Investigating Cell-type Specific Neuropathology in knock-in APP and tau Mouse Models of Alzheimer's Disease Correlated with Cognitive Deficits and Anxiety

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Declaration

I declare that the presented work here is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Content

Abstract

Alzheimer's disease (AD) is a debilitating chronic neurodegenerative condition characterised by progressive cognitive deficits, neuropsychiatric symptoms (NPS), and toxic amyloid-beta accumulation, shown to be correlated with selective dysfunction of cell types.

Currently, there are no disease modifying cures for cognition decline and NPS of AD, although there are drugs under clinical trials which slow down clinical decline, e.g. Donanemab (Eli Lilly); this could be related to the lack of mouse models that accurately recapitulate Aβ pathology and tauopathy as in the human condition. Therefore, the initial aim of this project was to generate a human tau and mice app gene knock-in mouse model of AD that harbours genes for microtubule-associated protein *tau* (*Mapt*^{hTau}) and β-amyloid precursor protein *App* (*App*$^{NL-F}$).

Using this model (*App* $^{NL-F}$ */MAPT* $^{htau/wt}$) in conjunction with *App*$^{NL-F}$ KI, an age-matched wild-type control, we performed behavioural studies combined with neurochemistry and confocal microscopy to investigate the impact of MAPT tau on anxiety level and AD progression in the CA1 region of brains, which are among the first to degenerate.

In conclusion, this project provides evidence that AD mouse models showed more severe cognition decline and anxiety levels compared to the age-matched wild-type mice. However, *MAPT* did not significantly affect the spread of the cellular hallmarks of AD: Aβ, neuroinflammation or the selective alteration of inhibitory interneurons (Calretinin, Parvalbumin, Cholecystokinin), which was previously shown to be correlated with synaptic dysfunction associated with the cognitive decline. Recent experimental data has suggested neuronal excitation-inhibition (E-I) imbalance as a critical regulator of AD pathology. GABA receptors play important roles in the balance. We found the downregulation of δ subunit of $GABA_A$ receptors(δ-$GABA_A$Rs) located in discreet neuronal circuitry of the hippocampus. Our data suggested a δ-subunit selective agonist increased levels of δ-$GABA_A$R, lowered inflammation and reduced anxiety in AD mice, which indicated a potential target to improve the quality of life of AD patients and caregivers.

Table 1. Table of Abbreviations

Aβ, amyloid-β

ACSF, artificial cerebrospinal fluid

AD, Alzheimer's disease

APP, β-amyloid precursor protein

CA1, Cornu Ammonis

CCK, cholecystokinin

DMSO, dimethyl sulfoxide

DG, dentate gyrus

ECM, extracellular matrix

EC, entorhinal cortex

GABA, gamma-aminobutyric acid

GAD67, glutamate decarboxylase

GC, Granule cells

GFAP, glial fibrillary acidic protein

MCI, mild cognitive impairment

NMDA, N-methyl-D-aspartate

PNN, perineuronal net

PAM, positive allosteric modulator

PC, principal cells

PV, parvalbumin

SEM, Standard Error of Mean

SP, stratum pyramidale

TBST, Tris-buffered saline with 0.1% Tween® 20 detergent

WFA, Wisteria floribunda agglutinin

Impact Statement

This study utilised a comprehensive approach to examine the physiological changes associated with cognitive impairment and anxiety indicators in preclinical mouse models of Alzheimer's disease (AD) called *App*$^{NL-F}$ KI, *App* $^{NL-F}$ */MAPT*$^{htau/wt}$ and *App*$^{NL-F}$*/MAPT* dKI. The study found that three specific types of inhibitory interneurons, which express calretinin, cholecystokinin, and parvalbumin, were affected differently in the disease. These interneurons also expressed δ-$GABA_A$ receptors, which play a crucial role in anxiety and memory. The observed pathological changes include the accumulation of amyloid beta plaques(Aβ) and a rise in neuroinflammatory markers, as evidenced by astrocytosis and microgliosis.

1. The behavioural trials conducted in this work served as the foundation for a continuing investigation within the research group. This investigation involves the use of a unique mouse model, the *App*$^{NL-F}$ KI crossbred with a tau model to produce *App* $^{NL-F}$ */MAPT*$^{htau/wt}$ and *App*$^{NL-F}$*/MAPT* dKI, with the objective of further replicating the pathology of Alzheimer's disease. This continuity demonstrates the significance of the work for the researchers within the same research group. The lack of evident pathological changes in *App*$^{NL-F}$ */MAPT* dKI mice makes it an ideal model for future investigations into tau protein dysfunctions and the progression of AD *in vivo*.

2. The results are valuable, as they contribute to the comprehension of the pathogenesis of Alzheimer's disease and enhance the understanding of potential treatment options in this field. The research conducted in this thesis contributed to a publication (Zhang et al., 2024) that suggested δ-subunit-containing $GABA_A$ receptors as a potential target for AD treatment.

3. Neuroinflammation, changes in neuronal density, anxiety, and cognitive impairment are not only specific to AD but also common in other neurodegenerative disorders. Therefore, the findings of this study have the potential to influence research in other disciplines outside AD.

1 Introduction

1.1 An overview of Alzheimer's disease (AD)

Currently, more than 55 million people have dementia worldwide, over 60% of whom live in low-and middle-income countries. Every year, there are nearly 10 million new cases of dementia, which results from a variety of diseases and injuries that affect the brain. AD is the most common form of dementia and may contribute to 60–70% of cases (World Health Organization, 2023). Around six in every 10 people with dementia are affected by AD in the UK (ARUK, 2023). AD is characterised by a slow onset, irreversible neurological damage and is a progressive neurodegenerative disease accounting for a significant health threat worldwide (Martínez-Nicolás et al., 2019).

Currently, the diagnosis of AD can be classified based on the age at which the ailment first manifests its initial symptoms. Early onset Alzheimer's disease (EOAD) refers to any type of AD that develops before the age of 65. It is characterised by a faster progression and a lower age of morbidity compared to late onset AD (LOAD) (Panegyres & Chen, 2013). Patients diagnosed with EOAD exhibit more pronounced metabolic abnormalities, along with heightened neuronal degeneration and synaptic dysfunction (Nochlin et al., 1993; Yasuno et al., 1998). This early onset AD has a vital familial component, indicating that individuals with EOAD are more likely to have a genetic predisposition, which is present in 15% of all cases (Awada, 2015).

LOAD is more prevalent than EOAD, accounting for 90-95% of all instances Researchers have specifically discovered that LOAD has a more significant influence on memory (Tellechea et al., 2018; Sá et al., 2012). LOAD has higher prevalence of psychiatric/behavioural symptoms (delirium, hallucinations, agitation, disinhibition, abnormal motor behaviour) compared to EOAD at same dementia severity (Snowden et al., 2007; Toyota et al., 2007).

The first AD symptoms include a gradual decline in short-term memory and the inability to remember new things. There are multiple types of cognitive impairment that occur during AD progression, including language, attention, and executive functions(Hampel et al., 2018; Nikolac Perkovic & Pivac, 2019).

