test (single comparisons). *p<0.05, **p<0.01.

5.2.2. Growth of dendritic spines following LTP induction is reduced both in response to Aβ and inhibiting Ca^{2+} release from the ER

Since ER Ca^{2+} signalling plays an important role in maintaining Ca^{2+} homeostasis and the mechanisms underlying LTP expression, I decided to subject CA1 hippocampal neurons in organotypic slices to a theta-burst stimulation (TBS) protocol, which induces the growth of dendritic spines. I hypothesized that thapsigargin and ryanodine might rescue the impairment of spine growth following Aβ incubation as the ER inhibitors were able to normalise peak Ca^{2+} levels following Aβ treatment. The TBS protocol caused an increase in dendritic spine size (1.654 ± 0.025 fold Δ volume at 60 mins, n = 5) and treatment with 200 nM of Aβ oligomers for two hours (one hour incubation pre stimulation and one hour during the duration of experiment) significantly reduced the growth of spines (1.312 ± 0.038 fold Δ volume at 60 mins, n = 5) ($p<0.01$) (Figure 5.2D). Treatment with thapsigargin (15 μM) and ryanodine (20 μM), applied prior to baseline recordings and present for entire experiment, also caused a reduction in the growth of spines compared to control conditions, and the inhibitors were not able to restore spine expansion when incubated with Aβ (Figure 5.2D). Glutamate photolysis was used to generate enlargement of dendritic spines in acute hippocampal slices from wild type and J20 mice. A robust expansion of spines was observed in wild type slices (1.589 ± 0.049 fold Δ volume at 30 mins, n = 4), while J20 mice exhibited significantly decreased spine growth (1.284 ± 0.032 fold Δ volume at 30 mins, n = 4) ($p<0.01$) (Figure 5.2G). Incubating both wild type and J20 slices with thapsigargin significantly reduced spine growth levels, comparable to the limited spine growth observed in J20 neurons (Figure 5.2G). Two different methods, glutamate photolysis and TBS, were compared to generate dendritic spine growth. Both methods produced equal results of control spine enlargement in organotypic and acute hippocampal slices (Figure 5.2D, G), however, with glutamate photolysis it was possible to restrict LTP at individual dendritic spines with high spatial precision.

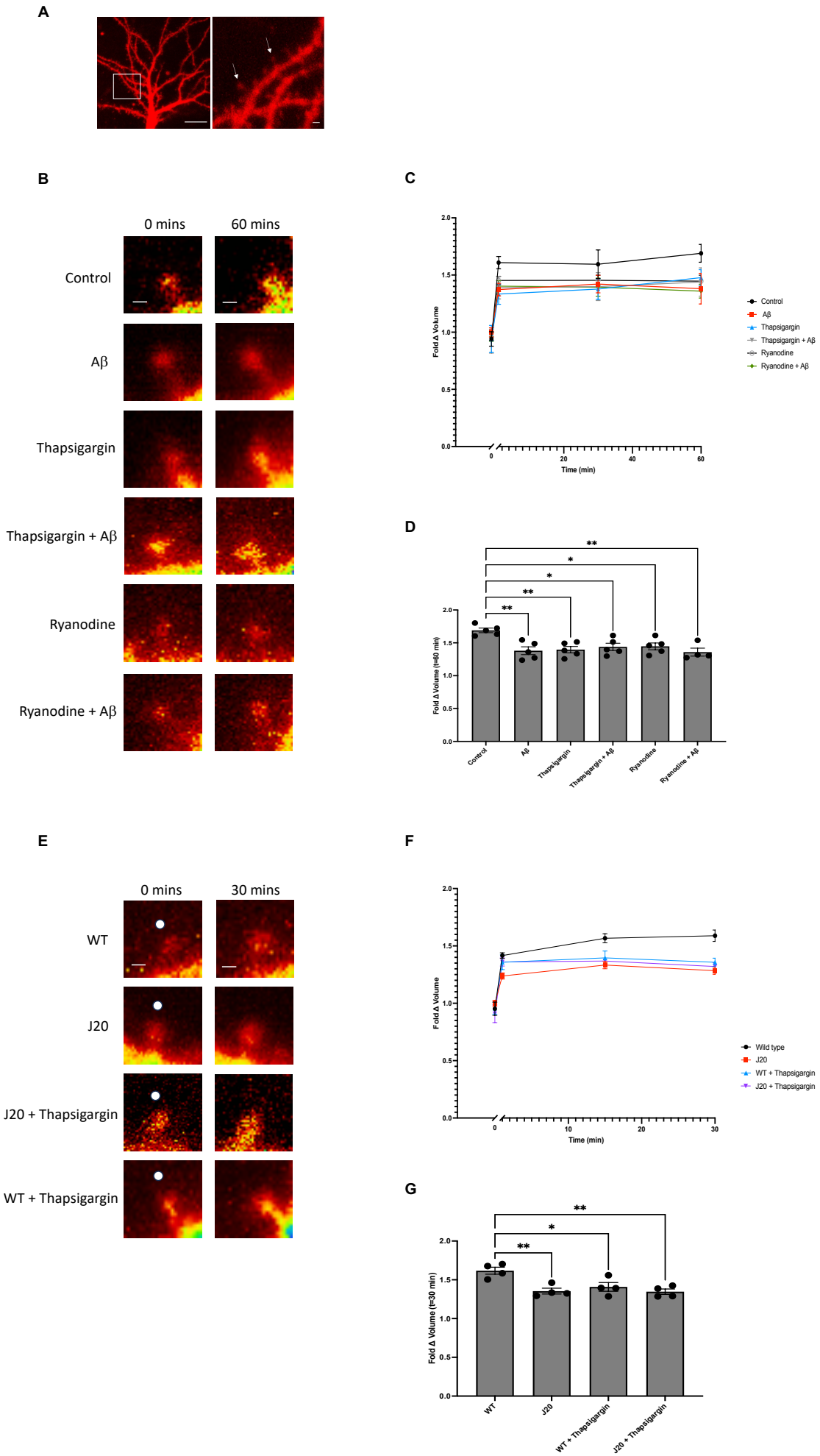

Figure 5.2: Aβ and ER Ca^{2+} release inhibitors reduce the growth of dendritic spines following

LTP induction. **a)** Representative image of dye-filled neuron with highlighted secondary dendrite and examples of typical spines selected for measurements; scale bar = 10 μm in the first panel and 1 μm in the second panel. **b,e)** Representative images of dendritic spines from the hippocampus of organotypic (a) or acute (d) slices at 0 and 60/30 mins after TBS/glutamate photolysis. White dot represents photolysis spot; scale bar = 1 μm. **c,f)** Fold Δ volume of dendritic spines over time (mins) under various conditions. **d)** Average fold Δ volume of dendritic spines at 60 minutes after TBS (n = 4-5 neurons per condition; 4-5 spines analysed within 20 μm of dendrite per neuron). One-way ANOVA with post-hoc Tukey's test. **g)** Average fold Δ volume of dendritic spines at 30 minutes after photolysis (n = 4 spines per condition). One-way ANOVA with post-hoc Tukey's test. *$p<0.05$, **$p<0.01$.

5.2.3. Aβ stimulates Ca^{2+} release from the ER by activating synaptic mGluR5 receptors

The hippocampus exhibits strong immunoreactivity for mGluR5 (Shigemoto et al. 1997). While mGluR5 is predominantly confined to perisynaptic nanodomains, it can be recruited to synapses, leading to an acute enhancement of synaptic Ca^{2+} responses (Scheefhals, Westra, and MacGillavry 2023). Publications have stated that soluble Aβ oligomers bind to metabotropic glutamate receptor 5 (mGluR5) and redistribute the receptor from perisynaptic zones to the synapse, facilitating an increase in receptor signalling, which is linked to the production of IP_3 that promotes Ca^{2+} release from the ER via IP_3Rs (Renner et al. 2010; Demuro and Parker 2013). The impaired Ca^{2+} mobilisation has also been shown to lead to the depletion of NMDARs and eventually, synapse loss (Zhang et al. 2015). I hypothesized that the interaction of Aβ with mGluR5 might be partly responsible for the recruitment of ER Ca^{2+} release following back-propagating action potentials. 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP), a selective allosteric antagonist of mGluR5, was applied to organotypic and acute hippocampal slices at 3 μM for 60 mins, to determine if MTEP was able to prevent the effects of Aβ on ER Ca^{2+} release. Basal electrophysiological parameters were unaltered following MTEP treatment (Supplementary Figure 4). The application of MTEP alone did not affect control levels of the Ca^{2+} signal, however, when MTEP was applied with Aβ, there was a significant decrease in peak Ca^{2+} signal, which returned to control levels (1.714 ± 0.083

fold Δ fluorescence, n = 4), compared with the treatment of slices with Aβ alone (2.171 ± 0.096 fold Δ fluorescence, n = 4) ($p<0.05$) (Figure 5.3A). The combination of MTEP, Aβ and thapsigargin produced a result comparable to the application of MTEP and Aβ together, and did not further diminish the peak Ca^{2+} signal (Figure 5.3A). Ca^{2+} imaging was then replicated in acute hippocampal slices from 6-9 month old wild type and J20 mice. Similar to the results obtained from organotypic slices, the application of MTEP in wild type slices did not produce a peak Ca^{2+} signal that deviated from control conditions. In contrast, the treatment of MTEP in J20 slices demonstrated no difference in peak Ca^{2+} signals and was comparable to J20 slices in control conditions, although, a trend was observed towards a decreased peak Ca^{2+} signal that was not significant (Figure 5.3B). However, the addition of thapsigargin and MTEP in J20 slices returned the peak Ca^{2+} signals to control levels (Figure 5.3B), as in Figure 5.1C.

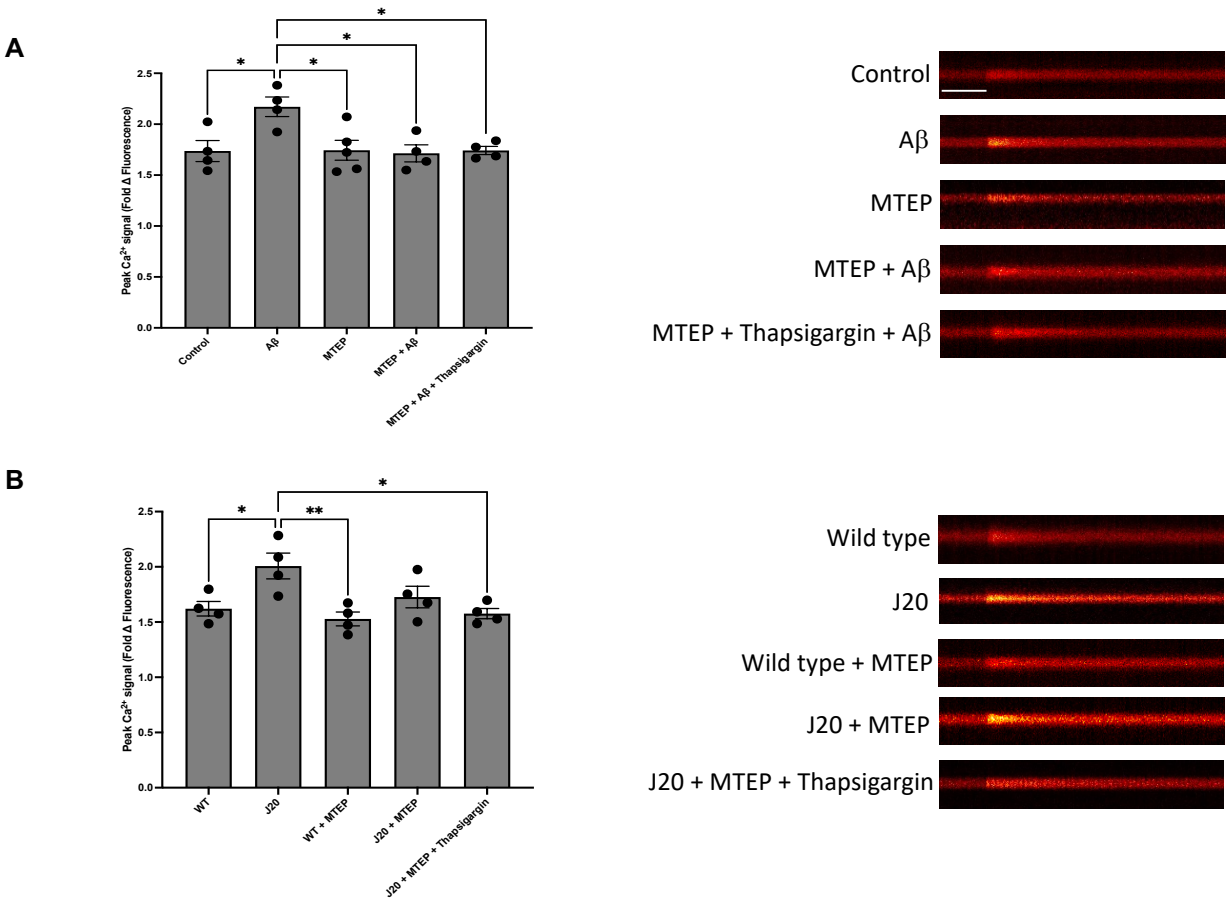

Figure 5.3: mGluR5 antagonist, MTEP, returns the Aβ-induced increase in peak Ca^{2+} signals to control levels in organotypic hippocampal slices. **a,b)** Average peak Ca^{2+} signals (fold Δ

fluorescence) measured across different conditions indicated (n = 4-5 neurons per condition, n = the average of 10 line scans per experiment) with examples of line scan images (scale bar = 50 ms). One-way ANOVA with post-hoc Tukey's test. *p<0.05, **p<0.01.

5.2.4. Inhibition of mGluR5 reduces growth of dendritic spines in response to LTP induction independent of Aβ

MTEP was able to rescue the Aβ-induced increase in peak Ca^{2+} signals and return them to control levels in organotypic slices. By acting upstream of Ca^{2+} release from the ER, MTEP was able to prevent Aβ oligomers from interacting and activating mGluR5. Next, I decided to examine the effects of MTEP on structural plasticity of dendritic spines, and to determine if the application of MTEP with Aβ would rescue the growth of spines. CA1 hippocampal neurons in organotypic slices were exposed to a theta-burst stimulation (TBS) protocol, which induces the growth of dendritic spines. As before, slices that were incubated with 200 nM of Aβ oligomers exhibited a significant decrease in the growth of dendritic spines (1.259 ± 0.050 fold Δ volume at 60 mins, n = 5) compared to the control growth of spines (1.643 ± 0.058 fold Δ volume at 60 mins, n = 5) ($p<0.01$) (Figure 5.4C). MTEP treatment (3 μM applied prior to baseline recordings and present for entire experiment) produced a similar significant reduction in the growth of spines (1.320 ± 0.044 fold Δ volume at 60 mins, n = 5) compared to control conditions (Figure 5.4C). The combination of MTEP and Aβ also significantly reduced spine growth (1.357 ± 0.059 fold Δ volume at 60 mins, n = 5) compared to control conditions. In acute hippocampal slices from wild type and J20 mice, glutamate photolysis was used to stimulate dendritic spine expansion. J20 mice displayed significantly diminished dendritic spine growth following stimulation (1.349 ± 0.048 fold Δ volume at 30 mins, n = 4) compared with the growth of spines in wild type mice (1.687 ± 0.057 fold Δ volume at 30 mins, n = 4) ($p<0.01$) (Figure 5.4F). Slices from both wild type and J20 mice incubated with MTEP showed significantly reduced growth of spines compared with control levels of spine growth (Figure 5.4F).

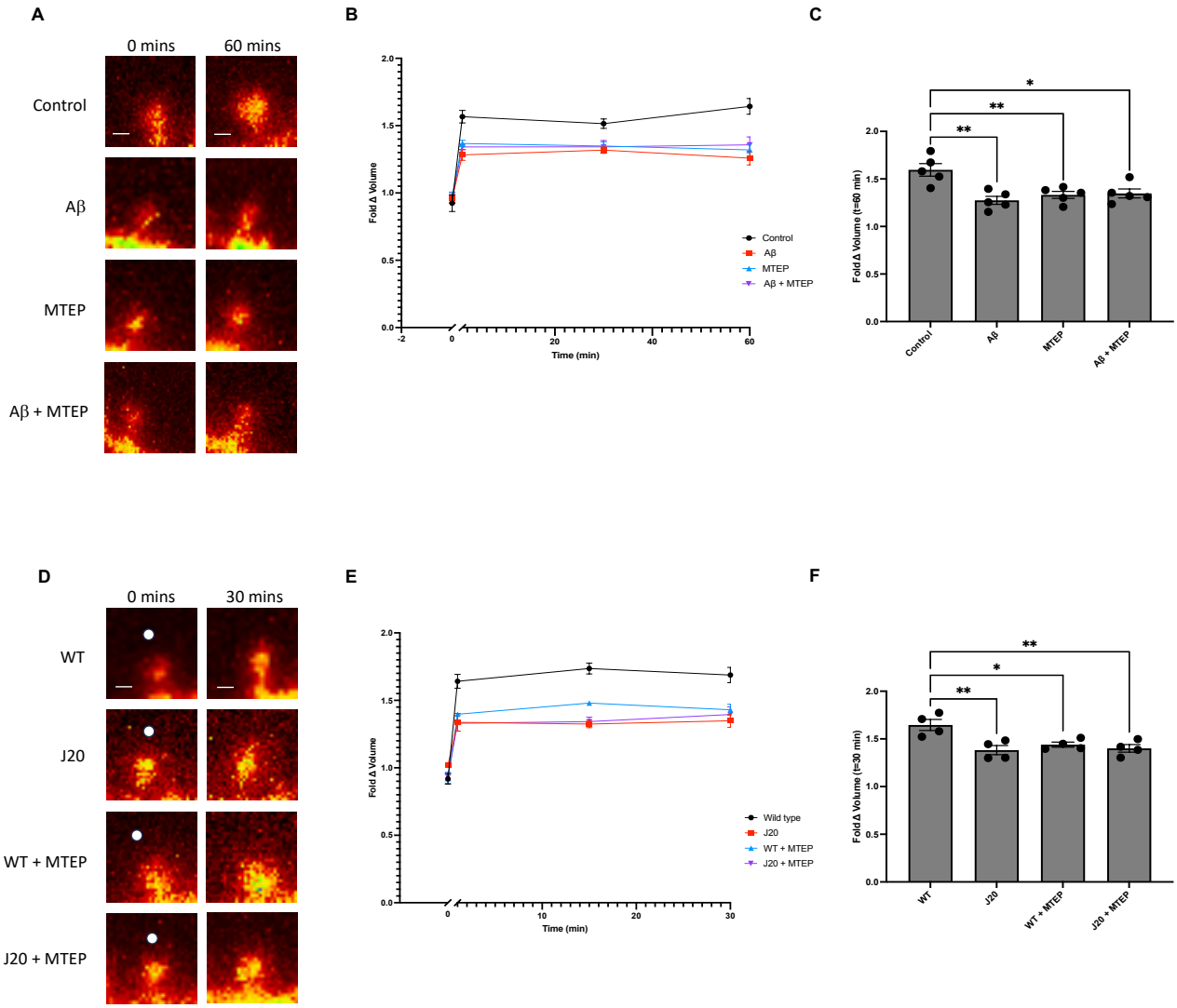

Figure 5.4: Inhibiting mGlur5 with MTEP reduces the growth of dendritic spines following LTP induction. **a,d)** Representative images of dendritic spines from the hippocampus of organotypic (a) or acute (d) slices at 0 and 60/30 mins after TBS/glutamate photolysis. White dot represents photolysis spot; scale bar = 1 μm. **b,e)** Fold Δ volume of dendritic spines over time (mins) under various conditions. **c)** Average fold Δ volume of dendritic spines at 60 minutes after TBS (n = 5 neurons per condition; 4-5 spines analysed within 20 μm of dendrite per neuron). One-way ANOVA with post-hoc Tukey's test. **f)** Average fold Δ volume of dendritic spines at 30 minutes after photolysis (n = 4 spines per condition). One-way ANOVA with post-hoc Tukey's test. *p<0.05, **p<0.01.

5.2.5. Aβ does not affect ER dynamics in dendritic spines following glutamate photolysis

Studies have discovered that the ER moves into spines experiencing high synaptic activity to inhibit runaway potentiation of dendritic spines (Perez-Alvarez et al. 2020). I wanted to investigate this process in organotypic hippocampal slices incubated with Aβ (200 nM for two hours), and determine if any changes in ER spine visits may provide an explanation for the Aβ-induced impairment in structural plasticity previously observed. Organotypic hippocampal slices were transfected with ER-GFP virus and imaged 10 days later. CA1 neurons expressing ER-GFP were patch-filled with Alexa Fluor 546 dye to visualise dendritic spine morphology, and structural LTP was induced at single spines using glutamate photolysis (Figure 5.5A,B). Glutamate uncaging was stimulated at spines lacking ER to observe ER movement into the spine. In control conditions, glutamate photolysis produced an increase in the volume of spines and transient ER entry into the dendritic spine, which peaked when spine volume was at its maximum and began to retract shortly after (Figure 5.5C). This observation is comparable to previous studies examining ER visits into dendritic spines following LTP induction (Perez-Alvarez et al. 2020). Incubation with Aβ oligomers (200 nM for two hours) reduced the expansion of spines following glutamate uncaging, however, there was no change in ER dynamics, which followed the same pattern of transiently visiting spines post-photolysis as in control conditions (Figure 5.5C). Comparing the peak fluorescence of spine volume and ER-GFP labelling at 1 min post-photolysis, there was a significant difference between the peak spine volume in control conditions (1.653 ± 0.063 peak fluorescence (AU), n = 5) and slices treated with Aβ (1.309 ± 0.032 peak fluorescence (AU), n = 5) ($p<0.01$) (Figure 5.5D). In contrast, there was no significant difference between the peak fluorescence of ER-GFP in control and Aβ treated slices (Figure 5.5D).

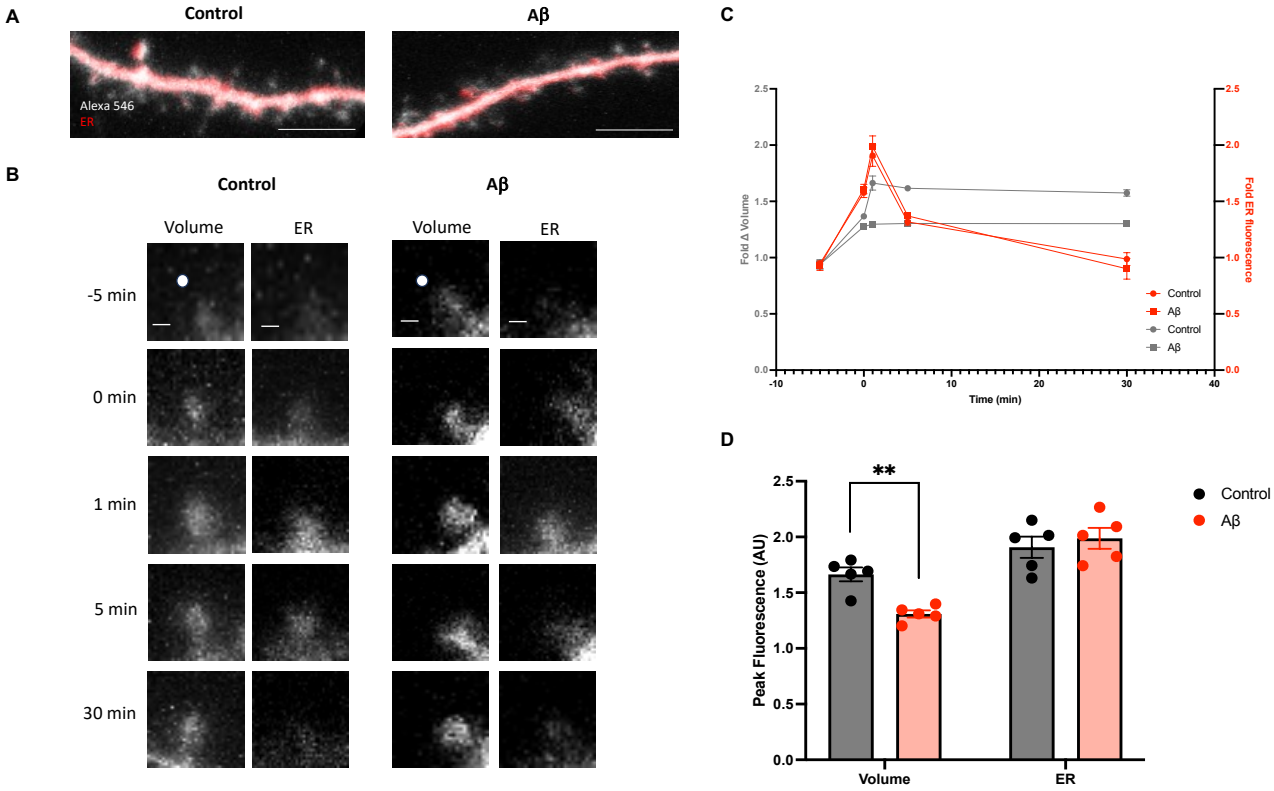

Figure 5.5: Aβ treatment does not affect ER movement into spines following glutamate uncaging and spine expansion. **a)** Representative images of a dendrite section from CA1 hippocampal neurons transfected with ER-GFP (red) and patch-filled with Alexa Fluor 546 dye (white); scale bar = 10 μm. **b**) Time-lapse images of dendritic spine volume and ER-GFP fluorescence during glutamate uncaging experiments. White dot represents photolysis spot; scale bar = 1 μm. **c)** Fold Δ volume of dendritic spines and ER-GFP fluorescence over time (mins) during a glutamate uncaging experiment. **d)** Average peak fluorescence of spine volume and ER-GFP at 1 min post-photolysis (n = 5 spines per condition). Unpaired Student's t-test (single comparisons). **p<0.01.

5.3. Discussion

The Ca^{2+} hypothesis of AD postulates that activation of the amyloidogenic pathway in neurons alters the mechanisms associated with Ca^{2+} homeostasis and signalling, resulting in the increase in intracellular Ca^{2+} concentration, eventually leading to cell death and the advancement of AD symptoms (Berridge 2010). The ER contains the largest store of releasable Ca^{2+} in neurons and is involved in regulating important cellular functions (Berridge 2002). Aβ oligomers play a significant role in Ca^{2+} dyshomeostasis throughout AD pathogenesis, and numerous studies have indicated that ER stress and dysregulated ER Ca^{2+} release and signalling contribute to synaptic pathology in AD (Chami and Checler 2020). In this Chapter, I decided to investigate the contribution of the ER to activity-dependent Ca^{2+} signalling in dendrites and structural plasticity of dendritic spines on CA1 hippocampal neurons, in response to acute Aβ treatment and chronic Aβ accumulation in J20 mice.

In neuronal dendrites, Ca^{2+} release from the ER is involved in regulating postsynaptic signalling and synaptic plasticity (Holbro, Grunditz, and Oertner 2009). Additionally, ER morphology is linked to changes in dendritic spine organisation, maturation and function (Holbro, Grunditz, and Oertner 2009). While NMDAR-mediated Ca^{2+} influx is typically required for the induction of LTP, it alone is not sufficient to maintain long-term forms of plasticity (Raymond and Redman 2006). ER Ca^{2+} release plays a crucial role in this process by amplifying and prolonging the duration of the initial Ca^{2+} signal (Ross et al. 2005). CICR from ER stores also ensures the appropriate spatial and temporal Ca^{2+} release patterns needed to activate downstream pathways essential for both LTP and LTD (Raymond and Redman 2006; Stutzmann and Mattson 2011). As a result, manipulation of ER Ca^{2+} signalling significantly influences the expression of synaptic plasticity (Berridge 2002). In this Chapter, the increase in peak Ca^{2+} signals following Aβ treatment was shown to arise from ER Ca^{2+} release, as the application of ryanodine or thapsigargin returned the

Ca^{2+} signal to control levels. The same results were observed in acute hippocampal slices from J20 mice, suggesting that the chronic accumulation of Aβ does not alter the response to ER inhibitors. In addition, Aβ oligomer treatment in J20 slices did not produce a difference in the peak Ca^{2+} signal (Supplementary Figure 3). The results suggest that Aβ may recruit CICR from the ER in response to back-propagating action potentials in dendrites, something that does not ordinarily occur in the dendrites of neurons (Emptage, Bliss, and Fine 1999), except in response to high-frequency stimulation (Miller et al. 1996; Alford et al. 1993). Aβ may alter the sensitivity of ER Ca^{2+} channels and enhance CICR in response to back-propagating action potentials. The overloading of ER Ca^{2+} stores, such as in AD models (Tu et al. 2006), may also stimulate the ER to release a significant amount of Ca^{2+}, contributing to the activity-dependent Ca^{2+} signal in dendrites.

In hippocampal neurons, the ER in the cell body and dendrites expresses both IP_3Rs and RyRs, however, ER networks located in distal processes and dendritic spines predominantly express RyR (Hertle and Yeckel 2007; Sharp et al. 1993). Ca^{2+} signalling involving these receptors may serve various roles in synaptic activity. Studies have shown that IP_3Rs may be implicated in gene transcription and protein synthesis, and extra-synaptic IP_3R may be engaged during synaptic spillover or require stronger activity inputs (Mellström and Naranjo 2001; Nakamura et al. 1999). Conversely, RyRs in dendritic spines may be strategically placed to directly regulate synaptic activity (Alford et al. 1993; Emptage, Bliss, and Fine 1999). RyR2 is the predominant RyR protein found in the brain, mainly expressed within axons of mossy fibres in the hippocampus, while RyR1 and RyR3 mRNAs are also expressed throughout the brain (Giannini et al. 1995; Shimizu et al. 2008). In the spines of hippocampal CA1 neurons, the Ca^{2+} influx via NMDARs is predominantly amplified by CICR mediated by RyRs (Emptage, Bliss, and Fine 1999). In AD, these processes may be compromised due to the dysregulation of ER Ca^{2+} signalling (Popugaeva and Bezprozvanny 2013). Studies have observed that elevated ER Ca^{2+} levels in the postsynaptic

compartment cause subsequent impairment of store-operated calcium entry, reduced CAMKII activity, and the loss of dendritic spines (Sun et al. 2014).

Since thapsigargin and ryanodine were able to normalise the increased peak Ca^{2+} signals evoked by Aβ, I postulated that these inhibitors may also rescue the Aβ-mediated structural plasticity impairments of dendritic spines, however, all treatments prevented spines from reaching control levels post-LTP induction. Thapsigargin depletes the ER Ca^{2+} store by blocking Ca^{2+} uptake through SERCA, and has been shown to block the induction of LTP (Harvey and Collingridge 1992). Thapsigargin seemingly inhibits LTP induction by preventing the NMDAR-mediated Ca^{2+} signal from being amplified by CICR from the ER (Harvey and Collingridge 1992). It has therefore been suggested that Ca^{2+} release from internal stores may be necessary for the induction of LTP (Behnisch and Reymann 1995). Similarly, other studies have revealed that treating neurons with ryanodine to block RyRs results in decreased spine density (Bertan et al. 2020). Furthermore, RyR2, the isoform highly expressed in the CNS, plays a crucial function in synaptic plasticity and memory formation (Mori et al. 2000). Studies have demonstrated that loss of RyR2 compromises the maintenance of structural plasticity in spines during memory acquisition (Bertan et al. 2020). In J20 mice, thapsigargin and ryanodine also had the same effect on the reduction of spine growth in response to LTP induction. Due to the evidence that ER Ca^{2+} release is essential for the induction of LTP, it may not be an appropriate target to prevent the effects of Aβ. Several AD mouse models have presented with elevated RyR expression and upregulated IP_3R-mediated Ca^{2+} release (Chakroborty et al. 2009; Leissring, Parker, and LaFerla 1999). It would be interesting to utilise partial antagonists of the IP_3R or RyR in future studies to determine if this may prevent the effect of Aβ-induced reduction in dendritic spine growth. Moreover, in these experiments, I did not account for Aβ plaque proximity in the acute slices from J20 mice. Future studies could incorporate fluorescent labelling of amyloid plaques to assess whether proximity to plaques influences the results and exacerbates impairment of structural plasticity. It is well-established that

impaired spine stability and spine loss are more pronounced in the vicinity of amyloid plaques (Spires-Jones et al. 2007; Bittner et al. 2012).

Growing evidence has suggested the involvement of mGluR5 in the neurotoxicity of Aβ. Aβ oligomers recruit mGluR5 to synapses and instigate enhanced G-protein-mediated IP_3 production, leading to augmented ER Ca^{2+} release via IP_3Rs and increased intracellular Ca^{2+} levels (Renner et al. 2010; Demuro and Parker 2013). Additionally, another study has shown that Aβ can form a scaffolding complex between PrP^C and mGluR5, activating pathological mGluR5 signalling in male but not female mice (Abd-Elrahman et al. 2020). Using MTEP to block mGluR5 resulted in the rescue of increased peak Ca^{2+} signals in response to Aβ treatment in organotypic slices, providing further support for the involvement of ER Ca^{2+} release in Aβ neurotoxicity. The addition of thapsigargin to MTEP and Aβ treatment did not further reduce the peak Ca^{2+} signal, suggesting that MTEP and thapsigargin might act on the same pathway. Conversely, in J20 neurons, MTEP was not able to return the enhanced peak Ca^{2+} signal to control levels, although a downward trend was observed. When thapsigargin was added to MTEP in J20 slices, the peak Ca^{2+} signal returned to control levels. With the accumulation of Aβ and developing pathogenesis in 6-9 month old J20 mice, the ER may be particularly overloaded with Ca^{2+}, and therefore, blocking SERCA with thapsigargin to deplete the Ca^{2+} store may be more effective than using MTEP to reduce the production of IP_3. Additionally, the intracellular baseline levels of Ca^{2+} in J20 mice might be excessively elevated, due to the contributions of various sources, for line-scan imaging to detect subtle differences attributed to mGluR5 function.

Treating hippocampal slices with MTEP prevented the growth of dendritic spines to control levels after the induction of LTP; the same result was observed in slices treated with Aβ alone, and Aβ with MTEP. A similar outcome was detected in wild type and J20 mice treated with MTEP. mGluR5 is mainly confined to perisynaptic domains, however, a minor fraction of mGluR5 is

relocated to the PSD, an activity-dependent process that regulates further downstream signalling (Scheefhals, Westra, and MacGillavry 2023). Although the activation of mGluR5 has been mainly associated with LTD, it has also been widely documented that metabotropic glutamate receptors are intrinsically involved in the modulation of hippocampal synaptic plasticity and spatial memory (Naie and Manahan-Vaughan 2004; Manahan-Vaughan 1997). Since mGluR5 plays an important role in LTP and LTD processes, it may be favourable to use a partial antagonist of the receptor, in future studies, to prevent the excessive activation by Aβ while maintaining normal levels of receptor function. mGluR5 has emerged as a target of Aβ oligomers in the early stages of AD, and postsynaptic mGluRs have been implicated in Aβ-induced LTP impairment and LTD enhancement (Li et al. 2009; Shankar et al. 2008). In one study, MTEP significantly improved Aβ-induced LTP impairment at CA3 synapses, although, this effect was limited to inhibiting presynaptic mGluR5 (He et al. 2019). The absence of correlation between studies investigating functional plasticity and structural changes following mGluR5 activation and LTD induction indicates that the two forms of plasticity may be governed by distinct neuronal mechanisms (Thomazeau et al. 2021). Although evidence points towards an interaction of Aβ oligomers with mGluR5, Aβ has been known to interact with multiple other receptors to impair the mechanisms of synaptic and structural plasticity (Mroczko et al. 2018). It is therefore challenging to focus on a single receptor to prevent downstream effects when the targets of Aβ are still entirely unclear.

Tracking ER movement into dendritic spines, following glutamate photolysis and the growth of spines, indicated that Aβ does not affect the capacity of the ER to move into spines experiencing high synaptic activity, which is associated with spine volume expansion (Perez-Alvarez et al. 2020). The Aβ-mediated significant increase in peak Ca^{2+} signals may arise from enhanced ER Ca^{2+} channel sensitivity or overloaded ER Ca^{2+} stores, rather than an impairment in ER movement and dynamics, which might disturb Ca^{2+} signalling domains within neurons.

ER Ca^{2+} dysregulation has been given significant attention as a contributing factor in AD pathogenesis, however, there are still gaps in our knowledge of ER Ca^{2+} dyshomeostasis in early AD. For example, PS1 mutations impair the physiological function of PS1, which act as Ca^{2+} leak channels on the ER membrane and prevent Ca^{2+} overload (Tu et al. 2006); although, in cases of AD without PS1 mutations or in models using Aβ oligomers, it is unclear whether the ER is equally overloaded with Ca^{2+}. The overloaded ER Ca^{2+} store may also impact other organelles, such as the mitochondria (Hedskog et al. 2013). Studies have shown that increased contact sites between the ER and mitochondria exist in AD models (Del Prete et al. 2017). Contact between the two organelles can promote excessive Ca^{2+} transfer from the ER to mitochondria, and lead to ROS generation, apoptosis and neurodegeneration (Zampese et al. 2011; Calvo-Rodriguez et al. 2020). Future studies are needed to investigate the key events triggering ER Ca^{2+} dysfunction in early AD, as well as how these impairments may impact downstream changes at later disease stages.

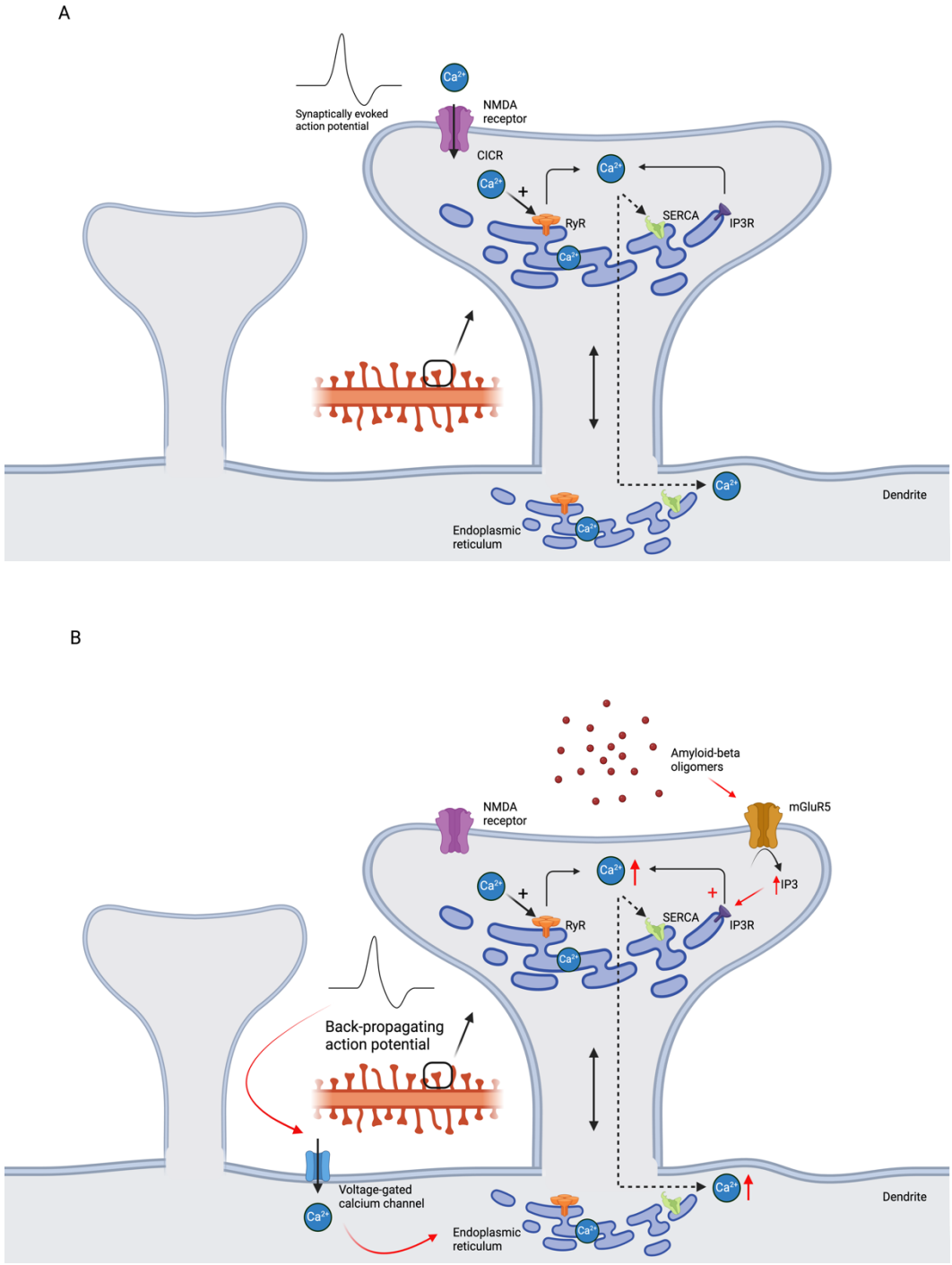

Figure 5.6: a) Following a synaptically evoked action potential, Ca^{2+} influx from activated NMDARs triggers CICR via the ryanodine receptor (RyR) on the ER. CICR can spread along the dendrite and the SERCA pump is responsible for transporting Ca^{2+} back into the ER. The ER itself can move between the dendrite and dendritic spine. **b)** In response to back-propagating action potentials in the presence of Aβ oligomers, Ca^{2+} influx via VGCCs recruits CICR from the ER. Aβ may also activate the mGluR5 receptor, stimulating the production of IP_3, and further potentiating Ca^{2+} release from the ER via IP_3 receptors (IP_3R). The red arrows denote processes impacted by pathogenic Aβ oligomers.