AD pathogenesis has generally been attributed to extracellular aggregates of β-amyloid (Aβ) plaques and intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein. High levels of Aβ fragments in the central nervous system activate microglia infiltration, triggering an innate immune response to these Aβ aggregations (Tiwari et al., 2019). Glial cells, including astrocytes and microglia, are part of the critical support system of the brain because they function as neuronal protectors by releasing cytokines that

initiate immune responses. Glial cells play fundamental roles in AD progression because they are thought to fail to maintain the homeostatic immune function, consequently exposing neurons to excitotoxicity and oxidising agents (Al-Ghraiybah et al., 2022; Uddin & Lim, 2022).

Although the main mechanisms of AD remain unknown, the key risk factors include age, genetic predisposition, gender (female dominance) and mild cognitive impairment (Nikolac Perkovic & Pivac, 2019). There are no completely- curing treatments for AD, although there are a few FDA-approved drugs to manage AD, e.g. Cholinesterase inhibitors and NMDA antagonists(National Institute On Aging, 2023). Therefore, there is a pressing need to investigate the disease mechanisms to shed light on potential targeted treatment regimens.

1.2 Risk factors of AD

1.2.1 Genes associated with AD

AD can be divided into two main types, sporadic and familial cases. The familial form is due to mutations in three major genes (amyloid precursor protein (APP) gene, presenilin1 (PSEN1) gene and presenilin 2 (PSEN2) gene). In contrast, many genetic and environmental factors may contribute to determining the sporadic AD form(Piaceri et al., 2013). Most AD cases are sporadic or called LOAD (late-onset AD) in nature (Piaceri et al., 2013), suggesting other disease-contributing factors such as genes and environment. Although there is a reported solid genetic risk to the disease, a large percentage of genetic risks remain unidentified (Barber, 2012). The ApoE gene is found on chromosome 19, coding for proteins that support lipid transfer in the bloodstream and injury repair in the brain. The 3 most found alleles are ApoE 2, ApoE 3 and ApoE 4. Carriers of ApoE 4 alleles are more prone to develop AD than those carrying ApoE 3. Contrastingly, ApoE 2 alleles reduce the risk of AD. ApoE isoforms control β-amyloid aggregation and clearance in the brain (Liu et al., 2013).

Familial AD (fAD) or EOAD (Early Onset AD) is a rare form of the disease with an onset at <65 years of age and accounts for 5% of all AD cases (Mendez, 2019). EOAD patients have more significant parietal atrophy, higher white matter abnormalities and reduced hippocampal volume loss than those suffering from LOAD (Mendez, 2019). Early-onset fAD has been associated with mutations in three genes: the amyloid precursor protein (Rosi et al. 2024) gene and presenilin (PSEN)-1 and -2 genes and has an autosomal dominant inheritance pattern (dominant Mendelian transmission) (Barber, 2012; Bekris et al., 2010). Early onset of the disease can occur in people as young as 30 years old and is seen in one in 20 people with Fad (ARUK 2020). Chromosome 21 codes for the amyloid precursor protein gene (Rosi et al. 2024), chromosome 14 codes for presenilin 1 (PSEN1), while chromosome 1 codes for presenilin 2(PSEN2) (Barber, 2012; Saunders, 2001). Presenilin proteins are one of the four proteins in the gamma-secretase complex, affecting beta-amyloid production. With mutations on these, all offspring in the same generation have a 50% chance of inheriting the condition if one of the parents harbours the gene.

The genetic factors start from a dominant Mendelian transmission in fAD to risk factors for a complex multifactorial and etiologically heterogeneous disease in LOAD. While many causal alleles have been identified for FAD, only roughly half of the genetic variation for LOAD has been conclusively identified. There is a pressing urgency regarding the identification of other risky loci and leading to effective treatment regimens (Barber, 2012).

Under this circumstance, several generations of genetically modified mouse models have been developed and contributed to the accurate mimicking of human AD. The following describes different generations of mouse models and their replication of human AD conditions.

1.2.2 Other risk factors of AD

Research has demonstrated that the occurrence of the condition is more common in female patients across all age groups from 65 to 95. However, in the later age range of 80 to 95, this disparity may be attributed to the longer lifespan of female patients rather than the actual development of the disease. Between the ages of 65 and 80, the death rate among men and women is relatively low. However, there are other factors that contribute to the higher prevalence of certain conditions in women during this age range.

Some researchers suggest that increased obesity, diabetes, and blood pressure among women may be major contributors to the higher incidence of these conditions(Lloret et al., 2019). The relationship between obesity and the development of AD is currently unclear, as research has shown varied and inconsistent results. A meta-analysis found a substantial and independent association between obesity (Body Mass Index - BMI ≥30 kg / m2) and the chance of acquiring AD (Profenno et al., 2010). However, a meta-analysis carried out by Fitzpatrick et al. (2009) found that being obese in middle age increases the likelihood of developing dementia (hazard ratio - HR: 1.39; 95% CI: 1.03–1.87)(Fitzpatrick et al., 2009). Conversely, in later stages of life, obesity is associated with a lower risk of dementia (HR: 0.63; 95% CI: 0.44–0.91). The authors have also found that being underweight (BMI < 20 kg / m2) is linked to a higher incidence of dementia (HR: 1.62, 95% CI: 1.02–2.64). Weight loss in older individuals often coincides with the presence of other medical conditions and is frequently a sign of poor health. In certain cases, it may even start before the onset of dementia within a decade. An additional meta-analysis (Anstey et al., 2011) demonstrated that being underweight, overweight, or obese throughout middle age is linked to an increased likelihood of acquiring AD in later life.

A longitudinal study has shown that hypertension can significantly elevate the likelihood of getting AD(Skoog et al., 1996). Additional research has corroborated this correlation, demonstrating that high blood pressure, mainly when occurring during middle age, has a detrimental impact on cognitive function in later years. Furthermore, this relationship weakens as individuals become older (Staessen et al., 2007). Hypertension can induce alterations in the blood vessel walls, resulting in reduced blood flow, insufficient oxygen supply, and brain oxygen deprivation. These factors contribute to the initiation of AD. Research indicates that cerebral ischemia can cause the buildup of APP and Aβ and

trigger the activation of presenilin, which is implicated in the manufacture of Aβ. Hypertension can also cause impairment in the blood-brain barrier, which is linked to the development of AD through mechanisms that have been previously addressed (Silva et al., 2019; Skoog & Gustafson, 2006).

The diagnosis of this illness has been challenging due to two primary factors. The primary challenge is distinguishing between the various types of dementia found in humans, as they exhibit similar symptoms, often leading to misdiagnosis of patients (Bradford et al., 2009). Furthermore, the precise aetiology of AD remains poorly comprehended and is thought to differ among individuals, rendering it challenging to identify using biomarker or neuroimaging techniques such as CT and MRI scans (Frisoni et al., 2010). The most effective method for identifying this disorder continues to be obtaining a comprehensive patient history to evaluate cognitive function and familial risk factors.

1.3 Symptoms of AD

1.3.1 Cognitive deficits

Alzheimer's disease is presently classified as one of the leading causes of mortality and the predominant cause of dementia in elderly individuals(Jack Jr et al., 2018).