6. Investigating the metabolome of the J20 mouse model of AD – monitoring the effects of age and disease progression

6.1. Introduction

The brain has a high metabolic demand, and in a pathogenic state, the balance of energy metabolites is disrupted, leading to neuronal damage and cognitive decline (Fiandaca et al. 2015; Watts, Pocock, and Claudianos 2018). Abnormal neuronal metabolism has been observed in the brains of AD patients and animal models (González-Domínguez, García-Barrera, and Gómez-Ariza 2014; González-Domínguez et al. 2015). The pathogenesis of AD increases metabolic demand due to a number of factors such as an increase in the generation of reactive oxygen species (ROS), inflammation, lipid peroxidation, excessive release of glutamate, and reduced production of ATP (Wang et al. 2014). Interestingly, reduced glucose utilisation and metabolic decline has been detected in the early stages of AD using fluorodeoxyglucose positron emission tomography (FDG-PET) (Marcus, Mena, and Subramaniam 2014), suggesting that altered metabolism may make an important contribution to AD pathogenesis.

Metabolomics is an emerging field and refers to the quantitative study of metabolites, which represent the end-products of cellular processes in biological samples (Patti, Yanes, and Siuzdak 2012). NMR-based metabolomics is particularly useful as a major analytical method, as it can provide quantitative information of many metabolites simultaneously (Patti, Yanes, and Siuzdak 2012). Specific metabolites may be sensitive to AD pathogenic processes, and as most metabolic pathways are conserved between mammalian species, metabolomics represents a powerful tool for the discovery of biomarkers in preclinical animal models, which may be translated to humans (Salek et al. 2007). The lack of clinically available biomarkers for AD diagnosis and treatment

monitoring is a major issue. Current research is focused on the development of non-invasive biomarkers, mainly blood-based biomarkers that measure Aβ peptides and phosphorylated tau (Varesi et al. 2022). These novel biomarkers may enable earlier and faster diagnoses, however, due to the heterogeneity of AD patients, a single biomarker may be insufficient to detect AD. By detecting longitudinal changes in metabolites, it is possible to gain insight into disease mechanisms and establish disease-associated metabolic biomarkers (Motsinger-Reif et al. 2014).

A recent study using post-mortem AD patient brain tissue samples has discovered that metabolic changes associated with cellular energy, glucose metabolism, and fatty acids, are linked to memory loss and the build-up of pathogenic tau (Batra et al. 2023). In another study, using a mouse model of AD, it was shown that metabolite alterations precede cognitive dysfunctions, and therefore, may drive disease progression (Chen et al. 2012). AD is a multifaceted disease with numerous pathways synergistically affected, ultimately converging on cognitive decline (Selkoe and Hardy 2016). For example, another metabolic multi-platform study in AD patients detected significant changes in the levels of several phospholipids, and perturbations in several metabolic networks including the TCA cycle, neurotransmission and inflammation, in both CSF and plasma (González-Domínguez, García-Barrera, and Gómez-Ariza 2015). Similar findings have been observed in other specific mouse models of AD, affecting mainly the hippocampus and cortex, with changes in membrane lipid metabolism and neurotransmitter amino acids (González-Domínguez, García-Barrera, and Gómez-Ariza 2015). Although many studies have provided a comprehensive insight into the metabolome of AD, most were conducted at a single time point, typically after the development of AD symptoms.

Incidence of AD is known to increase with age, however, age-related cognitive decline is a physiological process of normal aging and differs from the severe deterioration of cognition observed in AD (Salthouse 2012; Riedel, Thompson, and Brinton 2016). In this chapter, we were

interested in elucidating the differences between metabolic pathways involved in normal aging and AD pathogenesis. Identification of these changes would allow for a more accurate characterisation of pathogenic cognitive dysfunction, as well as support the discovery of novel biomarkers. Furthermore, we measured levels of metabolites at two different time-points, with wild-type and J20 mice being grouped into younger (< 5 months of age) and older (> 5 months of age) categories. The J20 mouse strain, a long standing and important model for AD research, overexpresses human APP with familial disease-linked Swedish and Indiana mutations, driven by the PDGF-β promoter (Mucke et al. 2000). This expression results in high Aβ immunoreactivity across the brain, especially in the neocortex and hippocampus (Wright et al. 2013). J20 mice exhibit elevated Aβ levels and develop significant amyloid plaques from five to seven months of age (Mucke et al. 2000). Associated synaptic changes include reduced basal synaptic transmission and impaired LTP, which underscores the model's relevance for studying synaptic dysfunction in AD, and these changes are accompanied by learning and memory deficits (Mucke et al. 2000; Wright et al. 2013). Therefore, we sought to discover whether we could differentiate between metabolic pathways affected in early and late disease stages.

Metabolite changes across age were examined in tissues of the central nervous system (CNS) (hippocampus and prefrontal cortex) and periphery (spleen, liver and serum). The prefrontal cortex (PFC) and hippocampus play an important role in learning and memory, and are affected by AD pathology from the earliest stages (Chen, Dang, and Zhang 2021). By investigating metabolite changes in the periphery, a more complete AD metabolomic dataset may be acquired, which accounts for various organs that may also be involved in pathogenic processes. Although most research has focused on the metabolic changes occurring in the CNS in AD, there is also substantial evidence of metabolic alterations in the peripheral system of AD mouse models (Hunsberger et al. 2020; Zheng et al. 2019). Research has established that connections exist between the brain and peripheral tissues, for example, liver-induced inflammation is able to cause

disturbances in CNS metabolism (Garcia-Martinez and Cordoba 2012). Additionally, a direct connection pathway exists between the brain and spleen, and the two organs may communicate via immune modulation (Rosas-Ballina et al. 2008). It is therefore important to investigate how the metabolic changes in peripheral tissues interact with CNS metabolism and impact the progression of AD. The development of accurate metabolomic datasets of relevance to AD may have a key impact to further our understanding of AD pathogenesis, diagnosis and treatment.

6.2. Results

6.2.1. J20 mice have altered CNS and peripheral tissue metabolite profiles

Untargeted H^1 NMR-based metabolomic profiling was performed on all tissues of young (< 5 months of age) and old (> 5 months of age) wild-type (WT) and J20 mice; the 'young' group had a median age of 2.2 months, with ages ranging from 1 to 4.4 months, while the 'old' group had a median age of 9 months, with ages ranging from 5.5 to 11.5 months. The J20 mice develop memory deficits and progressive plaque accumulation by 5 months of age (Mucke et al. 2000; Wright et al. 2013). Supervised orthogonal partial least square discriminant analysis (OPLS-DA) was performed to identify metabolite differences between groups, build predictive models and report the mean accuracy, sensitivity and specificity of the model.

Firstly, OPLS-DA models were built for each tissue to visualise variations between WT and J20 mice (Figure 6.1). The OPLS-DA models were able to differentiate between WT and J20 mice of all ages with significant mean accuracy ($p<0.0001$) sensitivity ($p<0.0001$) and specificity ($p<0.0001$) (Table 6.1) when compared to a model generated by random chance with an approximate accuracy, sensitivity and specificity of 50%. Accuracy of the model refers to the degree to which it accurately classifies samples as either WT or J20, sensitivity reflects the

competence of the model to identify true positives, and specificity signifies the correct detection of true negatives.

To determine the effect of normal aging and AD pathogenesis on the metabolite profiles, the groups were further split into WT/J20 young and WT/J20 old mice. Additional OPLS-DA models were built to detect metabolite differences between the following groups: WT young vs J20 young, WT old vs J20 old, WT young vs WT old, and J20 young vs J20 old. The models were able to discriminate between young and old mice of both genotypes (Figure 6.2), and the accuracy, sensitivity and specificity were significant in all models based on samples from the PFC, hippocampus and spleen (Table 6.2). However, some OPLS-DA models did not perform significantly better compared to the random chance model, possibly due to the lower number of samples used. In liver samples, the mean predictive accuracy, sensitivity and specificity reported from the OPLS-DA analysis of WT young vs J20 young mice was not significant compared to the statistics of the random chance model. Similarly, in serum samples, the mean predictive accuracy, sensitivity and specificity reported from the OPLS-DA analysis of J20 young vs J20 old mice was not significant. Additionally, the mean accuracy and sensitivity of the WT young vs J20 young model did not out-perform the random chance model.

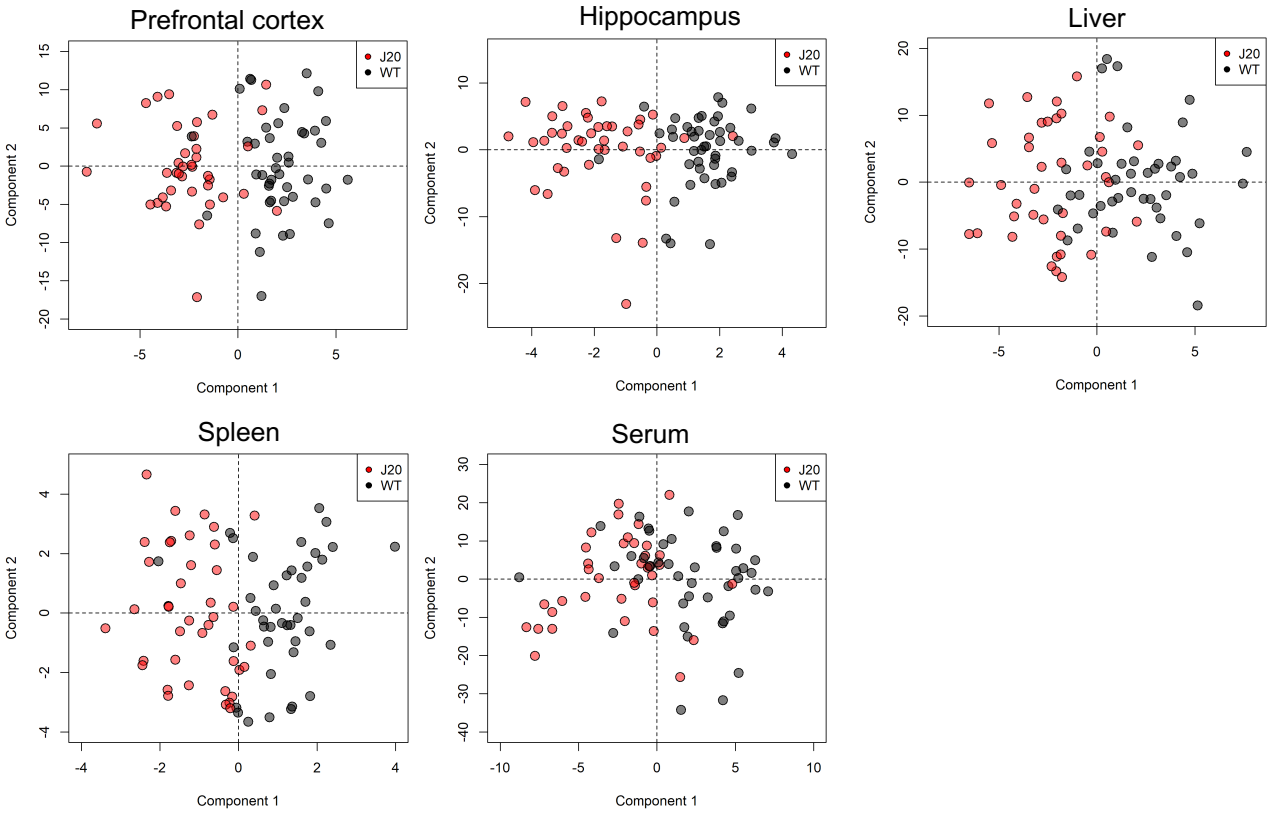

Figure 6.1: OPLS-DA plots used to visualise metabolite differences between WT and J20 mice of all ages. Figure reproduced with permission from Jaezah Zainal.

Sample	WT vs J20 (all ages)	
PFC	Sample number	Total (n= 81) J20 (n=38) WT (n=43)
	Accuracy (%)	79 ± 3 ****
	Specificity (%)	84 ± 6 ****
	Sensitivity (%)	75 ± 5 ****
Hippocampus	Sample number	Total (n= 90) J20 (n=43) WT (n=47)
	Accuracy (%)	74 ± 4 ****
	Specificity (%)	73 ± 5 ****
	Sensitivity (%)	75 ± 4 ****
Liver	Sample number	Total (n= 83) J20 (n=40) WT (n=43)
	Accuracy (%)	64 ± 4 ****
	Specificity (%)	63 ± 5 ****
	Sensitivity (%)	65 ± 6 ****
Spleen	Sample number	Total (n=84) J20 (n=41) WT (n=43)
	Accuracy (%)	73 ± 3 ****
	Specificity (%)	77 ± 5 ****
	Sensitivity (%)	70 ± 4 ****
Serum	Sample number	Total (n=80) J20 (n=37) WT (n=43)
	Accuracy (%)	64 ± 4 ****
	Specificity (%)	67 ± 5 ****
	Sensitivity (%)	63 ± 8 ****

Table 6.1: The sample number, mean accuracy, specificity and sensitivity ± standard deviation for the OPLS-DA models generated of WT and J20 mice of all ages. Mean accuracy, specificity and sensitivity were compared to a random model using an unpaired Student's t-test; ****$p<0.0001$. Table reproduced with permission from Jaezah Zainal.

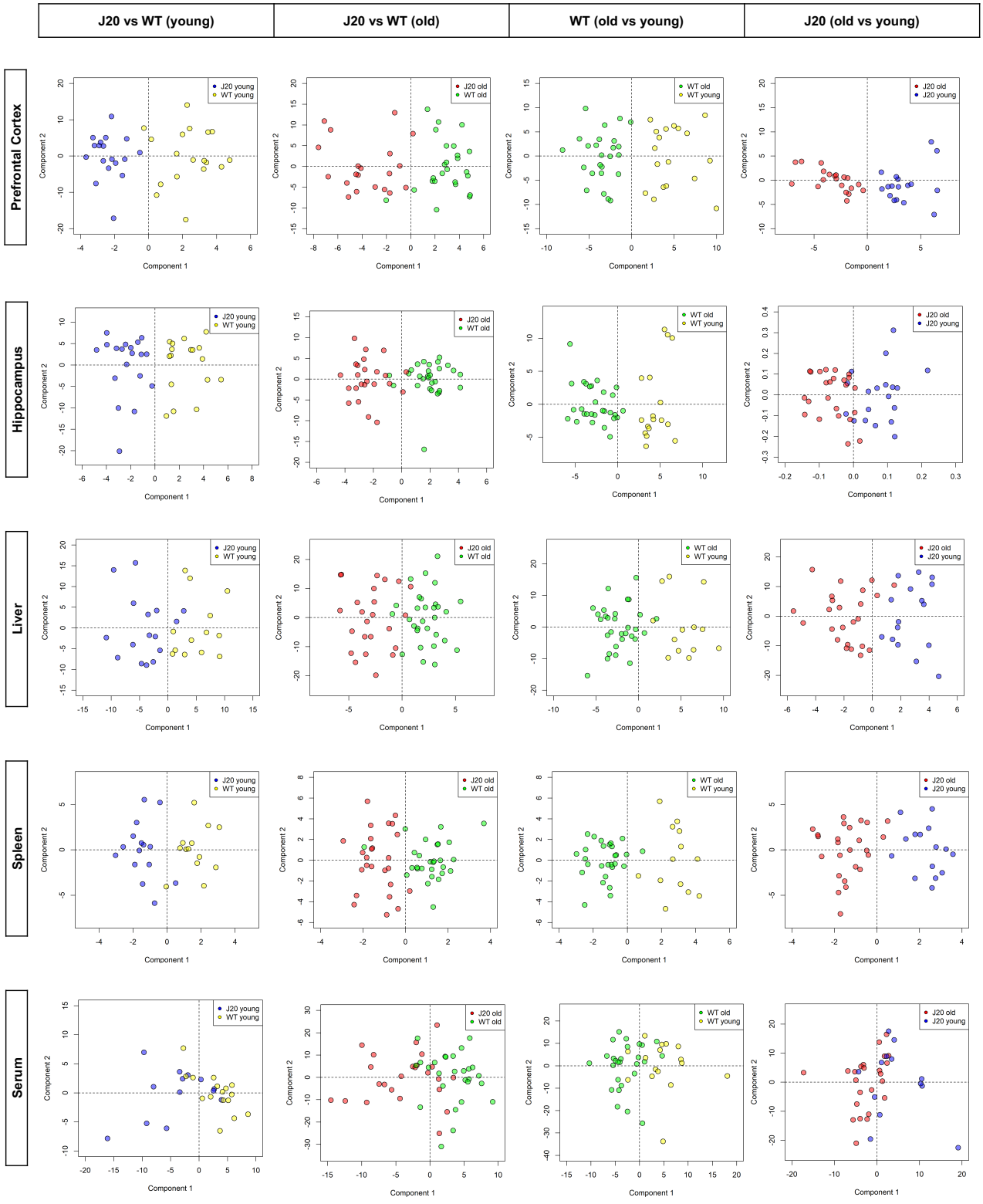

Figure 6.2: OPLS-DA plots used to visualise metabolite differences between WT and J20 mice split into young (< 5 months of age) and old (> 5 months of age) groups. Figure reproduced with permission from Jaezah Zainal.

		J20 vs WT (young)	J20 vs WT (old)	WT (old vs young)	J20 (old vs young)
PFC	Sample number	Total (n = 36) J20 young (n=18) WT young (n=18)	Total (n=45) J20 old (n=20) WT old (n=25)	Total (n=43) WT old (n=25) WT young (n=18)	Total (n=38) J20 old (n=20) J20 young (n=18)
	Accuracy (%)	75 ± 5 ****	72 ± 5 ****	85 ± 6 ****	71 ± 5 ****
	Specificity (%)	70 ± 7 ****	78 ± 7 ****	82 ± 10 ****	67 ± 8 ****
	Sensitivity (%)	81 ± 7 ****	67 ± 8 ****	89 ± 7 ****	76 ± 8 ****
HC	Sample number	Total (n= 36) J20 young (n=19) WT young (n=17)	Total (n=53) J20 old (n=24) WT old (n=29)	Total (n= 46) WT old (n=29) WT young (n=17)	Total (n= 43) J20 old (n=24) J20 young (n=19)
	Accuracy (%)	73 ± 4 ****	74 ± 4 ****	79 ± 6 ****	69 ± 5 ****
	Specificity (%)	82 ± 8 ****	80 ± 7 ****	86 ± 8 ****	71 ± 7 ****
	Sensitivity (%)	77 ± 6 ****	70 ± 7 ****	83 ± 7 ****	75 ± 8 ****
Liver	Sample number	Total (n = 29) J20 young (n=16) WT young (n=13)	Total (n=54) J20 old (n=24) WT old (n=30)	Total (n=43) WT old (n=30) WT young (n=13)	Total (n=40) J20 old (n=24) J20 young (n=16)
	Accuracy (%)	40 ± 7 (ns)	71 ± 5 ****	76 ± 8 ****	66 ± 7 ****
	Specificity (%)	30 ± 8 (ns)	74 ± 8 ****	73 ± 9 ****	70 ± 10 ****
	Sensitivity (%)	51 ± 11 (ns)	69 ± 7 ****	81 ± 10 ****	63 ± 10 ****
Spleen	Sample number	Total (n = 31) J20 young (n=16) WT young (n=15)	Total (n=53) J20 old (n=25) WT old (n=28) vs	Total (n=43) WT old (n=28) WT young (n=15)	Total (n=41) J20 old (n=25) J20 young (n=16)
	Accuracy (%)	67 ± 5 ****	75 ± 4 ****	88 ± 5 ****	90 ± 5 ****
	Specificity (%)	73 ± 8 ****	77 ± 6 ****	84 ± 7 ****	88 ± 7 ****
	Sensitivity (%)	63 ± 8 ****	74 ± 7 ****	91 ± 6 ****	93 ± 6 ****
Serum	Sample number	Total (n = 29) J20 young (n=13) WT young (n=16)	Total (n=51) J20 old (n=24) WT old (n=27)	Total (n=43) WT old (n=27) WT young (n=16)	Total (n=41) J20 old (n=24) J20 young (n=13)
	Accuracy (%)	53 ± 6 (ns)	70 ± 4 ****	69 ± 7 ****	50 ± 9 (ns)
	Specificity (%)	59 ± 11 ***	75 ± 7 ****	67 ± 10 ****	53 ± 11 (ns)
	Sensitivity (%)	48 ± 10 (ns)	66 ± 6 ****	72 ± 11 ****	50 ± 13 (ns)

Table 6.2: The sample number, mean accuracy, specificity and sensitivity ± standard deviation for the OPLS-DA models generated of WT and J20 mice, split into young and old age groups. Mean accuracy, specificity and sensitivity were compared to a random model using an unpaired Student's t-test; ***$p<0.001$ ****$p<0.0001$. Table reproduced with permission from Jaezah Zainal.

6.2.2. Tissue-specific metabolomic profiling of WT and J20 mice

To investigate which metabolites drive group separation in the OPLS-DA models of each tissue, variable importance in projection (VIP) scores were analysed to determine the contribution of specific metabolites to the models. VIP analysis reveals that metabolites with the highest VIP scores have the greatest contribution to driving the discrimination between groups.

Prefrontal cortex

Once the VIP metabolites were identified, the integral values (AU) were compared using two-way ANOVA with post-hoc analysis to determine significant main effects and which groups are significantly different. Citrate (Figure 6.3A) showed a significant effect of genotype ($p<0.0001$) and post-hoc analysis revealed that citrate levels were significantly decreased in young J20 mice compared with young WT mice ($p<0.01$). Similarly, old J20 mice also had significantly decreased citrate levels compared to old WT mice ($p<0.001$). Fatty acyl analysis (Figure 6.3B) showed an effect of genotype ($p<0.0001$) and age ($p<0.0001$) and specifically, old WT mice had increased levels of fatty acyl chain compared with young WT mice ($p<0.0001$). This difference between the age groups was also present in the J20 mice ($p<0.05$). Old J20 mice showed decreased levels of fatty acyl chain compared with old WT mice ($p<0.0001$). Lactate (Figure 6.3C), which had an effect of genotype ($p<0.001$) was decreased in old J20 mice compared with old WT mice ($p<0.05$). Sphingomyelin (Figure 6.3D) exhibited an effect of age ($p<0.05$) and was significantly increased in old WT mice compared with young WT mice ($p<0.05$). Histidine (Figure 6.3E) had an effect of age ($p<0.001$) and old J20 mice had decreased levels compared with young J20 mice ($p<0.05$). Glucose (Figure 6.3F) showed an effect of age ($p<0.05$) and a significant interaction between genotype and age ($p<0.01$), and glucose levels were increased in old WT mice compared with young WT mice ($p<0.01$), and decreased in old J20 mice compared with old WT mice ($p<0.05$). Glutamine (Figure 6.3G), which had significant interaction ($p<0.05$), was increased in old J20 mice

compared with old WT mice ($p<0.05$). Lysine (Figure 6.3H) had an effect of age ($p<0.0001$) and genotype ($p<0.01$), and demonstrated a similar trend to glucose levels, with an increase in old WT mice compared with young WT mice ($p<0.0001$), and a decrease in old J20 mice compared with old WT mice ($p<0.01$).

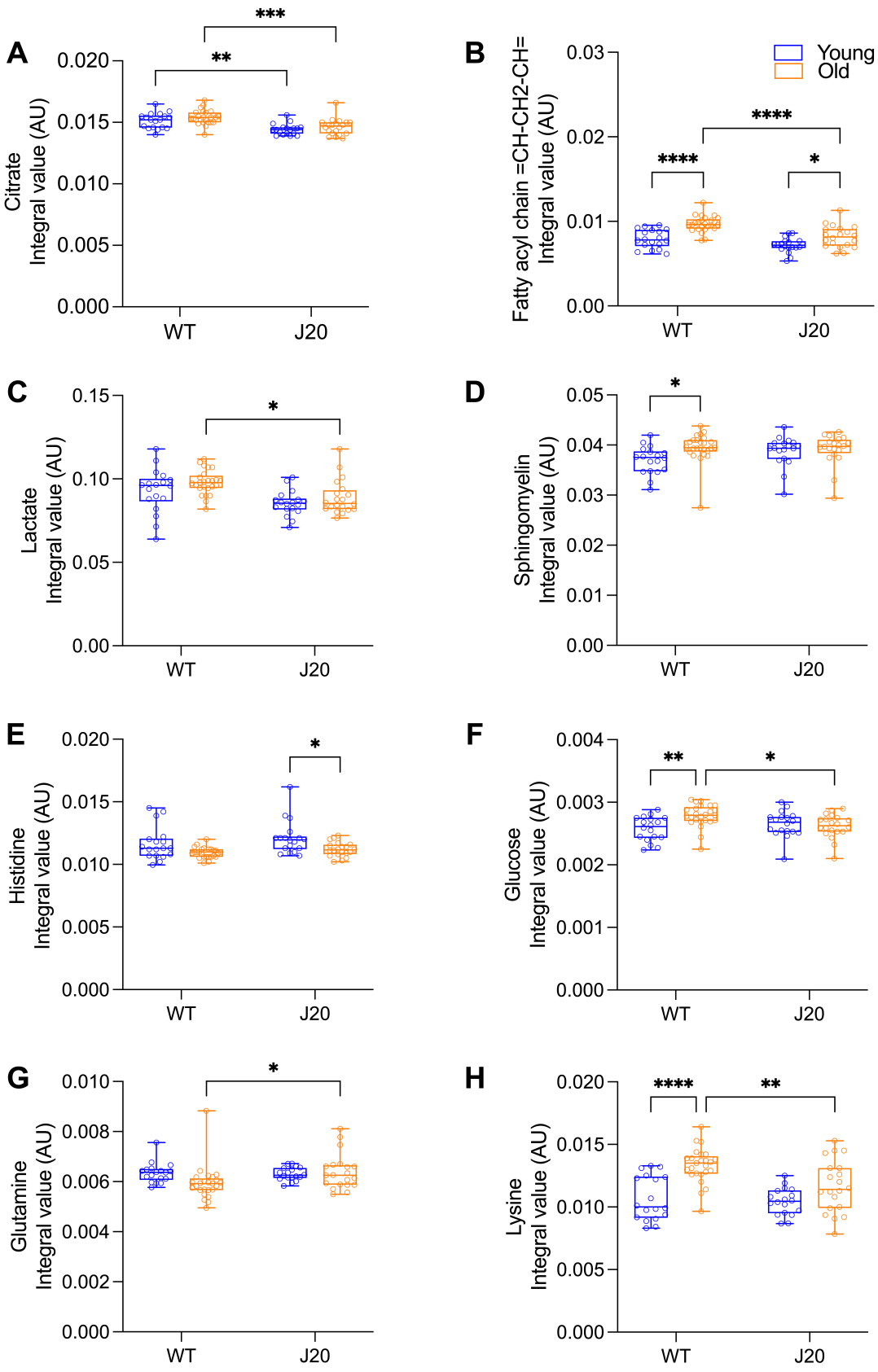

Figure 6.3: Prefrontal cortex metabolites altered by genotype and age. VIP analysis revealed discriminatory metabolites, which are **a)** citrate, **b)** fatty acyl chain =CH-CH2-CH=, **c)** lactate, **d)** sphingomyelin, **e)** histidine, **f)** glucose, **g)** glutamine, and **h**) lysine. n = 18 mice (WT young), 18 mice (J20 young), 25 mice (WT old) and 20 mice (J20 old). Data expressed as integral values (AU)

and presented as boxplots including the median, interquartile range, minimum and maximum data points. Two-way ANOVA with post-hoc Tukey's test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Figure panels a, b, c, f, g, h adapted with permission from Jaezah Zainal.

Hippocampus

Alanine (Figure 6.4A) was shown to have an effect of age ($p<0.001$), and was significantly decreased in WT old mice compared with WT young mice ($p<0.01$). Choline (Figure 6.4B) had an effect of genotype ($p<0.01$) and was increased in old J20 mice compared with old WT mice ($p<0.05$). Glutamate (Figure 6.4C) had an effect of age ($p<0.0001$) and was decreased in old J20 mice compared with young J20 mice ($p<0.01$). Glycerophosphocholine (Figure 6.4D) had an effect of age ($p<0.01$) and was increased in old WT mice compared with young WT mice ($p<0.01$). Lactate (Figure 6.4E) had an effect of genotype ($p<0.001$) and was decreased in young J20 mice compared with young WT mice ($p<0.01$). Similarly, old J20 mice had decreased lactate levels compared with old WT mice ($p<0.01$). Serine phosphoethanolamine (PETA) (Figure 6.4F) had an effect of genotype ($p<0.05$) and age ($p<0.0001$), and was decreased in old WT mice compared with young WT mice ($p<0.05$). The same result was observed in the J20 mice, with old mice exhibiting decreased Serine-PETA levels compared with young mice ($p<0.05$).

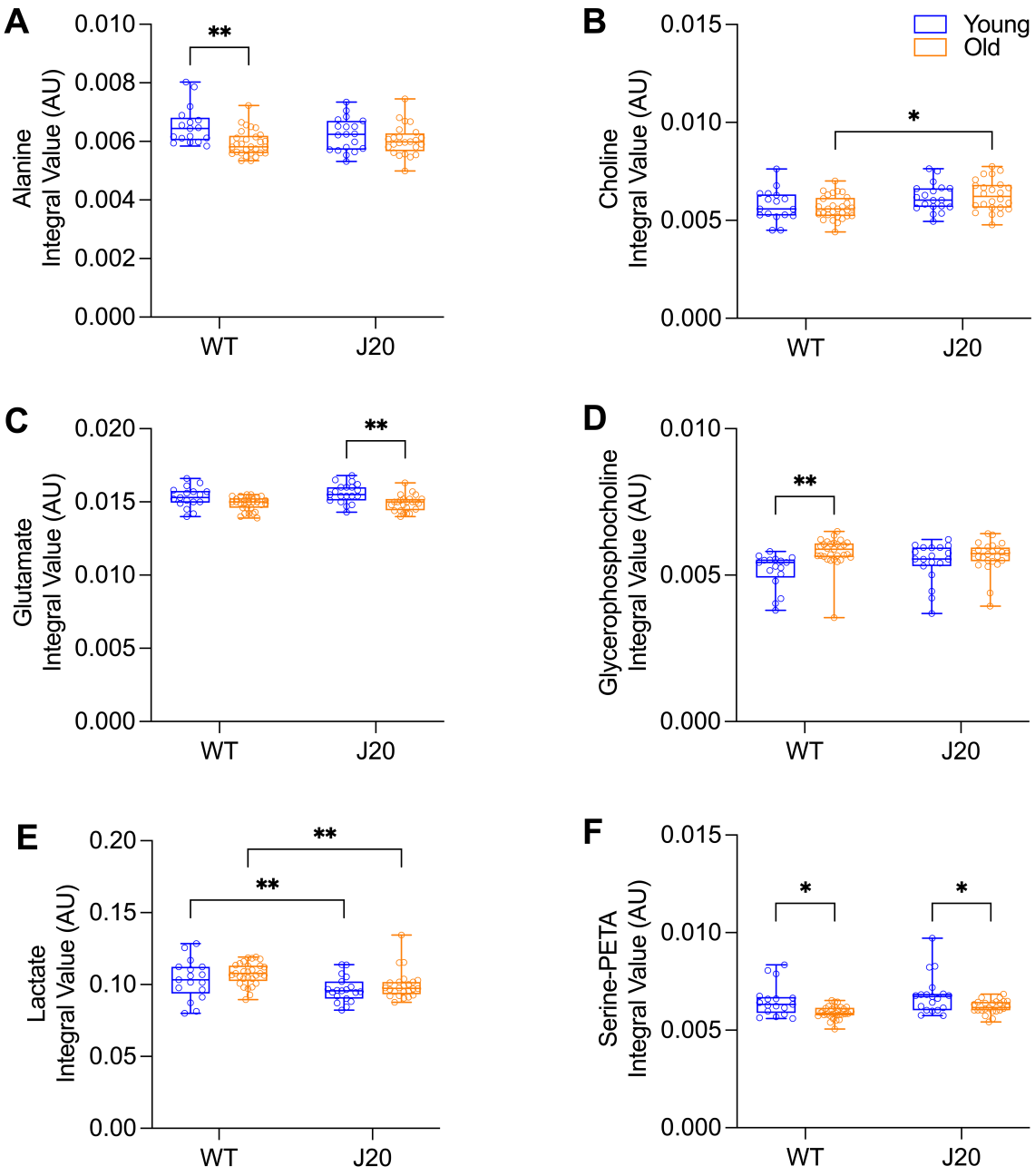

Figure 6.4: Hippocampus metabolites altered by genotype and age. VIP analysis revealed discriminatory metabolites, which are **a)** alanine, **b)** choline **c)** glutamate, **d)** glycerophosphocholine, **e)** lactate, and **f)** Serine-PETA. n = 17 mice (WT young), 19 mice (J20 young), 29 mice (WT old) and 24 mice (J20 old). Data expressed as integral values (AU) and presented as boxplots including the median, interquartile range, minimum and maximum data points. Two-way ANOVA with post-hoc Tukey's test. *p<0.05, **p<0.01. Figure panels b, e adapted with permission from Jaezah Zainal.

Liver

Citrate levels (Figure 6.5A) had significant interaction between genotype and age ($p<0.05$), and was decreased in old WT mice compared with young WT mice ($p<0.05$). However, citrate levels were increased in old J20 mice compared with old WT mice ($p<0.01$). Glutamine (Figure 6.5B) had an effect of age ($p<0.05$) and was increased in old J20 mice compared with old WT mice ($p<0.05$). Histidine (Figure 6.5C) had an effect of age ($p<0.01$) and levels were decreased in old WT mice compared with young WT mice ($p<0.05$). Lactate (Figure 6.5D) showed a similar trend and an effect of age ($p<0.001$). Lactate levels were decreased in old WT mice compared with young WT mice ($p<0.01$). Lysine (Figure 6.5E) had an effect of age ($p<0.05$) and interaction ($p<0.05$). Lysine levels were also decreased in old WT mice compared with young WT mice ($p<0.01$). Myo-inositol (Figure 6.5F) additionally showed the same result, with an effect of age ($p<0.01$) and with a decrease of myo-inositol levels in old WT mice compared with young WT mice ($p<0.05$). Threonine (Figure 6.5G) had an effect of age ($p<0.0001$) and genotype ($p<0.001$). Threonine levels were elevated in old WT mice compared with young WT mice ($p<0.001$), and in young J20 mice compared with young WT mice ($p<0.05$).

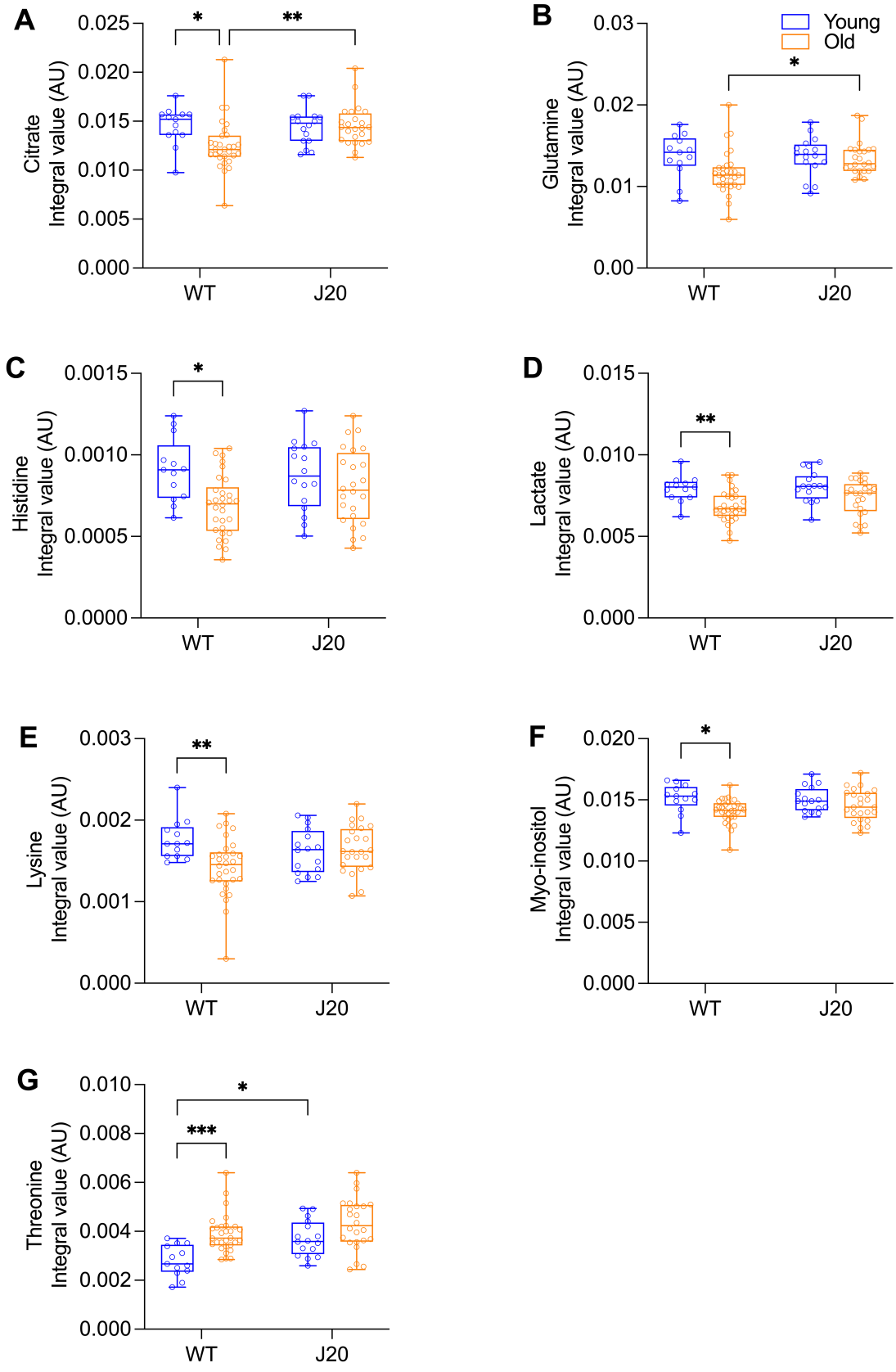

Figure 6.5: Liver metabolites altered by genotype and age. VIP analysis revealed discriminatory metabolites, which are **a)** citrate, **b)** glutamine **c)** histidine, **d)** lactate, **e)** lysine, **f)** myo-inositol, and **g)** threonine. n = 13 mice (WT young), 16 mice (J20 young), 30 mice (WT old) and 24 mice (J20 old). Data expressed as integral values (AU) and presented as boxplots including the median,

interquartile range, minimum and maximum data points. Two-way ANOVA with post-hoc Tukey's test. *p<0.05, **p<0.01, ***p<0.001. Figure panels a, b, c, e adapted with permission from Jaezah Zainal.