Dementia refers to the decline in cognitive functioning, encompassing thinking, remembering, and reasoning, along with a disruption in behavioural abilities, to the extent that it hampers an individual's everyday life and activities(Jenkins et al., 2021). Dementia varies in intensity, ranging from its initial impact on a person's functioning to the point where the individual becomes entirely reliant on others for assistance with fundamental everyday functions(Rosati et al., 2020).

Cognitive decline, often initially identified as memory impairment, is a characteristic manifestation of AD(Ávila-Villanueva et al., 2022). Neuropathological alterations within the cerebral cortex and limbic system result in impairments in various cognitive domains, including learning, memory, language, and visuospatial abilities(Davis et al., 2018; Jenkins et al., 2021). The specific characteristics of cognitive dysfunction in AD are determined by the pattern of pathological alterations in the brain. The factors mentioned above will exhibit variability over the continuum of disease severity and may also be influenced by the position of the disease along the spectrum of dementia(Soldan et al., 2017).

Based on the degree of cognitive impairment, AD is often divided into three stages: the preclinical stage, characterized by normal cognitive ability, the prodromal stage, characterized by mild cognitive impairment (Meshkat et al. 2023), and the dementia stage, with functional impairment (Albert et al., 2013; Burnham et al., 2019; Vermunt et al., 2019). The amnestic subtype of MCI is strongly associated with AD and some studies estimate that approximately 80% of people with amnestic MCI go on to develop Alzheimer's disease within 6 years (Figure 1) (Carmasin et al., 2021; Mohs et al., 2024). As a result, cognitive therapies have the potential to be advantageous during MCI in reducing the probability of AD progression(Verny et al., 2015). MCI is categorised into three subtypes: amnestic-MCI (a-MCI), multiple domain a-MCI (a-MCI+), and non-amnestic-MCI (na-MCI)(Albert et al., 2011; Saunders & Summers, 2011). Individuals with amnestic mild cognitive impairment (a-MCI) demonstrate measurable deficits in episodic memory. In addition to episodic memory problems, individuals with a-MCI+ also experience cognitive impairments in working memory, executive function, processing speed, and attentional processing. On the other hand, individuals with non-amnestic mild cognitive impairment (na-MCI) exhibit cognitive impairments that are unrelated to episodic memory. These

findings are supported by previous research(Saunders & Summers, 2011). Previously, it was thought that a-MCI had the highest likelihood of developing into AD due to the significant memory problems associated with AD. However, recent research indicates that early difficulties in visual memory, executive function, semantic language and memory, attention, and working memory are also reliable indicators of the progression from MCI to AD.

There is a general agreement that AD typically begins with patients experiencing memory difficulties. These difficulties can impact their ability to remember specific events (episodic memory), speak fluently, recall names, understand meanings (semantic issues), or have difficulty with visual perception. Memory is the cognitive process of encoding, retaining, and retrieving knowledge about external and internal events. It involves presenting information to the neurological system of an organism, which enables the organism to react and adapt to new stimuli. Various classifications of memory have been established, each with distinct neuroanatomical and neurophysiological associations: short-term memory versus long-term memory and implicit versus declarative memory. Short-term memory has a restricted capacity, confined to a small number of "chunks," and its duration is only a matter of seconds to minutes(Jahn, 2013). The localisation of this phenomenon is contingent upon the specific regions within the frontal and parietal lobes. Long-term memory, in contrast, exhibits nearly boundless storage capacity and the potential for an indefinite lifespan. The formation of different memory types relies on de novo protein synthesis and alterations in the molecular composition of the neural networks within certain cortical areas.

AD patients typically display a cognitive profile with impairments in multiple cognitive domains. This cognitive profile develops over time, and AD patients often start to show a progressive decay of working memory. The patients display increased sensitivity to distraction in memory tasks, the capacity of working memory measured, e.g., digit span is, however, at first still intact (Pepeu et al., 2013). The deficits in attention and working memory associated with damage to frontal subcortical circuits also influence executive functions in AD, impairing planning, problem-solving, and goal-directed behaviour, such as the ability to deploy response alternatives or modify behaviour. AD patients show impaired results in tests that require planning, problem-solving, or cognitive flexibility, e.g., the Wisconsin Card Sorting Test, the Stroop Test, or the Tower of London Test(Jahn, 2013).

Cognitive decline will be focused on results 3.1 of this thesis, where a more detailed introduction of how cognitive deficits are measured in rodent AD models.

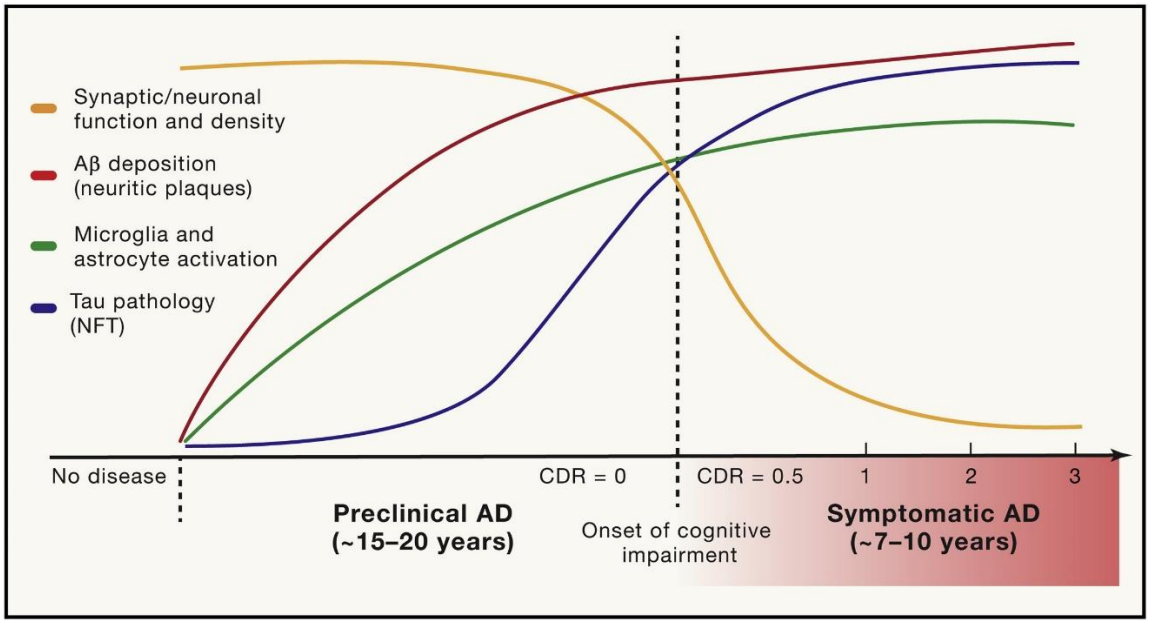


Figure 1 Major AD Pathophysiological Events' Timing in Connection with the Clinical Course. Early beginning of amyloid deposition is indicative of a long preclinical phase of the disease. A decrease in CSF and plasma levels of Aβ42 or an increase in the global signal on amyloid PET imaging are indicators of this. Early neuroinflammatory alterations (such microglial activation) occur concurrently. PK11195 PET imaging can be used to identify microgliosis longitudinally, however more effective drugs are required. The neurofibrillary tangle (NFT) tau disease then spreads into the neocortex from the medial temporal lobes. This shift in patients is shown by elevated CSF phospho-tau levels and an enhanced signal on tau PET imaging. When tau aggregates spread pathologically, it can lead to neurodegeneration, synapse loss, and synaptic dysfunction. Tracking neurodegenerative changes over time is made possible by imaging studies of cortical and hippocampus volumes. While amyloid deposition is not correlated with the onset and progression of cognitive impairment, tau accumulation and hippocampus volume reduction are. The Clinical Dementia Rating (CDR) scale can be used to assess the onset and severity of clinical symptoms in AD. A score of 0 denotes normal cognition, while scores of 0.5, 1, 2, and 3 denote doubtful, mild, moderate, and severe dementia, respectively.(Long & Holtzman, 2019).

1.3.2 Neuropsychiatric symptoms

While memory impairment and abnormalities in other cognitive domains are the primary symptoms of AD (Dubois et al., 2014), neuropsychiatric symptoms such as agitation, anxiety and depression to be present throughout the progress, thef the illness (Connors et al., 2018; Suárez-González et al., 2016). The prevalence of anxiety in AD ranges from 9.4% during the preclinical period to 39% in individuals with mild to severe deterioration (Becker et al., 2018; Zhao et al., 2016). Similarly, the incidence of depression in individuals with mild-to-moderate AD varies from 14.8% (Asmer et al., 2018) to 40% (Chen et al., 2018).

NPS in AD are associated with the underlying neuropathological processes of the disease (Ehrenberg et al., 2018). These symptoms manifest in several phases, starting from the preclinical stage with symptoms like anxiety and sadness (Masters et al., 2015) and progressing to symptomatic AD with symptoms such as agitation, delusions, and hallucinations. There is a hypothesis suggesting that agitation, which is a behaviour

observed in individuals with AD, may be a manifestation of anxiety, a subjective feeling. This implies that as dementia progresses, agitation may replace anxiety. Additionally, it is believed that experiencing anxiety early ib dementia could raise the likelihood of developing agitation later. Supporting this notion, anxiety commonly arises during the early stages of AD (Ehrenberg et al., 2018; Masters et al., 2015) and is less common in individuals with advanced AD (Seignourel et al., 2008; Breitve et al., 2016). On the other hand, agitation becomes more prevalent as the illness progresses and cognitive impairment worsens (Lyketsos et al., 2000; Steinberg et al., 2006; Sennik et al., 2017). Nevertheless, anxiety does not comprehensively include all the behavioural features of agitation, and the extent to which they overlap remains uncertain (Seignourel et al., 2008). Gaining insight into whether individuals with AD who exhibit early NPS, such as anxiety, are more likely to develop agitation later, would be beneficial for clinical decision-making and would encourage further investigation. This would include exploring the potential effects of early anxiety treatment to prevent the onset of agitation in the later stages of the disease.

Agitation is a distressing and hard-to-treat neuropsychiatric condition that is frequently observed in individuals with dementia. A widely accepted definition of agitation describes it as a persistent display or indication of emotional anguish accompanied by excessive motor activity, verbal or physical aggressiveness (Cummings et al., 2015; Liu et al., 2020). Agitation is prevalent in approximately 30% of patients with Alzheimer's disease in the community and 80% of those residents in care homes (Lyketsos et al., 2002; Zuidema et al., 2007). Agitation has a substantial negative impact on the overall quality of life and leads to premature placement in institutions (Okura et al., 2011). However, when it comes to treatment, the most reliable evidence supports the short-term utilisation of antipsychotic medications, which have limited effectiveness and the possibility of adverse consequences. Given that agitation in dementia can arise from various causes (Howard et al., 2001), including brain abnormalities associated with Alzheimer's disease (Liu et al., 2018; Rosenberg et al., 2015), it is imperative to have a deeper understanding of the factors that may contribute to an individual's susceptibility to developing agitation. This knowledge will enable the development of more effective and tailored approaches for preventing and treating agitation.

Anxiety is typically defined by an excessive amount of concern, tension, impatience, restlessness, and reduced participation in activities that were once enjoyable (Rossana Botto et al., 2022). The presence of anxious symptoms is correlated with greater impairments in everyday activities and more severe behavioural issues (Breitve et al., 2016). Anxiety can be viewed as a psychological reaction to a diagnosis of AD (Mormont

et al., 2014). The characteristic symptoms of depression in AD include sleeplessness, isolation from social activities, decreased engagement in goal-oriented actions, loss of interest in formerly enjoyable activities and hobbies, feelings of guilt, hopelessness, and melancholy (Nobis & Husain, 2018)..

The presence of anxiety in AD can be attributed to the shrinkage of the right precuneus and inferior parietal lobule, as well as the increased blood flow in the contralateral anterior cingulate cortex (Tagai et al., 2014). A negative correlation was observed between increased anxiety levels and decreased resting metabolism in several brain regions, including the bilateral entorhinal cortex, anterior parahippocampal gyrus, left anterior superior temporal gyrus, and insula (Hashimoto et al., 2006). Elevated levels of anxiety were found to be correlated with increased activity of the glycine receptor (GlyRS) that is sensitive to strychnine, as well as a specific decrease in the density of the NR2A subunit of the N-methyl-D-aspartate (NMDA) receptor (Tsang et al., 2008). Anxiety is manifested through the synchronised functioning of several brain pathways that involve various neurotransmitters. These neurotransmitters interact and are regulated by both nearby and distant synaptic connections. The inhibitory neurotransmitter GABA plays a crucial role in regulating anxiety. Benzodiazepines and similar medications target this neurotransmitter system to treat anxiety disorders. More details of GABA and GABA receptors will be illustrated in chapters 1.8&1.9.

Anxiety and depression frequently coincide, particularly in those with mild AD (Hynninen et al., 2012; Rossana Botto et al., 2022). Depression was linked to the presence of AD pathology, specifically lower levels of CSF Aβ42 and higher levels of t-tau and p-tau (Lebedeva et al., 2014; Wu et al., 2020). Depression can be identified through reduced cortical metabolism, the presence of neuritic plaques, and damage to neurons in the temporal cortex, which results in the disinhibition of the HPA-axis. Conversely, the correlation between depression, excessive activity of the hypothalamic-pituitary-adrenal (Olivares et al. 2012) axis, and cardiovascular illness can influence the evolution of AD. The onset of AD appears to disrupt the ongoing depressive state caused by memory and executive control impairments (Meynen, 2010). The manifestation of depressive symptoms and their emotional impact tend to diminish as AD advances (Milwain & Nagy, 2004; Wu et al., 2020). Dysthymia initially occurs in the early stages of AD as an emotional response to the gradual deterioration in cognitive function. On the other hand, significant depression may be influenced by biological causes and is a symptom of the neurodegenerative processes associated with AD (Heun et al., 2002).

Banning et al.(Banning et al., 2020) described anxiety in AD as an initial compensatory response, while sadness may be more associated with AD awareness or the

psychological response to AD, as well as relational and biological aspects (Banning et al., 2021).