Spleen

Alanine (Figure 6.6A) had an effect of genotype ($p<0.05$) and was significantly increased in old J20 mice compared with old WT mice ($p<0.05$). Aspartate (Figure 6.6B) had an effect of age ($p<0.0001$), with an increase in aspartate levels in old WT mice compared with young WT mice ($p<0.05$). The J20 mice also showed the same difference between age groups ($p<0.01$). Citrate (Figure 6.6C) exhibited an effect of age ($p<0.001$) and was increased in old WT mice compared with young WT mice ($p<0.01$). Choline (Figure 6.6D) showed an effect of age ($p<0.0001$), and both WT and J20 mice demonstrated decreased choline levels in old mice compared with young mice ($p<0.01$). Glycerophosphocholine (Figure 6.6E) had an effect of genotype ($p<0.0001$) and age ($p<0.001$), and was increased in young J20 mice compared with young WT mice ($p<0.01$). Glycerophosphocholine levels were also increased in old J20 mice compared with old WT mice ($p<0.001$), and within the J20 mice, levels were decreased in old J20 mice compared with young J20 mice ($p<0.05$). Phosphoethanolamine (Figure 6.6F) had an effect of age ($p<0.0001$) and was decreased in old J20 mice compared with young J20 mice ($p<0.01$). Threonine (Figure 6.6G) had an effect of genotype ($p<0.001$) and was increased in old J20 mice compared with old WT mice ($p<0.05$). Tyrosine (Figure 6.6H) had an effect of age ($p<0.0001$) and old WT mice had increased tyrosine levels compared with young WT mice ($p<0.0001$). The J20 mice also demonstrated significantly raised tyrosine levels in old mice compared with young mice ($p<0.001$). Valine (Figure 6.6I) had an effect of age ($p<0.0001$) and was decreased in old WT mice compared with young WT mice ($p<0.01$). This effect was also observed in the J20 mice, with lower levels of valine in old mice compared with young mice ($p<0.001$).

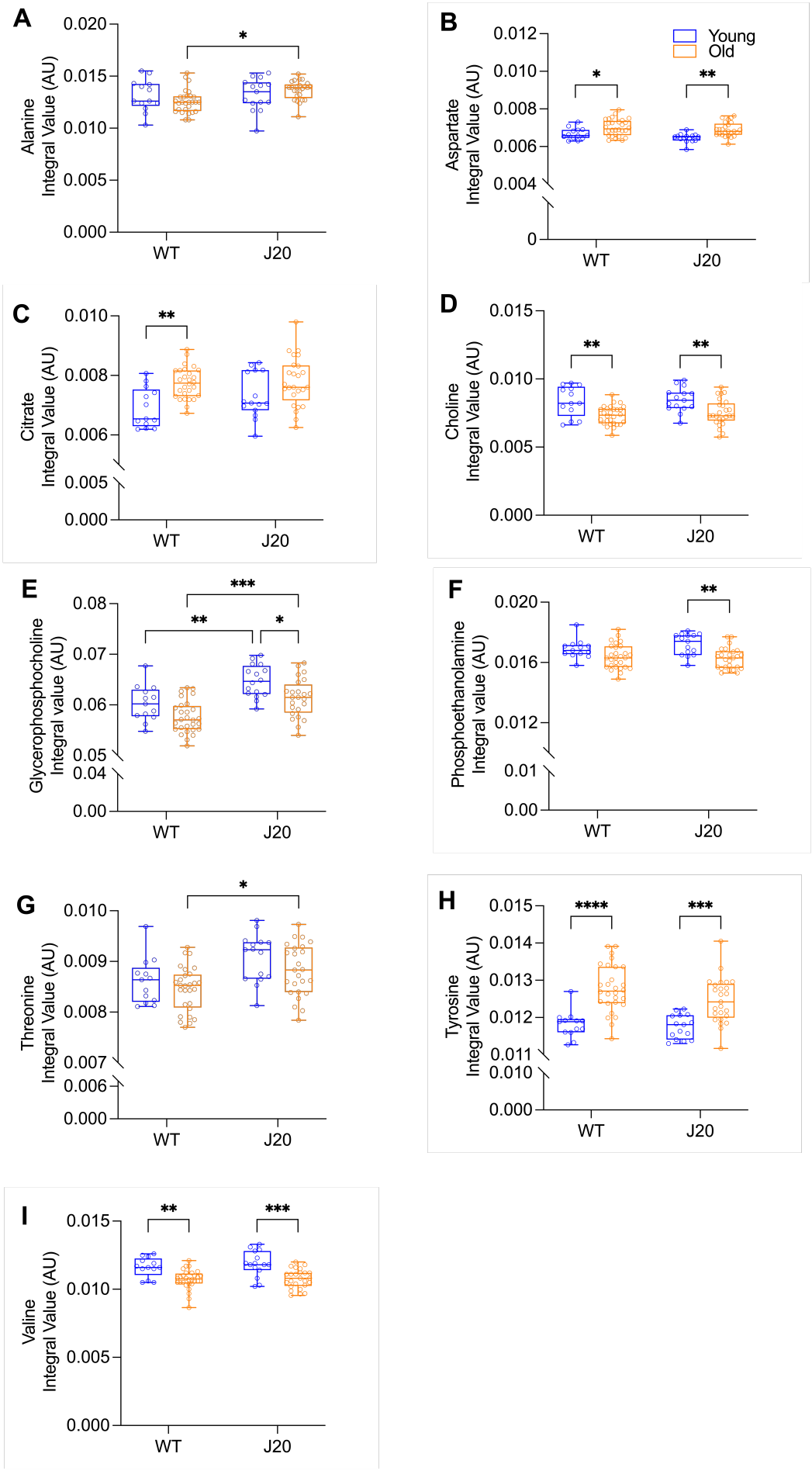

Figure 6.6: Spleen metabolites altered by genotype and age. VIP analysis revealed discriminatory

metabolites, which are **a)** alanine, **b)** aspartate, **c)** citrate, **d)** choline, **e)** glycerophosphocholine, **f)** phosphoethanolamine, **g)** threonine, **h)** tyrosine, and **i)** valine. n = 15 mice (WT young), 16 mice (J20 young), 28 mice (WT old) and 25 mice (J20 old). Data expressed as integral values (AU) and presented as boxplots including the median, interquartile range, minimum and maximum data points. Two-way ANOVA with post-hoc Tukey's test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Serum

Acetoacetate (Figure 6.7A) had an effect of genotype ($p<0.05$) and interaction ($p<0.01$), and was decreased in old WT mice compared with young WT mice ($p<0.01$). Acetoacetate levels were elevated in old J20 mice compared with old WT mice ($p<0.001$). Fatty acid βCH2 (Figure 6.7B) had an effect of age ($p<0.05$), genotype ($p<0.05$), and interaction ($p<0.001$). Levels of fatty acid βCH2 were decreased in old WT mice compared with young WT mice ($p<0.001$), and increased in old J20 mice compared with old WT mice ($p<0.0001$). Glucose (Figure 6.7C) had an effect of age ($p<0.05$) and interaction ($p<0.05$). Glucose levels were increased in old WT mice compared with young WT mice ($p<0.01$), and decreased in old J20 mice compared with old WT mice ($p<0.01$). Lipoprotein HDL (Figure 6.7D), which had an effect of interaction ($p<0.05$), was decreased in old WT mice compared with young WT mice ($p<0.05$), and increased in old J20 mice compared with old WT mice ($p<0.05$). Lipoprotein VLDL levels (Figure 6.7E) had an effect of interaction ($p<0.001$) and demonstrated the same differences between groups as lipoprotein HDL, which is a decrease in old WT mice compared with young WT mice ($p<0.01$), and an increase in old J20 mice compared with old WT mice ($p<0.001$). Lysine (Figure 6.7F) had an effect of interaction ($p<0.01$) and was elevated in young J20 mice compared with young WT mice ($p<0.05$). Within the J20 mice, lysine levels were decreased in old J20 mice compared with young J20 mice ($p<0.01$). Myo-inositol (Figure 6.7G) had an effect of interaction ($p<0.01$) and was decreased in old J20 mice compared with old WT mice ($p<0.001$). Polyunsaturated fatty acid (PUFA) (Figure 6.7H) showed an effect of interaction ($p<0.001$) and was decreased in old WT mice compared

with young WT mice ($p<0.01$). PUFA levels were also increased in old J20 mice compared with old WT mice ($p<0.01$). Proline (Figure 6.7I) showed an effect of interaction ($p<0.01$) and levels decreased in old WT mice compared with young WT mice ($p<0.01$), and increased in old J20 mice compared with old WT mice ($p<0.001$). Threonine (Figure 6.7J) had an effect of interaction ($p<0.05$) and was decreased in old J20 mice compared with young J20 mice ($p<0.05$).

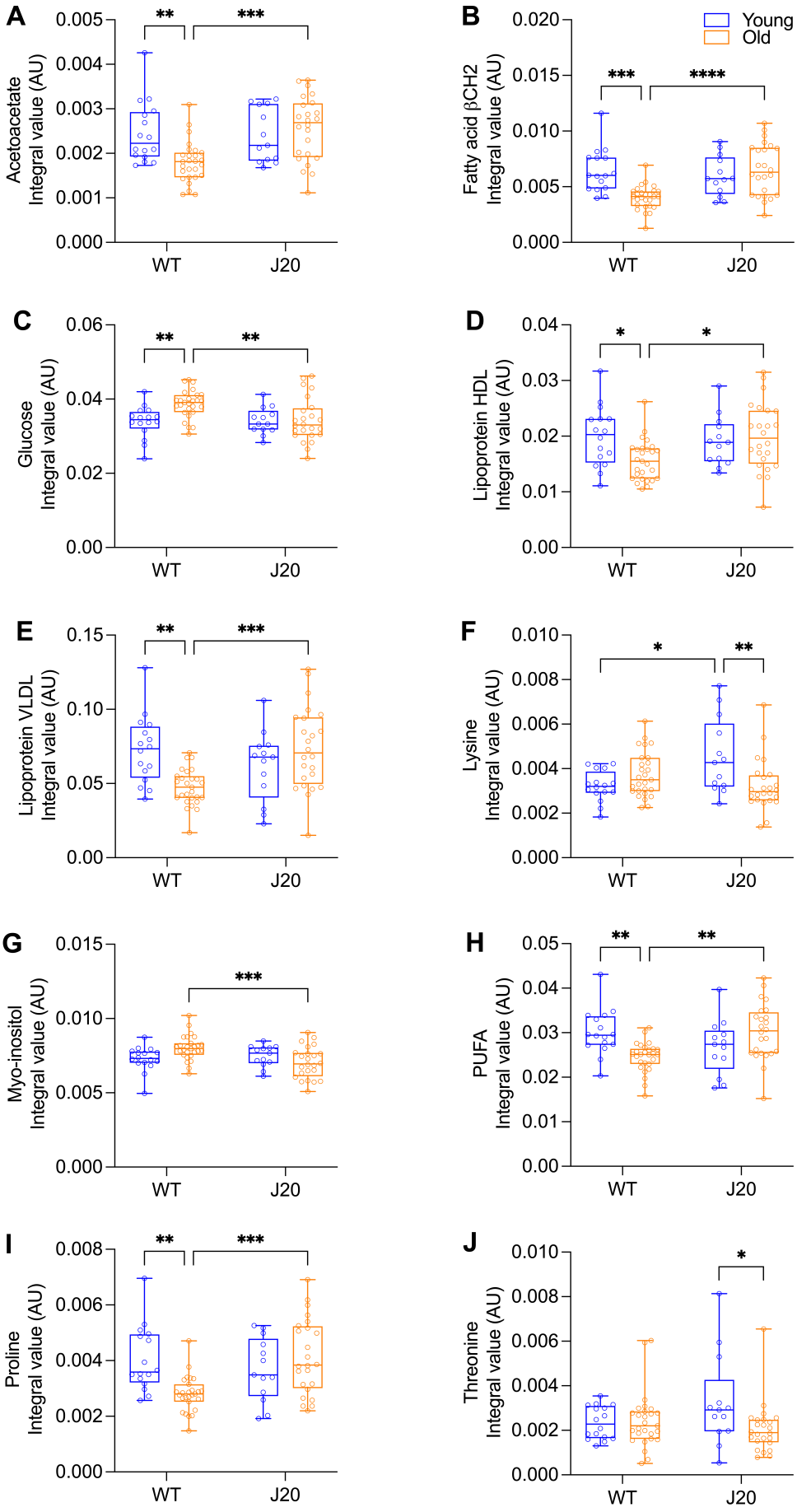

Figure 6.7: Serum metabolites altered by genotype and age. VIP analysis revealed discriminatory

metabolites, which are **a)** acetoacetate, **b)** fatty acid βCH2, **c)** glucose, **d)** lipoprotein HDL, **e)** lipoprotein VLDL, **f)** lysine, **g)** myo-inositol, **h)** PUFA, **i)** proline, and **j)** threonine. n = 16 mice (WT young), 13 mice (J20 young), 27 mice (WT old) and 24 mice (J20 old). Data expressed as integral values (AU) and presented as boxplots including the median, interquartile range, minimum and maximum data points. Two-way ANOVA with post-hoc Tukey's test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Figure panels a, b, c, d, e, g, h, i adapted with permission from Jaezah Zainal.

6.2.3. Metabolite correlations in CNS and peripheral tissues

Metabolite correlation analysis, between the VIP metabolites discovered in all tissues, was performed to infer potential interactions between CNS and peripheral tissues, which share common metabolites altered in normal aging or AD pathogenesis. A Pearson correlation heatmap matrix (Figure 6.8) was produced to visualise correlations, and after false discovery rate (FDR) correction for multiple testing, the correlations that remained significant were displayed above the diagonal. Investigating the correlations between metabolites altered in CNS and peripheral tissues, several metabolites were shown to have highly significant correlations across tissues. Most notably, a negative correlation between alanine in the hippocampus and citrate in the spleen ($r = -0.82$, $p<0.001$), as well as lactate in the hippocampus and histidine in the PFC ($r = -0.79$, $p<0.001$). Significant positive correlations were also observed between lactate in the hippocampus and lactate in the PFC ($r = 0.84$, $p<0.001$), glycerophosphocholine in the hippocampus and sphingomyelin in the PFC ($r = 0.88$, $p<0.001$), and serine-PETA in the hippocampus and histidine in the PFC ($r = 0.78$, $p<0.001$).

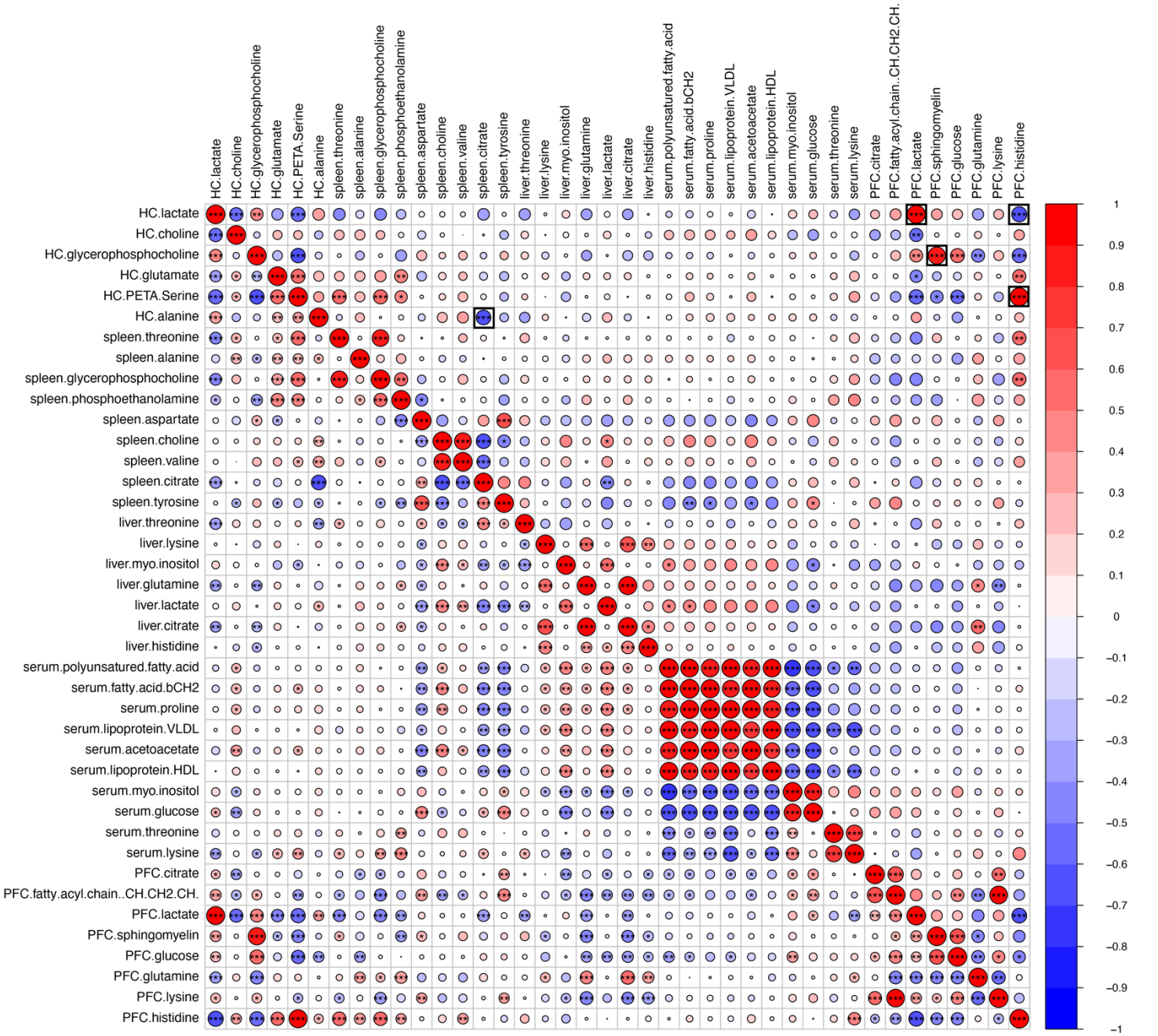

Figure 6.8: Pearson correlation matrix of VIP metabolites in all tissues. A heatmap was used to visualise significant correlations after FDR correction above the diagonal. Highly correlated metabolites between tissues are highlighted with a black square. *p<0.05, **p<0.01, ***p<0.001.

6.3. Discussion

In this chapter, we sought to identify changes in metabolites associated with the normal aging process as well as AD pathogenesis, and to discover potential biomarkers for early disease diagnosis. Metabolomics was used to examine metabolite alterations in the CNS and peripheral tissues of WT and J20 mice, separated into 'young' and 'old' age groups. Our results indicated that both regular aging and AD induce a metabolic signature across tissues, as evidenced by alteration in the levels of a range of metabolites. The significant changes in VIP metabolites may provide insight into the mechanisms underlying aging and AD pathogenesis.

Several key metabolites, identified in this chapter, correlated with previous studies on AD pathogenesis and dementia symptoms. This suggests that the J20 mouse model is particularly useful for metabolomic screening, and the characterisation of tissue-specific metabolic perturbations in this model might assist the examination of pathological mechanisms of AD. Since numerous metabolic pathways are conserved between mice and humans, these findings might contribute to a more accurate understanding of the AD metabolome, and narrow the gap between preclinical studies and future patient clinical trials.

Citrate, an intermediate of the tricarboxylic acid (TCA) cycle, is produced in the mitochondria by the enzyme citrate synthase (Iacobazzi and Infantino 2014). Citrate is involved in fatty acid synthesis, and many studies have reported a decrease in citrate levels in elderly individuals and AD patients (Pan et al. 2022; Paglia et al. 2016). Additionally, low citrate synthase activity has been observed in AD, which is related to a reduction in citrate production and low cellular energy (Wilkins et al. 2017). Since the TCA cycle operates primarily in mitochondria, and AD is associated with the downregulation of TCA cycle enzymes, this may further exacerbate mitochondrial dysfunction in the disease, leading to a reduction in ATP production and an increase in the levels

of ROS (Yan et al. 2020). Our results show a reduction of citrate levels in the PFC of young and old J20 mice compared with WT mice. Citrate was also decreased in the liver of old WT mice compared with young WT mice, conversely, citrate levels were elevated in the spleen of old WT mice compared with young WT mice, and in the liver of old J20 mice compared with old WT mice. The raised citrate levels in the spleen and liver of these mice might be reflective of the progressive failure of these tissues during aging and disease processes.

Lactate, a key metabolite, which showed altered levels across several tissues, is commonly regarded as a metabolic waste product, however, it is crucial for energy production and works as a major energy source (Li et al. 2022). Lactate is also important for molecule signalling and plays a role in gluconeogenesis (Li et al. 2022). Lactate present in the hippocampus is released from astrocytes, and mediates the molecular changes accompanying synaptic plasticity (Margineanu et al. 2018). Studies using the APP/PS1 mouse model describe reduced lactate levels (Zhang et al. 2018), which compare to our results that show a decrease in lactate content in the PFC, hippocampus and liver, associated with aging and AD progression. Conversely, other studies have shown increased lactate levels in APP/PS1 mice above 12 months of age (Harris et al. 2016), and furthermore, elevated lactate has been observed within the brain and CSF of AD patients, a finding correlated with memory decline (Liguori et al. 2016). Lactate may be beneficial for memory function, although, the excessive production of lactate in aging and disease is harmful. It is possible that the inconsistencies in previous studies may be explained by the use of various AD mouse models at different time points.

Histidine levels were decreased with age in the liver of WT mice and in the PFC of J20 mice. Histidine levels were shown to be reduced in the CSF of AD patients, and the metabolite has since been identified as a potential biomarker of AD (Ibáñez et al. 2012). Histidine has a variety of functions, including in the synthesis of enzymes and inflammatory responses (Brosnan and

Brosnan 2020). Histidine is known to exhibit neuroprotective effects, and AD mouse studies have shown changes in the histidine pathway, which correlated positively with memory retrieval impairment (Hunsberger et al. 2020).

The dysfunction of the glutamatergic system is also related to aging and neurodegeneration linked to AD (Cox et al. 2022). Glutamine levels were increased in J20 mice in the liver and PFC, while glutamate levels were reduced in the hippocampus with AD progression. Astrocytes convert glutamate to glutamine via the enzyme glutamine synthetase, additionally, glutamate can be oxidised via glutamate dehydrogenase (Mahmoud et al. 2019). Glutamine is released extracellularly and transported to neurons, where it acts as a precursor for the synthesis of glutamate and GABA, as well as an energy substrate (Waagepetersen, Sonnewald, and Schousboe 2003). In agreement with our study, glutamate levels decrease with AD progression (Wang and Reddy 2017).

Our results also revealed alterations in lysine levels, with the general trend being a decrease in levels with age and disease progression. Studies have shown that lysine metabolism is compromised in the early stages of AD in plasma and CSF of patients (Trushina et al. 2013). Furthermore, lysine supplementation may have beneficial effects to reduce the incidence of AD (Suzuki et al. 2020), although there are currently no prospective studies of the benefits of lysine supplementation. Lysine plays a key role in fatty acid metabolism, as it serves as the precursor for carnitine, which facilitates the transport of fatty acids to the mitochondria, where they undergo oxidation to release energy (Longo, Frigeni, and Pasquali 2016).

Changes in glucose levels observed in our results also corresponded to previous studies, which describe hyperglycaemia in older individuals that is associated with the development of dementia (Grillo and Colombatto 2008). However, during the late stages of AD, patients become hypoglycaemic (Balendra, Esposto, and Reich 2021). Glucose levels were increased in WT mice in

regular aging, and old J20 mice had decreased levels of glucose in the PFC and serum compared with old WT mice. Recent studies have indicated that sphingolipids, including sphingomyelin, accumulate in the brains of elderly individuals (Couttas et al. 2018). Our results corroborate this observation, demonstrating elevated levels of sphingomyelin during the normal aging process in WT mice in the PFC. Additionally, aging and neurodegeneration in disease is associated with heightened levels of circulating free fatty acids (FFAs), which trigger inflammation and insulin resistance, eventually leading to increased glucose levels and bioenergetic dysfunction in the brain (Mandal et al. 2012; Marrano et al. 2023). Our results showed an increase in FFAs with fatty acyl chains in the PFC during normal aging and with AD progression.

Alanine, a non-essential amino acid, is involved in gluconeogenesis and has been observed to decline with age (Le Couteur et al. 2010; Canfield and Bradshaw 2019). Our findings in the hippocampus support this decline in alanine levels, although, in the spleen, there was a notable increase in alanine in old J20 mice compared with old WT mice. This difference might be attributed to reduced levels of alanine transaminase in AD, an enzyme responsible for converting alanine to pyruvate (Le Couteur et al. 2010).

Glycerophosphocholine, an important metabolite that was affected by aging and disease progression in several tissues in our study, serves as a source of choline, which is a precursor to acetylcholine and other phospholipids crucial for cell membrane structure (Walter et al. 2004). AD patients commonly exhibit choline deficiency (Judd et al. 2023), and studies have shown that choline supplementation may be beneficial for cognitive function in the elderly (Liu et al. 2021). Our findings indicate reduced choline levels in the spleen in normal aging and disease progression, yet an elevation in choline levels in old J20 mice compared with old WT mice in the hippocampus. Both aging and AD are linked to increased glycerophosphocholine levels, potentially indicating compromised brain membrane integrity and reduced acetylcholine synthesis, which is

characteristic of AD (Perry 1980; Walter et al. 2004). Glycerophosphocholine levels were increased during normal aging in WT mice in the hippocampus, and decreased in AD progression in the J20 mice in the spleen. Additionally, glycerophosphocholine was increased in young and old J20 mice compared with their WT counterparts in the spleen. Comparably, phosphoethanolamine, a byproduct of phospholipid metabolism, decreased with AD progression in the spleen. Serine-PETA, another metabolite involved in phospholipid metabolism, decreased during regular aging and AD progression in the hippocampus. These reductions in metabolites associated with phospholipid metabolism parallel observations in AD patients and are associated with neuronal death (Mutlu, Duffy, and Wang 2021; Pettegrew et al. 2001).

Myo-inositol, a metabolite altered in the peripheral tissues, plays a role in signal transduction and metabolic regulation (Holub 2003). We observed a reduction in myo-inositol levels in the liver during typical aging, and a decrease in the serum of old J20 mice compared with old WT mice. Reduced levels of myo-inositol have been reported in patients with mild cognitive impairment (Tumati et al. 2018). Low levels of myo-inositol may contribute to age-related tissue pathology (Stokes, Gillon, and Hawthorne 1983). Furthermore, supplementation of myo-inositol has been shown to enhance liver-related markers (Arefhosseini et al. 2023).

Threonine, an amino acid implicated in lipid metabolism and protein synthesis, has shown a positive correlation with AD traits (Figueira et al. 2019). Disturbed threonine metabolism at the pre-diagnostic stage of AD makes it an attractive biomarker candidate, and recent studies have focused on developing the clinical validity of blood-based biomarkers, such as threonine, in AD patients (Figueira et al. 2019). However, contradictory findings in animal models of AD have proposed threonine supplements as a potentially beneficial therapeutic approach (Tournissac et al. 2018). Our results showed no clear trend in fluctuating threonine levels across tissues as a result

of aging and AD progression. Once again, these discrepancies may stem from variations in AD mouse models used and the time points measured in studies.

Additional metabolites that were perturbed in peripheral tissues in our study included valine, a metabolite associated with energy metabolism (Xiong et al. 2022). Valine levels were decreased during both aging and the progression of AD in the spleen. Reduced valine concentrations have been linked to aging and cognitive decline (Le Couteur et al. 2020; Xiong et al. 2022). Conversely, levels of aspartate and tyrosine increased during both normal aging and AD progression in the spleen. Several studies have highlighted mitochondrial aspartate metabolism dysregulation in AD, characterised by reduced aspartate levels in the brain of autopsied AD patients (Paglia et al. 2016; Sasaki et al. 1986). The relationship between aspartate and the aging process remains uncertain, and the role of aspartate in the spleen requires further investigation. Tyrosine, a precursor of dopamine, displays increased levels with age and AD progression, while dopamine itself is reduced (Fonteh et al. 2007; Hunsberger et al. 2020).

Lastly, key metabolites identified in the serum consistently exhibited a similar pattern – a decline during normal aging and an increase in old J20 mice compared with old WT mice. The VIP metabolites in the serum primarily play roles in energy production and lipid metabolism (Shepherd 1994). Aging is typically correlated with heightened levels of ROS and diminished ATP production (Seo et al. 2019). Older individuals are prone to disruptions in lipid metabolism, contributing to the progressive degeneration of various organs (Seo et al. 2019). In AD, these pathways may also undergo dysregulation over time. Imbalances in lipid composition and metabolism in AD can lead to disrupted energy metabolism, breakdown of the blood-brain barrier (BBB) and increased inflammation (Chew, Solomon, and Fonteh 2020).

A growing body of research has contributed to investigating the intricate relationship between the brain and peripheral organs, such as the spleen and liver. The function of these peripheral organs can significantly impact an individual's susceptibility to cognitive decline and contribute to the development of neurodegenerative diseases (Estrada et al. 2019; Wei et al. 2022). Inflammation appears to play a pivotal role in linking liver and spleen function to brain health (D'Mello and Swain 2011; Rasouli et al. 2011). The liver is involved in metabolising various substances, including inflammatory mediators, and has been implicated in AD-related pathology (Nho et al. 2019). Studies using AD mouse models have shown that liver dysfunction is coupled with elevated oxidative stress, inflammation and hepatomegaly (Lam et al. 2021). Inflammatory molecules have the ability to traverse the BBB and influence brain function, thereby contributing to cognitive impairment (Sartori et al. 2012). Moreover, conditions characterised by liver fibrosis have been linked to reduced brain volume in specific regions and diminished cognitive abilities in affected patients (Parikh et al. 2023). The spleen-brain axis may also play an important role in the progression of AD, as splenomegaly has been observed in previous AD studies using mouse models (Yang et al. 2015). Spleen dysfunction was also associated with altered cytokine levels in both the brain and plasma (Yang et al. 2015; Wei et al. 2022; Rasouli et al. 2011). Communication between the brain and spleen is thought to be facilitated by the vagus nerve, which transmits bidirectional signals to maintain physiological homeostasis (Rosas-Ballina et al. 2008). Furthermore, both the liver and spleen contribute to peripheral degradation of Aβ, however, in advanced AD, spleen and liver dysfunction results in decreased antioxidant defences and elevated circulating Aβ (Maarouf et al. 2018; Yu et al. 2022; Cheng, Tian, and Wang 2020).

To summarise, our study revealed numerous alterations in metabolites, predominantly associated with energy metabolism, in the brain and peripheral tissues of WT and J20 mice across two age groups. Many of the changes in metabolites we observed are supported by similar reports in AD patients or mouse models. Interestingly, some of these metabolite changes occurred during early

AD stages in young J20 mice when compared to young WT mice, suggesting their potential capability in predicting disease onset. These metabolites were: citrate in the PFC, lactate in the hippocampus, threonine in the liver, glycerophosphocholine in the spleen, and lysine in serum. We observed some similar alteration patterns in metabolites across the various tissues examined and identified significant correlations between metabolites from different tissues. Sphingomyelin in the PFC and glycerophosphocholine in the hippocampus were significantly positively correlated. Sphingomyelin production involves the transfer of phosphocholine from glycerophosphocholine to ceramide, facilitated by sphingomyelin synthase (Kerwin, Tuininga, and Ericsson 1994). Both metabolites are constituents of phospholipids in cell membranes, and are increased during aging and AD progression (Walter et al. 2004; Couttas et al. 2018). Conversely, citrate in the spleen and alanine in the hippocampus were significantly negatively correlated. Alanine can be converted to pyruvate, which after further changes becomes oxaloacetate and can enter the TCA cycle to produce citrate (Gray, Tompkins, and Taylor 2014). Imbalances in the TCA cycle have been associated with aging, accompanied by decreases in TCA enzyme activity and impaired cycle flux (Brière et al. 2006; Kang et al. 2021). Furthermore, histidine in the PFC and lactate in the hippocampus were also negatively correlated. Histidine follows several metabolic pathways and can undergo conversion through transaminase activity to produce imidazole-pyruvic acid, which can be further metabolised by reduction, leading to the formation of imidazole-lactate (Moro et al. 2020). Imbalances in lactate and histidine levels have also been observed in aging and AD (Ibáñez et al. 2012; Liguori et al. 2016), although it is still unclear how the pathway between histidine and lactate is affected by aging and disease. It's important to note that these correlations are merely observational and may not imply direct causation. Future research should focus on pharmacologically modifying specific metabolites to assess the impact on AD pathogenesis at different disease stages. Exploring how metabolites originating from various tissues interact with each other is also essential to further our understanding of metabolic mechanisms. Additionally, using a targeted metabolomic approach could be useful to validate our findings in other AD

models, such as those that incorporate both tau and APP mutations. Addressing sex differences in the AD metabolome is also critical, as it remains unclear whether metabolic changes occur equally in males and females. Understanding these differences will be vital for developing more tailored and effective treatments in the future.

Chapter acknowledgments

This study was conducted in collaboration with Jaezah Zainal in the Anthony lab, who collected the metabolomics data from liver, PFC and serum samples.

Metabolite	Organ	WT (young vs old)	J20 (young vs old)	WT vs J20 (young)	WT vs J20 (old)	Primary function(s)
Citrate	Prefrontal cortex, liver, spleen	↓ ↑		↓	↓ ↑	Intermediate in the TCA cycle, fatty acid synthesis
Lactate	Prefrontal cortex, hippocampus, liver	↓		↓	↓ ↓	Energy source, signalling molecule
Histidine	Prefrontal cortex, liver	↓	↓			Precursor to histamine (local immune responses)
Glutamine	Prefrontal cortex, liver				↑ ↑	Neurotransmitter related to glutamate and GABA metabolism, nitrogen metabolism in the liver, pH homeostasis
Lysine	Prefrontal cortex, liver, serum	↑ ↓	↓	↑	↓	Fatty acid metabolism, proteinogenesis
Glucose	Prefrontal cortex, serum	↑ ↑			↓ ↓	Energy source, precursor for the synthesis of several important substances (starch, cellulose, glycogen)
Sphingomyelin	Prefrontal cortex	↑				Sphingolipid found in cell membranes, signal transduction
Fatty acyl chain =CH-CH2-CH=	Prefrontal cortex	↑	↑		↓	Energy production (ATP)
Alanine	Hippocampus, spleen	↓			↑	Linked to metabolic pathways such as glycolysis, gluconeogenesis, and the TCA cycle
Glutamate	Hippocampus		↓			Excitatory neurotransmitter, precursor for the synthesis of GABA
Choline	Hippocampus, spleen	↓	↓		↑	Precursor of the neurotransmitter acetylcholine, transformed to different phospholipids and found in cell membranes, epigenetic regulation
Glycerophosphocholine	Hippocampus, spleen	↑	↓	↑	↑	Source of choline, precursor to acetylcholine
PETA-Serine	Hippocampus	↓	↓			Product of phospholipid metabolism, signal transduction
Myo-inositol	Liver, serum	↓			↓	Secondary messenger in several intracellular signal transduction pathways, participates in osmoregulation
Threonine	Liver, spleen, serum	↑	↓	↑	↑	Metabolized to a variety of products (glycine, acetyl CoA, pyruvate)
Valine	Spleen	↓	↓			Source of energy and precursors to replenish TCA cycle intermediates
Aspartate	Spleen	↑	↑			Metabolite in the urea cycle, participates in gluconeogenesis
Phosphoethanolamine	Spleen		↓			Precursor and byproduct of phospholipid biosynthesis, plays a role in myelination
Tyrosine	Spleen	↑	↑			Precursor to hormones and neurotransmitters, signal transduction
Acetoacetate	Serum	↓			↑	Energy source in TCA cycle
Lipoprotein VLDL	Serum	↓			↑	Lipid trafficking and metabolism
Proline	Serum	↓			↑	Signalling molecule, energy production, osmolyte
Fatty acid βCH2	Serum	↓			↑	Energy production (ATP)
Lipoprotein HDL	Serum	↓			↑	Lipid trafficking and metabolism
Polyunsaturated fatty acid	Serum	↓			↑	Component of cell membranes, provides substrates for synthesis of lipid signalling molecules, regulators of lipid metabolism

Table 6.3: Summary of metabolite changes in all tissues of WT and J20 mice and their functions.

7. General discussion

Despite a growth in the wealth of knowledge in AD research over the past few decades, numerous unanswered questions persist in the AD field, particularly in securing an understanding of the changes that occur in the early stages of AD. Addressing these questions will be imperative to identify novel therapeutic targets, which are urgently needed. Many studies have contributed to the establishment of the 'amyloid hypothesis', which states that extracellular deposits of $A\beta_{1-42}$ oligomers are both necessary and sufficient for the onset of disease. Aβ initiates a cascade of events, including the recruitment and hyperphosphorylation of tau, culminating in the loss of synapses and ultimately, neurodegeneration (Hardy and Higgins 1992). One of the central predictions of the amyloid hypothesis is that reducing Aβ levels will mitigate the symptoms of AD, and potentially, slow down or halt its progression (Hardy 2009). Consequently, there has been a significant investment of time and resources in the development of amyloid-depleting agents (Musiek, Gomez-Isla, and Holtzman 2021; van Dyck et al. 2022). These efforts have primarily focused on monoclonal antibodies targeting Aβ peptide itself, including antibodies specifically designed to target particular pathological forms of Aβ, such as small oligomers (Zhang et al. 2023). The majority of these antibodies have proven to be ineffective and this has sparked intense debate in the field about the validity of the hypothesis (Zhang et al. 2023). Advocates of the amyloid hypothesis believe that Aβ remains an important therapeutic target in AD, and they argue that the failures of clinical trials merely indicate that the antibodies used have not effectively engaged with the target or have not been administered at the optimal stage in AD progression (Selkoe 2021). Other researchers question the value of Aβ as a therapeutic target, given the multitude of clinical trial failures, and raise doubts about the central role of Aβ in AD pathogenesis (Morris, Clark, and Vissel 2014). Although, the most common stance acknowledges the critical role of Aβ in AD but suggests that its involvement lies further upstream in the pathogenic cascade, before clinical

symptoms manifest, and Aβ may not be the immediate cause of cognitive decline (Morris, Clark, and Vissel 2014).

Much of the AD research effort has been redirected to understanding the contribution of a variety of other factors implicated in AD pathogenesis. These factors include neuroinflammation and the role of microglia and astrocytes, the genetic risk factor APOE4, hyperactivity in neuronal networks, sleep disturbances, and hyperphosphorylated tau (Knopman et al. 2021). Due to their multifaceted functional roles, it remains unclear which functions are applicable to AD pathogenesis. Although, all factors have been shown to exert a significant impact on the level or activity of Aβ, providing further support that Aβ continues to play a central role in AD pathogenesis (Weglinski and Jeans 2023).