Patients with early-onset AD experience higher levels of anxiety and depression compared to those with late-onset AD. This can be attributed to several factors specific to early-onset AD, including significant changes in lifestyle, roles, and responsibilities, difficulties in social adjustment, cognitive impairment, the severity of dementia, and a faster disease progression (Baillon et al., 2019; Kaiser et al., 2014; Panegyres et al., 2014; Tanaka et al., 2015).

In this thesis, anxiety examined in our rodent models will be the focus of chapter 3.2.

1.4 Alzheimer's disease pathology

1.4.1 Beta-amyloid plaque deposition

Amyloid fibrils are protein homopolymers with diverse cross β conformations (Knowles & Buehler, 2011). The normal physiological function of naturally occurring amyloid fibrils is to perform specialised functions, including pigment formation (Berson et al., 2003; Pavlopoulos et al., 2011). Moreover, peptides and protein hormones in secretory granules of the endocrine system are stored in the pituitary, secreting granules in amyloid-like β sheet conformation. Functional amyloids in the pituitary and other organs can contribute to normal cell and tissue physiology (Maji et al., 2009). However, numerous amyloid fibrils perturb cellular processes and induce systemic amyloidosis, leading to neurodegeneration (Edwards et al., 2019).

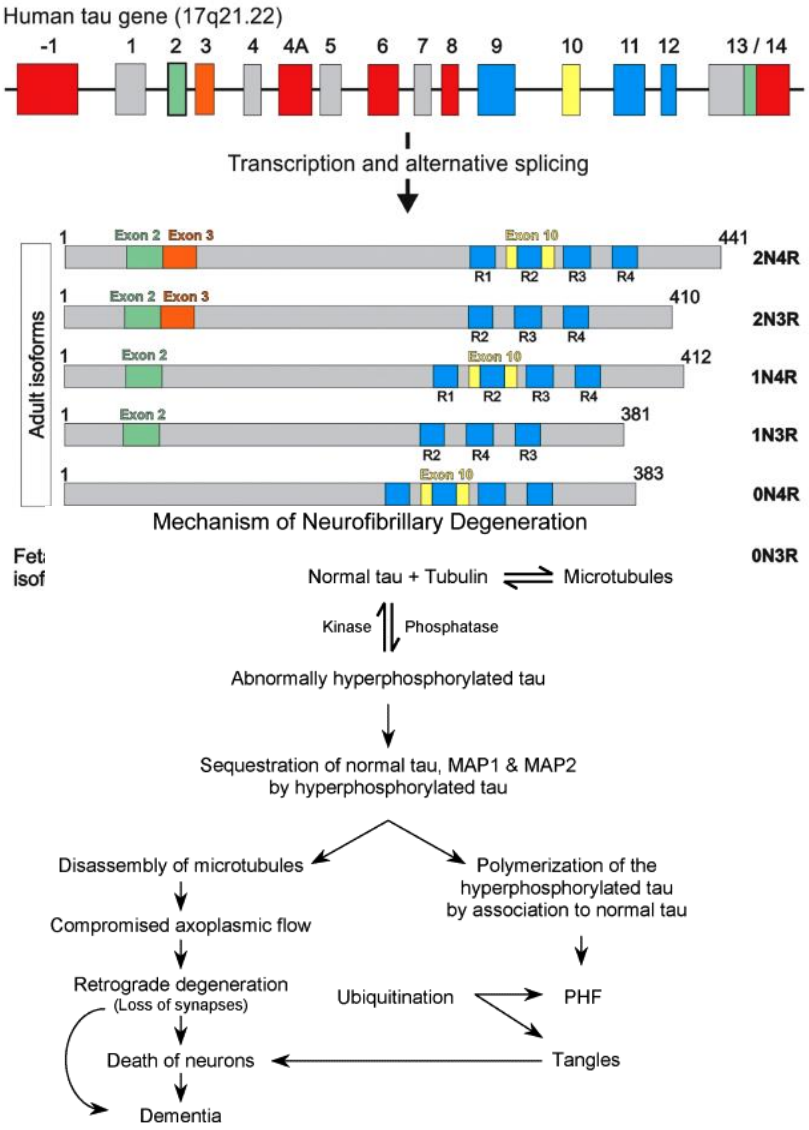
The amyloid cascade theory is based on the identification of beta-amyloid plaques and gene mutations on presenilin 1(PSEN1), presenilin 2(PSEN2) (Levy-Lahad et al., 1995; Nikolac Perkovic & Pivac, 2019) and amyloid precursor proteins (Goate et al., 1991). In 1992, Hardy and Higgins (Hardy & Higgins, 1992) first proposed the β-amyloid theory that the accumulation of beta-amyloid was the critical process in the progression of AD, causing neurofibrillary tangles, cell loss, vascular damage, and dementia. The beta-amyloid has been recognised as the main molecule for the last few decades. Previously accumulated data have supported the crucial role of this molecule in memory function.

The standard form of amyloid in the brain is a protein composed of 695 amino acids. Sequential cleavage of α-secretase and γ-secretase produces non-pathogenic filaments called P3. If β-secretase (BACE) first acts on the sequence instead of α-secretase, beta-amyloid monomers are produced, which aggregate to oligomers and form plaques inside the brains of AD patients (Ricciarelli & Fedele, 2017). β-secretase cleaves amyloid precursor proteins (APPs) at the N-terminal end of the protein sequence while γ-secretase regulated by PSEN1 and PSEN2 cleaves at the transmembrane domain of APP. The mutation in APP, PSEN1 and PSEN2 result in the production of a longer β-amyloid (Aβ) peptide, which is more likely to self-aggregate (Kepp, 2016). Self-aggregating Aβ monomers form Aβ oligomers, potentially triggering various synaptic dysfunctions, including altered synaptic plasticity and memory loss. Aβ has been proved to interact with multiple targets leading to different mechanisms, Ca^{2+} homeostasis dysregulation, oxidative stress, mitochondrial damage, all contributing to the neurotoxic effect (Garwood et al., 2017; Kamat et al., 2016).

1.4.2 Neurofibrillary tangle formation

Tau is the major microtubule-associated protein (Edwards et al. 2019) of a mature neuron, identified in 1975 by Weingarten et al. as a heat-stable protein that stabilises neurons through the interaction with tubulin and the promotion of microtubule assembly regulated by its phosphorylation extent (Weingarten et al., 1975). Tau proteins have 6 isoforms in the human brain, from the shortest, 352 amino acids, to the longest having 441 amino acids. The longest tau proteins are made up of two inserts (exons 2 and 3) (Figure 2A) and 4 repeats (R1, R2, R3 and R4), while the shortest tau proteins are made of 3 repeats (R1, R3, R4) devoid of any inserts (Pîrşcoveanu et al., 2017). Tau isoforms with 3 repeats (3R) display a lower affinity binding with microtubules than 4R tau isoforms (Lu & Kosik, 2001). One of the main functions of tau is to stabilise axonal microtubules. Tau is active in the distal parts of axons making microtubules stable and flexible. This change is crucial for axonal growth and effective axonal transport.

In AD patients and patients afflicted by other tauopathy diseases, tau protein is hyperphosphorylated and aggregated into bundles. The main point of the tau hypothesis is that hyperphosphorylated tau changes normal tau into PHF-tau (paired-helical tau) and NFTs (neurofibrillary tangles). Hyperphosphorylated tau proteins disrupt the microtubule assembly leading to the formation of neurofibrillary tangles (NFTs) made of normal tau proteins, microtubule-associated protein 1 and 2 and ubiquitin. Abnormal microtubules and NFTs result in neuronal cell death, eventually starting the onset of dementia (Iqbal et al., 2005). Hyperpolarised tau proteins accentuate the need to conduct a thorough investigation to comprehend their aggravating effect on AD progression.



1A

1B

Figure 2. A) organisation of the tau gene and transcription result of 6 isoforms. Exon 2 and 3 form inserts (N), and there could be 4 repeats towards the COOH end (Pîrşcoveanu et al., 2017). Exon-1 is part of the promotor, which is not translated. Exons 1,4,5,7,9,11,12,13 are constitutive exons. Exon 14 is found on mRNA but not translated into final proteins. Exons 2,3 and 10 are alternatively translated and specific to the adult brain. The alternation combination of these exons produces 6 isoforms of human tau. B) Mechanisms of Tau protein affecting the progress of AD. Hyperphosphorylated tau breaks up the tau microtubule bundle to make free tau proteins which stick together into neurofibrillary tangles (NFTs) and cause neuron death (Iqbal et al., 2005).

1.4.3 Neuroinflammation during AD

An optimal interplay between neurons, glial cells, soluble mediators, and the immune system is crucial for the proper functioning of the human brain, including cognitive and behavioural abilities. CNS has conventionally been considered an immune-privileged zone, safeguarded by specialised barriers. Nevertheless, it lacks immunological isolation from the peripheral immune system (Sandrone et al., 2019; Wong-Guerra et al., 2023) and is not impervious to harm signals from the peripheral organs. Neuroinflammation is an inflammatory reaction that occurs inside the CNS and can be triggered by several internal pathogenic insults (such as ischaemia, cellular signals, and chemicals) or external stimuli (such as infections, trauma, and toxins) (Fernandez et al., 2008). Neuroinflammation is defined by the activation of innate immune cells, specifically microglia and astrocytes, as well as the permeability of endothelial cells and the infiltration of blood cells. This inflammation can be caused by biochemical or mechanical damage to the brain structures or the blood-brain barrier (BBB) (DiSabato et al., 2016; Gilhus & Deuschl, 2019). Neuroinflammation stimulates the generation and discharge of inflammatory agents such as cytokines (IL-1β, IL-6, IL-18) and tumour necrosis factor (TNF), chemokines (CCL1, CCL5, CXCL1), small-molecule messengers (prostaglandins and nitric oxide), and reactive oxygen by various immune system cells (DiSabato et al., 2016). More details of astrocytes and glial cells will be shown in chapter 1.6.1.

The hypothesis of the relationship between inflammation and AD pathogenesis was proposed around three decades ago by Eikelenboom et al. (1994) and Rogers et al. (1996) (Eikelenboom et al., 1994; Rogers et al., 1996). A study conducted in 1994 revealed that chronic inflammation in rats replicated certain aspects of the neurobiology of AD (Hauss-Wegrzyniak et al., 1998). Subsequent research further confirmed this by investigating the entire sequence of reactions resulting from microglial activation due to damage signals (Fernandez et al., 2008; Rojo et al., 2008). Various studies provide evidence that Alzheimer's disease, characterised by inflammation, begins several decades prior to the onset of significant cognitive decline (Di Benedetto et al., 2017). Microglial activation precedes the start of Alzheimer's disease by several years (DiSabato et al., 2016; Eikelenboom et al., 1994).

Research has shown that a state of neuro-inflammation starts many years before the complete cognitive decline shown in people with Alzheimer's disease (Robbins et al., 2021). Moreover, there exists a robust correlation between neuroinflammation, the buildup of amyloid and tau proteins in the human brain (Leng & Edison, 2021; Martyn, 2003; Mcgeer et al., 1990; Jun Wang et al., 2015). The existing evidence has prompted

inquiry into the potential of neuro-inflammation as a source of novel pharmaceutical targets for combating AD(Fernandez et al., 2008; Maccioni et al., 2009; Rojo et al., 2008).

Within the framework of anti-inflammatory treatments for Alzheimer's disease (AD), numerous research endeavour to provide proof of the effectiveness of anti-inflammatory medications in managing inflammation in the peripheral areas of the body(McGeer et al., 2018). Nevertheless, none of these discoveries have had a tangible impact on the development of treatments for Alzheimer's disease. The investigation of nutraceuticals with anti-inflammatory properties, such as quercetin(Maccioni et al., 2022) and the Andean shilajit, has generated hopeful prospects. Recent clinical trials have supported the efficacy of the Andean shilajit(Leonardo Guzman-Martinez et al., 2021). Hence, it is reasonable to believe that natural multicomponent formulae are viable, as they align with the concept of multitarget therapy for a complex disease like AD (L. Guzman-Martinez et al., 2021).

1.5 The hippocampal-parahippocampal regions implicated in AD

The encoding, consolidation, and retrieval of mnemonic information rely heavily on a complex network of interconnected regions, which includes neocortical association regions, subcortical nuclei, the medial temporal lobe (MTL), parahippocampal areas, and the hippocampal formation. The hippocampus is regarded as the major hub in this circuit. The entorhinal cortex (EC) gets input from nearly all neocortical association areas through the perirhinal and parahippocampal cortices (Bartsch & Wulff, 2015; Chevaleyre & Piskorowski, 2016; Small et al., 2011; Van Strien et al., 2009). The hippocampus is a three-layered allocortical structure that exhibits reciprocal connections with several cortical and subcortical regions. The primary neurons of the hippocampus are arranged in layers and receive one-way polymodal input from the entorhinal cortex (EC), where layer II neurons transmit signals through the perforant path to granule cells in the dentate gyrus (DG) (Strange et al., 2014). The trisynaptic pathway, consisting of mossy fibres connecting the DG to CA3 and Schaffer collaterals connecting CA3 to CA1, is the primary feed-forward circuit responsible for information processing in the hippocampus. In addition, layer III neurons originating from the entorhinal cortex (EC) send direct projections to CA1 neurons through the temporoammonic pathway (also known as the perforant pathway to CA1). CA1 pyramidal cells, which are the primary output neurons, send projections through the subicular complex to the deep layers of the entorhinal cortex

and to other places in the subcortical and cortical regions (Murray et al., 2011). The areas CA1–CA3 are divided into four distinct layers, namely pyramidal, stratum oriens, stratum lucidum, and stratum radiatum(Cooper & Ritchey, 2019). The configuration of this unidirectional circuit, with its restricted duplication, could be crucial for the processes of acquiring knowledge and retaining information. The structure of this feed-forward circuit with its limited redundancy may be critical for learning and memory but may also contribute to its vulnerability during insults.

Nevertheless, CA1 serves as the main output of the hippocampus and, together with the subiculum, are the initial hippocampal regions impacted in AD (Lavenex & Amaral, 2000; Li et al., 2019; Masurkar, 2018; Mufson et al., 2015). CA1 contains tightly packed principal neurons called granule cells at the end of the hippocampus. The CA1 region is filled with pyramidal cells like those found in the neocortex, and is critically involved in hippocampal memory, *e.g.,* acquisition, consolidation, and recall (Broussard et al., 2023).