Although Aβ is recognised as a key protein involved in AD, physiological Aβ contributes to the normal functioning of synapses (Kent, Spires-Jones, and Durrant 2020). Neuronal activity positively regulates the concentration of Aβ (Cirrito et al. 2005), and picomolar concentrations of Aβ have been shown to modulate the probability of neurotransmitter release and LTP (Puzzo et al. 2008). Several studies have demonstrated that Aβ is critical for the expression of LTP (Puzzo et al. 2011; Abramov et al. 2009). In vivo experiments in mice have demonstrated that administration of an anti-Aβ antibody impairs LTP and inhibits short-term memory formation in contextual fear conditioning tests (Puzzo et al. 2011). The high concentrations of Aβ in the brains of AD patients and mouse models impairs the physiological function of Aβ and leads to the impairment of synaptic function (Hardy and Higgins 1992). Aβ oligomers are associated with network hyperactivity and the upregulation of the probability of release (Zott et al. 2019; Brito-Moreira et al. 2011). There is evidence suggesting that Aβ may directly induce toxic effects on synapses by binding to specific receptors on neurons, and several candidate receptors that mediate this function have been proposed (Viola and Klein 2015). Although, none have thus far

persuasively demonstrated to be sufficient to fully account for all features of Aβ synaptotoxicity (Wilcox et al. 2011). It is generally accepted that Aβ oligomers depress glutamatergic synapses, regardless of their initial binding target (Mucke and Selkoe 2012). This occurs through the pathological induction of LTD, and many studies have suggested mechanisms by which this occurs (Shankar et al. 2007; Hsieh et al. 2006). Some suggest that the synaptic weakening is due to a partial blockade of NMDARs by Aβ (Shankar et al. 2007), whereas others propose that elevated extracellular glutamate, due to neuronal hyperactivity and reuptake failure, can activate extra-synaptic NMDARs and/or mGluRs, which enlist LTD signalling pathways (Talantova et al. 2013; Li et al. 2009). Furthermore, the induction of LTD processes by Aβ can drive phosphorylation of tau (Taylor, Emptage, and Jeans 2021). It is apparent that Aβ-mediated synaptic weakening serves as the initial stage of a pathway that ultimately culminates on synapse loss and neurodegeneration (Shankar et al. 2007; Hsieh et al. 2006). Synaptic changes represent some of the earliest observed alterations in AD, and numerous studies have provided evidence that synaptic dysfunction emerges prior to the formation of amyloid plaques and evident neurodegeneration (Mucke and Selkoe 2012). Synapse loss precedes neuronal loss, and correlates with cognitive decline in AD (Coleman and Yao 2003).

In this Thesis, I have chosen to mainly focus on the effect of soluble Aβ oligomers on different aspects of synaptic function in hippocampal neurons to elucidate early pathological events in AD, in which therapeutic intervention might be achievable with the goal of preventing disease development. In many experiments, I have used both soluble Aβ oligomer treatment and slices from 6-9 month old J20 mice, an AD model, to determine if the application of Aβ is a valid in vitro model, and if there are any differences between the acute treatment of Aβ and the chronic accumulation of Aβ that eventually forms amyloid plaques in J20 mice. Transgenic mouse models have largely been based on the amyloid hypothesis, featuring FAD mutations in APP and presenilins, and provide valuable insights into the pathophysiology of Aβ toxicity (Yokoyama et

al. 2022). J20 mice, which overexpress human APP with two mutations associated with FAD, develop elevated levels of Aβ between 6 and 36 weeks of age, and amyloid plaque becomes widespread by 5-7 months (Mucke et al. 2000). Interestingly, impairment in basal neurotransmission, LTP deficits, and spatial memory perturbances in J20 mice occur before the appearance of plaques, an observation consistent with the notion of an early toxic role for soluble Aβ oligomers (Saganich et al. 2006; Wright et al. 2013).

The principal findings in this thesis include:

- The heterozygous knockout of the presynaptic Ca^{2+} channel, $Ca_v2.1$ (*Cacna1a*$^{+/-}$), which mediates Aβ-induced increased neurotransmitter release, is able to prevent the effects of Aβ and rescue the impairment in LTP, whilst preserving other aspects of physiological synaptic neurotransmission.
- The introduction of the *Cacna1a*$^{+/-}$ genotype in J20 mice was able to rescue several AD-related phenotypes despite the persisting neuroinflammation and elevated numbers of microglia and astrocytes.
- Aβ oligomers disrupt lysosomal membrane fusion and decrease MMP-9 activity levels in hippocampal neurons. Restoring MMP-9 levels did not rescue the Aβ-induced reduced growth of dendritic spines. In J20 mice, MMP-9 levels were increased, supporting the role of MMP-9 in inflammatory pathways linked to AD.
- Aβ recruits CICR from the ER in response to back-propagating action potentials in dendrites of CA1 hippocampal neurons.
- mGluR5 contributes to the Aβ-induced increased peak Ca^{2+} signals in organotypic slices. In J20 mice, inhibiting mGluR5 did not prevent the increased peak Ca^{2+} signal.
- Metabolomics was able to unveil a multitude of metabolite changes in CNS and peripheral tissues associated with normal aging and AD pathogenesis.

7.1. The effects of Aβ on presynaptic function

Studies exploring the impact of Aβ on synaptic transmission have primarily concentrated on the postsynaptic compartment, while overlooking the presynaptic compartment (Chen, Fu, and Ip 2019). The wider aim of the project, of which the results in Chapter 3 contribute to, was to examine the molecular mechanisms responsible for the dysregulation of presynaptic neurotransmission induced by Aβ, with the goal of detecting potential novel targets for therapeutic intervention (Jeans et al., Unpublished). Although several presynaptic mechanisms of Aβ toxicity have been uncovered, it is unclear how significant these synaptic changes are in the wider context of AD, which arises as a culmination of complex interactions among diverse cell types, such as neurons and glial cells (Busche and Konnerth 2016). Furthermore, there is uncertainty regarding which of the multitude of changes observed at early stages in AD contribute to downstream pathogenesis, such as synaptic loss and cognitive decline (Mucke and Selkoe 2012). In the study by Jeans et. al, Aβ oligomers produce enhancement of neurotransmitter release probability by the functional upregulation of $Ca_v2.1$ channels, and this leads to postsynaptic depression and eventually, deficits in learning and memory. In Chapter 3, I show that the heterozygous knockout of the gene encoding $Ca_v2.1$ (*Cacna1a*) can ameliorate the effects of Aβ on LTP impairment, while conserving other aspects of basal synaptic neurotransmission, providing further validation for the specific manipulation of $Ca_v2.1$ and its potential as a therapeutic target. Furthermore, *Cacna1a*$^{+/-}$ was able to rescue specific AD phenotypes, whilst neuroinflammation remained unchanged in *Cacna1a*$^{+/-}$/J20 mice, suggesting that restoring physiological presynaptic function in the early stages of AD may not mitigate certain downstream pathological phenotypes, although, the presynaptic manipulation can prevent the development of cognitive decline.

This research provides support for the crucial role that Aβ plays at the presynaptic compartment, and the significance of these changes in downstream alterations related to AD pathogenesis. While

this study comprehensively elucidates the mechanism related to upregulated presynaptic release mediated by Aβ, and demonstrates the resulting impact on the development of cognitive decline (Jeans et al., Unpublished), the pathways activated between the early and late stages of AD still need to be clarified. As previously mentioned, enhanced glutamate release following Aβ treatment can activate extra-synaptic NMDARs and induce the expression of chronic LTD, which firstly causes postsynaptic weakening and eventually, synapse loss in later stages of AD (Li et al. 2009). This may represent one mechanism through which Aβ-induced presynaptic facilitation leads to subsequent cognitive decline downstream.

The signalling pathway identified in this study highlights several important targets in AD pathogenesis, which may be amenable to therapeutic intervention. Other than playing a significant role in Aβ-induced toxicity, $Ca_v2.1$ has been associated with several conditions. Mutations in the *Cacna1a* gene result in modified Ca^{2+} influx, potentially triggering cortical spreading depression, which is believed to be the underlying mechanism of migraine aura (Van Den Maagdenberg et al. 2004). Conversely, reduced $Ca_v2.1$ channel activity may cause absence epilepsy and ataxia (Ophoff et al. 1998). These channels have also been considered as a drug target for pain (Yaksh 2006). Targeting a certain splice variant expressed in the region of interest could represent an advanced strategy for drug development aimed at circumventing off-target effects. For example, variant $\alpha_{1A\text{-}b}$, which exhibits specific expression in the hippocampus, holds promise as an intriguing target for the development of AD therapeutics (Bourinet et al. 1999). A number of compounds have been shown to modulate P/Q-type VGCCs, such as $Ca_v2.1$, although none are entirely selective for $Ca_v2.1$ (Nimmrich and Gross 2012). The only selective P/Q-type channel blocker, ω-agatoxin, isolated from the venom of various spiders (Adams 2004), may not be appropriate for use in patients. The pharmacokinetic properties of ω-agatoxin are incompatible with oral administration, and achieving sufficient brain availability remains challenging due to the blood-brain barrier

(Nimmrich and Gross 2012). Additionally, use of ω-agatoxin may lead to toxic effects, since it irreversibly blocks the channel (Adams et al. 1993), however, this toxin may serve as a foundation for developing selective and more suitable analogues. Another approach that is progressively integrated into drug discovery efforts involves the development of state-dependent compounds. The compounds are engineered to selectively bind to the inactivated state of the channel, thereby targeting channels that are overactive while preserving normal synaptic function (Mezler et al. 2012).

Another molecule involved in the novel mechanism is GSK-3β, which is central to AD pathogenesis, and its gain of function in AD stimulates the hyper-phosphorylation of tau, increased Aβ production, and activation of pro-inflammatory pathways (Hooper, Killick, and Lovestone 2008). However, Jeans et. al has shown that a loss of GSK-3β function mediates the presynaptic phenotype induced by Aβ during the early stages of disease. This finding is likely to be relevant for continuing efforts to modulate this enzyme for therapeutic purposes. Further upstream in the mechanism, ENaC also serves a function in the presynaptic domain in response to Aβ, and the use of amiloride to block ENaC was able to rescue the effects of Aβ on LTP inhibition (Jeans et al., Unpublished). The correlation between use of antihypertensive medications, such as amiloride, and a decreased risk of dementia has sparked interest in further investigations related to repurposing amiloride for the prevention of AD (Chuang et al. 2014). Studies have turned to investigate the function of ENaC in the brain (Giraldez, Dominguez, and de la Rosa 2013), and future studies could build upon the foundational principles observed in this study (Jeans et al., Unpublished) to design experiments utilising ENaC as a novel AD therapeutic target.

7.2. The effects of Aβ on postsynaptic intracellular Ca^{2+} signalling from internal stores

Recently, the calcium hypothesis of AD has begun to gain recognition, as it states that dysregulation of Ca^{2+} signalling in neurons during the early stages of AD is the pivotal event that elicits synaptic dysfunction and neurodegeneration (Berridge 2010; Khachaturian 1994). Furthermore, the advancement of Ca^{2+} imaging techniques has facilitated extensive research in this area, yielding new and captivating results (Grienberger et al. 2022). Since disruptions in neuronal Ca^{2+} signalling occur during the initial stages of AD, it has become an attractive area for developing therapeutics (Popugaeva, Pchitskaya, and Bezprozvanny 2018). In the postsynaptic compartment, AD is associated with elevated intracellular Ca^{2+} levels, which are detrimental to neurons and initiate subsequent pathological mechanisms, which contribute to the development of AD (Berridge 2010; Bezprozvanny 2009). Since Aβ oligomers are the initial toxic species of AD, numerous studies have focused on demonstrating that Aβ induces an elevation in neuronal Ca^{2+} concentration (Arbel-Ornath et al. 2017; Pellistri et al. 2008). However, the precise mechanism by which Aβ disrupts Ca^{2+} homeostasis is still unclear and being actively researched. Studies have described numerous receptors and signalling molecules that are affected by Aβ, and it has also been observed that Aβ can directly form Ca^{2+}-permeable channels in the plasma membrane, contributing to the elevated Ca^{2+} concentration in neurons (Arispe, Rojas, and Pollard 1993). NMDARs are probably among the most extensively studied targets of Aβ (Mota, Ferreira, and Rego 2014). In particular, research has demonstrated that Aβ can heighten the susceptibility of neurons to excitotoxicity, which arises from NMDAR overactivation and enhanced Ca^{2+} influx into neurons (Ferreira et al. 2012). By activating receptors and channels present on the surface of neurons, Aβ can alter intracellular signalling and impair the activity of internal Ca^{2+} stores (Briggs, Chakroborty, and Stutzmann 2017). The connection between extracellular Aβ and internal Ca^{2+} stores remains unclear, but several potential mechanisms have been discovered, and this Thesis

seeks to enhance our understanding of these mechanisms and their potential relevance to AD pathology.

I have shown that Aβ contributes to the rise in peak intracellular Ca^{2+} levels in response to back-propagating action potentials in hippocampal neuronal dendrites. Although the exact sites of Aβ action remain elusive, it has been shown that Aβ leads to mGluR5 activation and induces IP_3R-mediated ER Ca^{2+} release via IP_3 production (Renner et al. 2010; Demuro and Parker 2013). I have demonstrated that this interaction may also contribute to the elevated activity-dependent Ca^{2+} levels in neurons. Another potential mechanism of elevated Ca^{2+} levels in response to Aβ is that Ca^{2+} release by RyRs can be triggered by CICR, facilitated by the actions of Aβ on NMDARs or VGCCs (Goussakov, Miller, and Stutzmann 2010). Other studies conducted show that Aβ can enhance ER Ca^{2+} release by over-activating RyRs, which can lead to deleterious downstream changes such as ROS generation, disruption of the mitochondrial network, and apoptosis (Sanmartín et al. 2017). AD models have also demonstrated that Aβ can accumulate intracellularly, which may also contribute to destabilising ER Ca^{2+} signalling (Ji et al. 2016).

A significant portion of our understanding regarding impaired Ca^{2+} signalling in AD stems from research examining FAD mutations. These studies investigate how mutations in crucial FAD genes can influence Ca^{2+} homeostasis. For example, AD mouse models with PS1 mutations have demonstrated that presenilins can directly interact with RyRs and alter the receptor's gating properties, leading to enhanced RyR activity (Payne, Kaja, and Koulen 2015; Hayrapetyan et al. 2008). Mutations in PS1 disrupt the normal function of presenilins as ER Ca^{2+} leak channels (Tu et al. 2006), and it has been proposed that the increased Ca^{2+} release via RyRs may initially function to compensate for ER Ca^{2+} overload (Popugaeva, Pchitskaya, and Bezprozvanny 2018). In addition, dantrolene, a negative allosteric modulator of RyR, has demonstrated efficiency in reducing amyloid pathology, normalising ER Ca^{2+} signalling, restoring synaptic plasticity, and

improving performance in cognitive tests in mouse models of AD (Peng et al. 2012; Oulès et al. 2012). The sensitivity of IP_3R to its agonist, IP_3, was also significantly enhanced in cells expressing mutant presenilins (Cheung et al. 2008). Suppression of IP_3R expression was able to normalise the elevated Ca^{2+} levels observed in hippocampal neurons from PS1-M146V knock in and 3xTg mice, suggesting that this could represent another potential therapeutic approach (Shilling et al. 2014).

Store-operated Ca^{2+} entry is used to refill the ER store, however, it has also been shown to participate in important functions related to LTP and the stability of dendritic spines (Sun et al. 2014). Ca^{2+}-permeable channels that function in store-operated Ca^{2+} entry are downregulated in AD models (Leissring et al. 2000), perhaps due to the Ca^{2+}overfilled state of the ER. However, molecules that activate Ca^{2+}-permeable channels and participate in refilling ER Ca^{2+} have been shown to induce LTP recovery and improve memory functions (Zhang et al. 2015). It appears that there exists a complex interplay between Ca^{2+} levels and synaptic/structural plasticity, involving various sources of Ca^{2+} that exert significant influences on the process. Additionally, I've discovered that both insufficient and excessive Ca^{2+} levels can negatively impact the normal expression of structural plasticity, and Ca^{2+} signaling from the ER and lysosomes is crucial for this process.

Most of the research related to lysosomal biology in AD has focused on enhancing autophagy to degrade Aβ, however, lysosomal Ca^{2+} signalling also greatly contributes to AD pathogenesis (Zhang et al. 2022). Although lysosomes were shown not to play a role in the Aβ-induced significant rise in peak Ca^{2+} levels in response to back-propagating action potentials in Chapter 4, Aβ was able to disrupt lysosomal fusion with the plasma membrane and reduce the levels of MMP-9 expression. Since previous reports have stated that MMP-9 is essential for the maintenance of structural plasticity (Nagy et al. 2006), I hypothesised that the application of MMP-9 would be able to rescue the effects of Aβ on structural plasticity impairment. However, Aβ may act at various

sites in neurons to disrupt structural plasticity of spines, and the addition of MMP-9 may be deleterious in this context. MMP-9 levels are elevated in AD patients and mouse models (Lorenzl et al. 2003), comparable to the increased levels of expression and MMP-9 activity exhibited in J20 mice in this Thesis. It is possible that MMP-9 expression in AD may contribute to blood-brain barrier breakdown and inflammatory cell migration (Wang et al. 2014), therefore, the pathway involving lysosomal release of MMP-9 and the subsequent maintenance of dendritic spine growth may not be an appropriate target in the context of AD (Padamsey et al. 2017), where MMP-9 is already over-activated. The different results in MMP-9 activity in organotypic slices treated with Aβ and acute hippocampal slices from J20 mice reveal that some aspects of AD pathogenesis may be altered during disease progression, with the increased accumulation of Aβ into plaques. Although the J20 model is based on the over-expression of mutated APP, to increase production of Aβ, the model also includes a cascade of AD-related events, such as enhanced gliosis and amyloid plaque build-up (Wright et al. 2013).

Lysosomes in AD exhibit raised pH, linked to autophagy deficits, and lysosomal Ca^{2+} efflux (Lee et al. 2015). In vitro experiments have demonstrated that several lysosomal cathepsins function in the production of Aβ, while cathepsin B can degrade Aβ peptides (Simans et al. 1993). Given this information, various lysosomal mechanisms and their components emerge as potential targets for AD therapeutics. Several small molecules have already been approved for use in lysosomal storage disorders, mainly used for substrate reduction therapy and lysosomal enzyme inhibition (Bonam, Wang, and Muller 2019; Radin 1996). Preclinical investigations have demonstrated that modulating cathepsin activity can effectively ameliorate pathological features in certain diseases, such as AD (Hook, Kindy, and Hook 2008). Although, despite numerous attempts to develop specific cathepsin inhibitors, significant concerns persist regarding off-target effects, including activity against other cathepsins (Bonam, Wang, and Muller 2019). However, these inhibitors continue to be of great interest and could inform future therapeutic strategies.

When considering intracellular Ca^{2+} stores, such as the ER and lysosomes, as targets, it is crucial to emphasize the requirement for specificity. Drugs will have to selectively target those organelles that are dysfunctional in specific organs or cells (Bonam, Wang, and Muller 2019). It is also important to develop therapeutics that do not disturb basal organelle function and particular signalling pathways, which may be important for other neuronal mechanisms, such as structural plasticity. Another issue relates to the delivery method, specifically into neurons. Numerous nanomaterials have been engineered to act as vectors, however, safety remains a concern, as these vectors are often associated with nanotoxicity (Stern and Johnson 2008; Peynshaert et al. 2014). Further research is needed to characterise the role of these organelles in AD, in the hope that the development of a wide array of therapeutic applications could emerge from such research.

7.3. The application of metabolomics to investigate normal aging and AD

Although recent progress in the AD field has witnessed several drugs, which are capable of slowing disease progression (Selkoe 2021), progress through clinical trials, AD remains without a cure. These novel drugs may demonstrate their optimal effectiveness when administered before onset of symptoms, although, existing diagnostic techniques may not identify AD early enough for these drugs to significantly impact the course of the disease (Cummings, Morstorf, and Zhong 2014). The development of accurate and accessible biomarkers at the early stage of AD could profoundly impact treatment outcomes for affected individuals. Several studies have stated that a combination of AD biomarkers should be evaluated, due to the complex nature of the disease, to provide improved diagnostic accuracy (Bloudek et al. 2011; Craig-Schapiro et al. 2011). It is critical to investigate novel biomarkers beyond amyloid and tau, and study their longitudinal changes across AD pathogenesis (Wilkins and Trushina 2018).

Metabolomics represents a systems biology approach and involves measuring levels of small molecular metabolites in biological samples (Johnson, Ivanisevic, and Siuzdak 2016). Changes in metabolite levels can offer insights into disease processes (Johnson, Ivanisevic, and Siuzdak 2016). Furthermore, given that metabolic pathways are mostly conserved across species, metabolomics can facilitate the translation of preclinical findings in mouse models to humans (Peregrín-Alvarez, Sanford, and Parkinson 2009). Brain hypometabolism typically arises approximately 20 years before the onset of clinical AD symptoms, indicating that metabolic dysfunction plays an important role in the development of AD (Mosconi, Pupi, and De Leon 2008). Metabolic networks that are disrupted early in patients with AD include the TCA cycle, lipid metabolism, and metabolites associated with neurotransmission and inflammation, however, the most pronounced changes are related to energetic pathways (Trushina et al. 2013). Several metabolites that have been identified correlate with AD symptom progression, for example, N-acetylaspartate is reduced in AD patients and may be associated with neuronal and mitochondrial dysfunction, leading to memory impairment (Moffett et al. 2007). Fatty acid biosynthesis and lipid metabolism are also consistently altered in AD, as they contribute to neuronal structure and cell signalling (Liu and Zhang 2014). In addition, the identification of APOE4 as a major genetic risk factor for AD further reinforced the implication of lipid dyshomeostasis in the disease (Huang et al. 2017).