The hippocampus is surrounded by the parahippocampal gyrus, which is part of the limbic system (Lew & Semendeferi, 2017). The parahippocampal gyrus is made up of several regions, including the primary olfactory cortex at the anterior pole, presubiculum and entorhinal cortex at the posterior (Van Hoesen, 1982), whose function is to send afferent impulses to the hippocampus and are implemented in memory regarding various kinds of information, such as memory for spatial relations. While previous studies demonstrated that the hippocampus is critical to the construction of a cognitive map, both lesion and fMRI studies have shown an involvement of the RPH in the learning of spatial configurations of objects but not object identity. This occurs in a part independent of the hippocampus (Bohbot et al., 2015). Research concerning regions involved in the mechanism of memory loss and neurodegeneration would ultimately help in the identification of appropriate and effective treatment regimens.

1.6 Support cells of cortical regions

1.6.1 Astrocytes and glial cells

Astrocytes are star-shaped glial cells and are the most abundant in the CNS (Placone et al., 2015). Astrocytes are developed from radial glial cells. Mature astrocytes differ by functional and morphology proprieties (Taverna et al., 2014). They have a regulatory role in cerebral functions involved in neurogenesis and synaptogenesis, controlling blood-brain barrier permeability, and maintaining extracellular homeostasis (Siracusa et al., 2019). Astrocytes perform various physiological processes, including neurotransmitter transport, ion homeostasis, oxidative stress regulation and dopaminergic neuron protection (Catts et al., 2014; McCullumsmith et al., 2016). One essential astrocyte function is to deliver energy to neurons through the astrocyte-neuron lactate shuttle (Sherwood et al., 2006). Moreover, astrocytes modulate Ca^{2+} variations affecting neuronal activity, mediating gliotransmitters and other neurotransmitters uptake by excitatory transporters 1 and 2 (EAAT1 and 2) (Peteri et al., 2019). Regulation of astrocyte functions affects several brain pathologies such as AD. Recent studies found astrogliosis in an AD mouse model and AD patients where active astrocytes were surrounded with Aβ accumulation by phagocytosis of degenerated dendrites and synapses (Sajja et al., 2016). Reactive astrocytes also stimulate the secretion of the inflammatory cytokines (IL-1, IL-6, TNF-α), leading to neurodegeneration in AD.

Microglia cells, accounting for nearly 10% of all CNS cells, are considered the first-line of defence against invading pathogens (Solito & Sastre, 2012). In the past, microglia were believed to differentiate in the bone marrow from embryonic hematopoietic precursor cells. However, recent studies reported that microglial cells were grown from the progenitors in the embryonic yolk sac (Ginhoux et al., 2013). In response to an insult, microglia can shift into different functional states, modifying their proliferation (Gomez-Nicola & Perry, 2015), morphology (shortened processes) (Cuadros & Navascués, 1998), phagocytic activity (Sierra et al., 2013), antigen presentation (Bachiller et al., 2018) and release of inflammatory factors such as cytokines and chemokines (Kettenmann et al., 2011). The primary function of microglia is the monitoring of pathogens and host-derived ligands in CNS, including pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) (Fakhoury, 2016). When microglia are activated by invading pathogens, they undergo morphological changes such as the enlargement of the soma and the shortening of cellular processes. (Town et al., 2005). Activated microglia regulate neuroinflammation through pathogen phagocytosis, debris clearance and lesioned site cell degeneration. Coupled to phagocytosis, microglia also communicate with T cells by presenting antigens that alter the defence from an innate response to an adaptive immune

response (Figure 3). Various studies have shown the activation of glial cells, including microglia and astrocytes, plays a vital role in initiating neuroinflammatory pathways in neurodegenerative diseases*, e.g.,* AD (Fakhoury, 2018; J. Wang et al., 2015).

However, activated microglia also caused detrimental effects on neurons. Cytokines, chemokines, inducible nitric oxide synthase, COX-2, and free radical species are produced by microglia during neuroinflammation, which could potentially cause neuron dysfunctions and cellular damage.

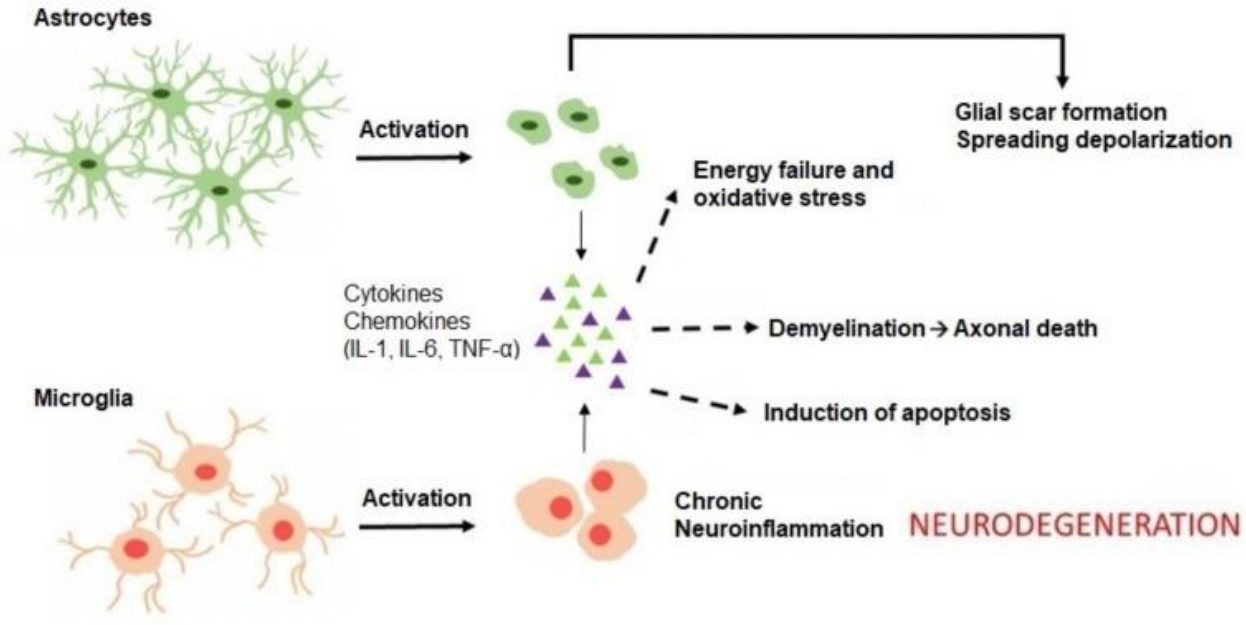


Figure 3. Astrocytes and microglia regulate neuroinflammation (Fakhoury, 2018). Astrocytes and microglia activate inflammatory cytokines and chemokine release, which leads to chronic neuroinflammation and eventually neurodegeneration.