In this Thesis, I have investigated changes in Ca^{2+} homeostasis, which occur at the level of neurons, to changes in the metabolomics of several tissues from an AD mouse model. Bioenergetics are systemically disrupted in AD, and together with alterations occurring at the level of neurons, metabolic alterations were detected in multiple tissues in J20 mice. In Chapter 6, our study unveiled numerous alterations in metabolites, primarily linked to energy and phospholipid metabolism, throughout the brain and peripheral tissues, which correlated with regular aging and AD pathogenesis. Many of these observed changes in metabolites are consistent with similar findings reported in AD patients or other mouse models, specified in Chapter 6. Although the OPLS-DA

models that were generated in Chapter 6 were able to separate metabolites associated with normal aging in WT mice from AD progression in J20 mice, many metabolites showed similar trends in both groups. This is not surprising given that age is the greatest risk factor for AD (Riedel, Thompson, and Brinton 2016), however, regular aging processes also significantly differs from AD (Salthouse 2012). By detecting systemic changes in aging and AD, this study could offer insights into novel disease mechanisms and advance the discovery of biomarkers.

Currently, researchers have been striving to develop blood-based metabolomic biomarkers for the diagnosis and monitoring of several diseases (Angioni et al. 2022). These tests show potential for accuracy, cost-effectiveness and accessibility, enabling accurate detection for a wider population and facilitating faster diagnosis (Hampel et al. 2023). Although there have been many studies contributing to understanding the metabolome of AD, findings can vary due to multiple factors. In human studies, limited sample availability can hinder statistical power, and insufficient representation of males and females can introduce bias due to sex-specific alterations (Wilkins and Trushina 2018). Furthermore, confounding factors, such as medications or comorbidities, can further obscure data interpretation, and additional variables, such as environment and diet, can also impact an individual's metabolome (Bermingham et al. 2021). In animal studies, many variables can be controlled for, although, it is worth noting that the overexpression of human mutations in mice, compared to natural disease progression, may lead to the emergence of different metabolites (Wilkins and Trushina 2018).

The development of metabolomic profiling and related biomarkers is still in the early stages of development, but it holds promise for elucidating mechanisms of disease, and could pave the way for more efficacious biomarkers for diagnosis and monitoring therapeutics in various diseases.

7.4. Limitations and future studies

In the past few decades, extensive research using synthetic soluble Aβ oligomers in various cellular models has supported the early toxic role Aβ plays in AD (Hayden and Teplow 2013). Based on many in vitro studies, it is clear that the treatment of neuronal cultures with synthetic Aβ is able to accurately replicate the earliest changes observed in AD (Fontana et al. 2020). Since most of the experiments in this Thesis were first conducted with the use of Aβ oligomers, it is critical to discuss the assembly states of Aβ. It is still unclear what physiological and pathological functions are linked to the different assembly states of Aβ, such as oligomers and larger Aβ species, in AD pathogenesis (Chen et al. 2017). Studying the different states of Aβ peptide poses an experimental challenge as the conformation of Aβ is dependent on its environment, and various assembly states can coexist in equilibrium, making it difficult to isolate a pure Aβ preparation (Viola and Klein 2015). In the synthetic Aβ oligomers used in this Thesis, data has shown that most Aβ oligomers range from 50 to 100 kD in size, however, Aβ monomers, trimers and tetramers may also be present in trace amounts (Sakono and Zako 2010). Furthermore, although Aβ is studied as an extracellular peptide, it can also be found intracellularly and impacts synaptic function and plasticity (Ripoli et al. 2014). In this Thesis, I did not examine intracellular Aβ, although, it would be interesting to determine what role it might play in the intracellular Ca^{2+} signalling mechanisms I have investigated.

Despite the abundance of studies utilising synthetic Aβ oligomers as a primary model of AD, it is highly valuable to study a disease, such as AD, in transgenic mouse models, as it is a complex and systemic disease that involves many processes and various cell types and tissues (De Strooper and Karran 2016). Transgenic mouse models have significantly advanced our understanding of AD. The J20 mouse model used here is based on the overexpression of human APP, and although it has been used widely in research, it has been presumed that some deficits in these mice may arise from the non-physiological expression and processing of APP, rather than an increase in Aβ

production (Drummond and Wisniewski 2017). Currently, there has been a shift towards the use of 'knock-in' models, where the endogenous mouse APP gene is replaced by the human version carrying one or more FAD mutations (Saito et al. 2014). The mutations are expressed under the control of an endogenous APP promoter, and therefore, do not exhibit transgene overexpression (Saito et al. 2014). These mice represent a more accurate portrayal of AD, however, no mouse model has been able to completely recapitulate all AD characteristics (Drummond and Wisniewski 2017). In this Thesis, I have not researched the effects of tau, the other pathological species of AD, which is recruited by Aβ, and is essential for many AD-related phenotypes, such as synaptic and behavioural deficits (Medeiros, Baglietto-Vargas, and Laferla 2011). Although many mouse models have incorporated the gene MAPT, encoding tau mutations, in combination with mutated APP, there are no mutations in MAPT associated with AD (Elder, Gama Sosa, and De Gasperi 2010). MAPT mutations are more commonly associated with frontotemporal dementia, and produce hyper-phosphorylated tau that is biochemically distinct from tau deposits found in AD (Wang and Mandelkow 2016). Studies are currently attempting to directly inoculate mouse models with AD-associated tau deposits (Robert, Schöll, and Vogels 2021). Additionally, several mouse models have introduced additional mutations in certain genes, such as TREM2 and APOE4, which have both been linked to the development of sporadic AD (Song et al. 2018; Kotredes et al. 2021). In future studies, inducible mutations in genes would be useful to enable manipulation of the timing and level of gene expression, facilitating the exploration of important questions in AD progression (Jankowsky et al. 2005).

AD is almost twice as common in females compared to males, and additionally, studies have shown that female hormones affect synaptic plasticity (Li, Cui, and Shen 2014; Viña and Lloret 2010). This may have consequences for the translation of preclinical research in mouse models to patient trials. In this Thesis, I have used a mixed population of J20 mice, comprising approximately equal

numbers of males and females. In the future, it will be important to analyse male and female mice independently to contribute to our understanding of the sexually dimorphic characteristics of AD.

In most of the Chapters, I have only investigated changes in CA1 pyramidal neurons in the hippocampus, mainly at the level of single synapses in neuronal slices. Since AD affects multiple brain regions, it would be beneficial to our understanding of disease mechanisms to determine if some of the observations made in hippocampal neurons also apply to other brain regions, such as the prefrontal cortex. It is also vital to determine the precise relationship between synapse dynamics in AD and cognition. Although most of the images acquired during this Thesis were taken with a confocal microscope, the current advancement of in vivo imaging tools, including two-photon imaging, has notably enhanced our capacity to visualise synapse dynamics in vivo (Meng et al. 2019). To relate neuronal activity to higher-order brain functions, such as cognition, it is essential to measure neural activity across different brain regions (Roth and Ding 2020). Recently, a technique utilising large-scale cranial window in awake mice has been developed to expand two-photon imaging to all layers of the cerebral cortex and measure fluorometric changes in intracellular Ca^{2+} (Takahashi et al. 2024).

In conclusion, this Thesis has contributed to our understanding of the role of Aβ oligomers in early stages of AD, associated with synaptic dysfunction and dysregulated intracellular Ca^{2+} signalling in both the presynaptic and postsynaptic compartment. Additionally, bioenergetic impairments were considered at a systems-level to identify distinct metabolite changes associated with AD. Although several targets linked to Aβ effects were identified here, it currently remains a challenge to efficiently target molecules related to essential signalling pathways in neurons. However, this work may form the basis for future research to explore the potential of these pathways in the development of disease-modifying AD therapeutics.

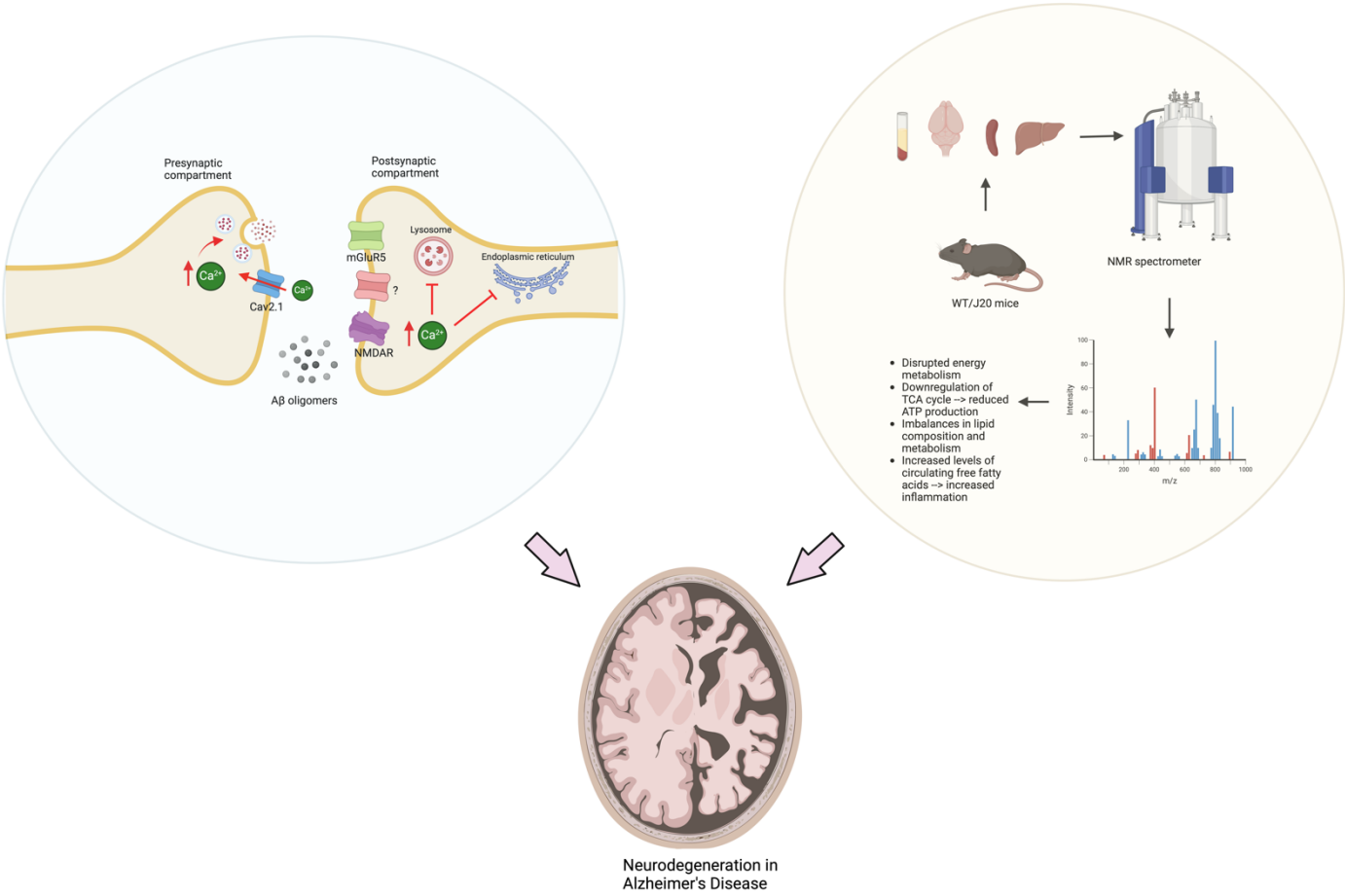

Figure 7.1: Summary of Thesis. Investigating changes in synaptic Ca^{2+} signalling in response to Aβ oligomers, and examining metabolite alterations in AD model mice to determine how these processes may culminate in neurodegeneration observed in AD.

8. References

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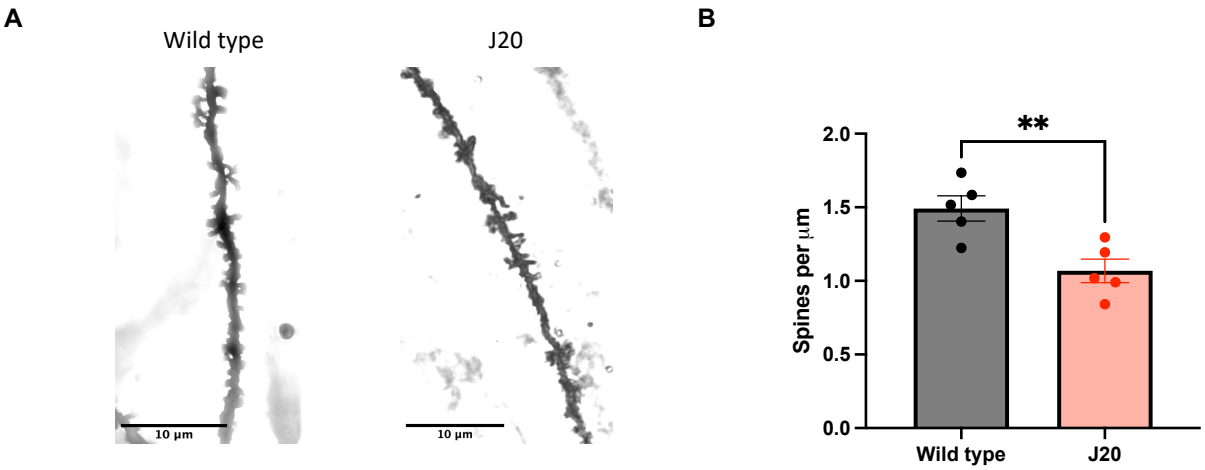
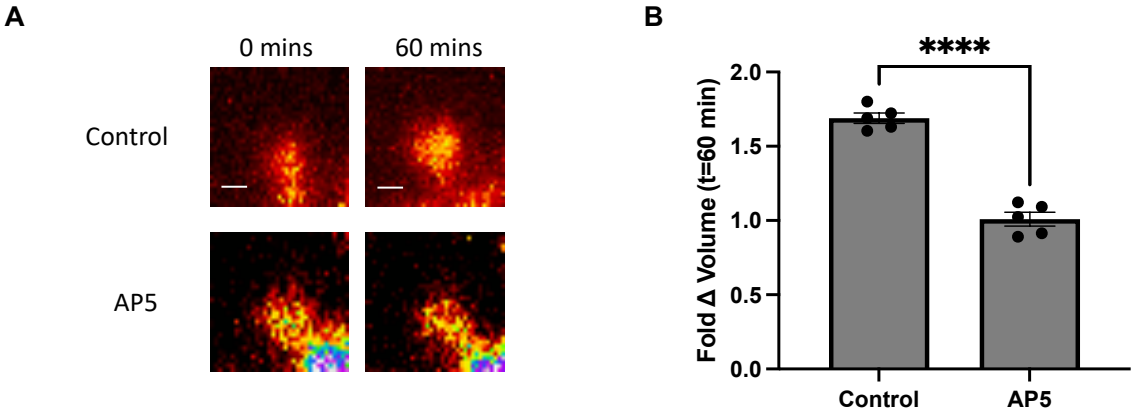
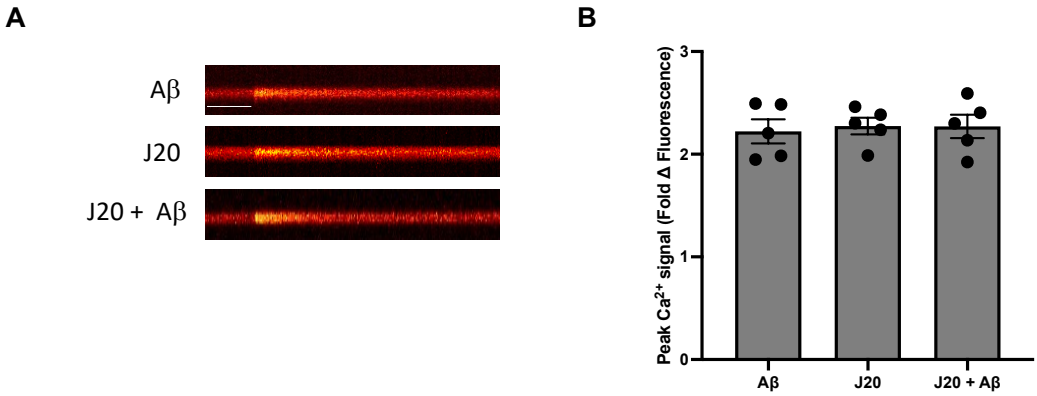
9. Appendix



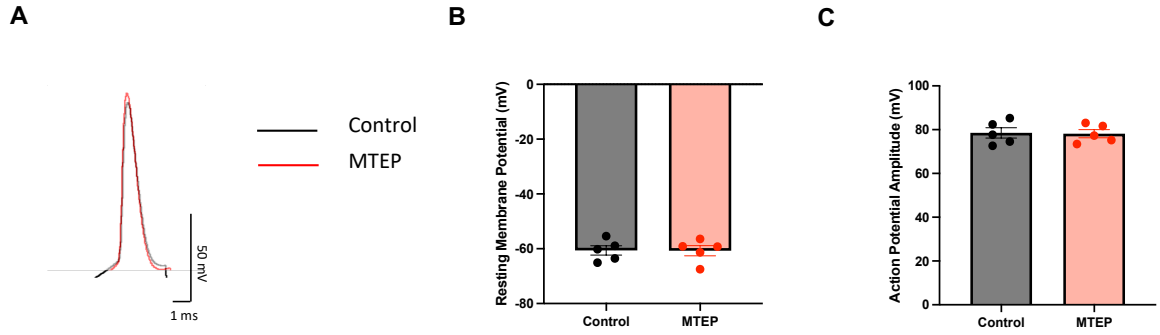
Supplementary Figure 1. Golgi-cox staining of CA1 hippocampal neurons. Reduced spine density observed in neurons from J20 mice. **a)** Representative images of Golgi-stained dendrites in hippocampus CA1 pyramidal neurons of 9 month old wild type and J20 mice; scale bar = 10 µm. **b)** Dendritic spine density (spines per µm) of CA1 hippocampal neurons (n = 5 dendrites, spines counted within 50 µm of dendrite, from 2 animals per genotype). Unpaired Student's t-test. **p<0.01.



Supplementary Figure 2. Dendritic spine growth following TBS to induce LTP in control conditions and AP5 treatment, an NMDAR antagonist, to prevent LTP induction and dendritic spine enlargement as a negative control. **a)** Representative images of dendritic spines of CA1 hippocampal neurons at 0 and 60 mins after TBS; scale bar = 1 µm. **b)** Average fold Δ volume of dendritic spines at 60 minutes after TBS (n = 5 neurons per condition; 4-5 spines analysed within 20 µm of dendrite per neuron). Unpaired Student's t-test. ****p<0.0001.



Supplementary Figure 3. Ca^{2+} line scans following back-propagating action potentials. All three conditions produce similar increases in peak Ca^{2+} signals. Importantly, Aβ oligomer treatment in J20 slices did not produce a difference in the peak Ca^{2+} signal. **a)** Representative images of Ca^{2+} line scans in different conditions; scale bar = 50 ms. **b)** Average peak Ca^{2+} signals (fold Δ fluorescence) measured across different conditions indicated (n = 5 neurons per condition, n = the average of 10 line scans per experiment). One-way ANOVA.



Supplementary Figure 4. Electrophysiological characterisation of CA1 hippocampal neurons following MTEP treatment. **a)** Representative action potential waveform. **b)** Average neuron resting membrane potential (mV). **c)** Average action potential amplitude (mV) (n = 5 neurons per condition). None of these parameters were altered following MTEP treatment. Unpaired Student's t-test.