1.7 Alteration of glutamatergic and GABAergic cells of the cortical region in AD

1.7.1 Excitatory principal cells of cortical regions in health and in AD

Pyramidal cells and granule cells occur in the hippocampus and use the neurotransmitter glutamate for communication. Pyramidal neurons are named after their shape, with a pyramidal soma body. Apical dendrites emerge from the apex of pyramidal cells and form dendrite arbours that integrate synaptic inputs into the cell. They receive excitatory inputs from the striatum, entorhinal cortex, amygdala *etc.* Excitatory pyramidal cells subsequently encode representations of spatial and other episodic memories and provide glutamatergic output to other cortical and subcortical areas (Klausberger & Somogyi, 2008). In CA1, primary excitatory inputs use glutamatergic CA3 Schaffer collaterals to contact dendritic spines on the apical and basal dendrites in strata radiatum and oriens (Engel et al., 2008). Other excitatory inputs originate from synapses on distal apical dendrites in the stratum lacunosum-moleculare by the temporoamonic system. CA3 pyramidal cells have complex dendritic arbours, receiving synaptic information from numerous sources: 1) fibres from ipsi and contra-lateral CA3 pyramidal neurons which synapse on basal and mid-apical dendrites in the stratum oriens and stratum radiatum and mossy fibres from granule cells of the dentate gyrus which synapse on the apical region, and the stratum lucidum. Excitatory inputs exclusively terminate on the dendritic spines, while inhibitory inputs terminate on dendritic shafts, the soma and even the axon. Pyramidal neurons can be excited by glutamate and are inhibited by GABA (García-López et al., 2006). In AD, Pyramidal cells are disproportional killed which lead to further neurodegeneration and cognitive decline (Mattson & Kater, 1989).

Granule cells (GCs) arise from a progenitor population with a radial morphology and are slowly integrated into the DG (dental gyrus) neural circuit, contributing to the overall DG circuit. Granule cells release glutamate onto target cells in the hilus and Cornus ammonis (CA3) region and receive glutamatergic and γ-aminobutyric acid (GABA)ergic inputs that tightly control their spiking activity (Toni & Schinder, 2016). These cells play vital roles in synaptic plasticity, which is fundamental for learning and memory (Wang et al., 2018), and is thought to be markedly altered during AD pathogenesis. AD patients exhibit a decrease in the number of dendritic spines and the overall length of dendrites in their GCs(de Ruiter & Uylings, 1987; Llorens-Martín et al., 2013). Patients diagnosed with frontotemporal dementia have been found to exhibit similar modifications (Terreros-Roncal et al., 2019). Interestingly, the proportion of GCs exhibiting multiple primary apical

dendrites is elevated in AD patients and mice (Llorens-Martín et al., 2013). The diseased phenotype referred to as the "V-shape" is distinctly different from the typical "Y-shape" phenotype exhibited in healthy control persons and normal mice(Llorens-Martín et al., 2013). It is still unclear whether the observed changes in the structure and function of GCs during the course of AD are caused by an atypical form of dendritic plasticity or if this plasticity is a result of the disease. The number of immature DGCs decreases gradually throughout AD progression (Cole et al., 2020). This alteration could be related to brain over-excitation. While activated glutamate NMDA receptors have critical roles in promoting neuron survival, hyperactivated NMDA receptors cause neurotoxicity, damaging or killing neurons. In addition to acute effects, studies have also reported the detrimental effect of hyperactivated NMDA receptors(NMDAR) in delayed, slowly evolving neurodegeneration (Liu et al., 2019). The partial NMDAR antagonist, memantine , has been proved to normalize the glutamatergic system and ameliorate cognitive and memory deficits (Olivares et al., 2012). Supplemental evidence suggests that neurotoxicity is due to high Ca^{2+} influx through the high Ca^{2+} permeability of NMDA receptors (Jung et al., 2019). The pathological Ca^{2+} level results in gradual neuron cell loss and synaptic dysfunction, correlating with clinical findings in AD patients: cognitive and memory decline and pathological neuron anatomy (Danysz & Parsons, 2003; Wenk et al., 2006). Inhibitory interneurons modulate excitatory cells through the timing of their spike activity to modulate their network behaviour.

Despite the subcellular region, impaired inhibitory interneuron functions cause severely altered excitatory cells. One GABAergic inhibitory interneuron could potentially receive excitation from various excitatory interneurons and output its inhibitory effect to numbers of principal cells in a short time (Ghatak et al., 2019). The investigation of GABAergic interneuron dysfunction could yield promising results in AD treatment because the imbalance between excitatory and inhibitory neurons could cause neurotoxicity and neuron death. (more details will be explained in the next chapter).

1.7.2 GABAergic interneurons of the hippocampus

The GABAergic system and cells that release this neurotransmitter have become the subject of attention in the AD field due to evidence suggesting the roles they play in memory and learning and rrecently research has evidenced GABAergic interneurons are selectively vulnerable during neurodegeneration in animal models (A. Shi et al., 2020) and in post-mortem brains from AD patients (Mattson, 2020). In health inhibitory interneurons function to control pyramidal cells behaviour, managing timing of synaptic communication and generate oscillatory behaviour (Roux & Buzsáki, 2015). Furthermore, inhibitory interneurons have specific firing properties, including, fast-spiking, burst-firing and adapting firing (Tzilivaki et al., 2023). These properties are important for timing of synaptic communication in the circuitry.

Inhibitory interneurons contribute to the stability of principal cell populations through two distinct mechanisms: feedforward and feedback inhibition. Each dendritic domain of the primary cells is associated with certain interneurons that receive excitatory input from distinct afferents. The interneurons selectively inhibit the same places that they receive input from, thus establishing a model for feedforward inhibition (Buzsáki, 1984). In addition to dendritic inhibition, interneurons with somatic targets (basket cells) (shown in Figure 4) or axon initial segment targets (chandelier or axo-axonic cells) can also form feed-forward circuits. Feed-forward inhibition can decrease the spike responses of principal neurons by either competing with dendritic excitation or lowering output spiking (Figure 4). For example, cholecystokinin (CCK) positive basket cells play a key role in the feed-forward inhibition of hippocampal CA1 pyramidal cells (Roux & Buzsáki, 2015; Basu et al., 2013). Within a feedforward circuit, despite the inhibitory action being disynaptic, various approaches exist to expedite the inhibitory action. Those consist of a reduced firing threshold and larger and more effectiveness of excitatory synapses on interneurons (Isaacson & Scanziani, 2009; Stokes & Isaacson, 2010). Consequently, feed-forward inhibition can occur prior to the primary cell's membrane reaching the threshold for charging, so preventing the generation of a spike, or at least the generation of multiple spikes (Buzsàki & Eidelberg, 1981). This technique efficiently reduces the time frame in which the principal cell reacts, resulting in a remarkably precise timing of induced spiking (Kitamura et al., 2014; Owen et al., 2013).

GABAergic local circuit inhibitory interneurons, which make up approximately 10-15% of the overall neuronal cell population. These cells forms a strikingly heterogeneous population of interneuronal types that were defined over the course of more than a century of research(Fishell & Kepecs, 2020; Pelkey et al., 2017; Tremblay et al., 2016; Tzilivaki

et al., 2023), based on morphology (e.g., basket cells [BCs]), electrophysiological properties (e.g., fast-spiking [FS]), connectivity/targeting (e.g., axo-axonic [AAC]), or the expression of particular molecular markers (e.g., somatostatin [SOM]). Extensive research conducted in the CA1 region of the mouse hippocampus, spanning several decades, involved the study of both anaesthetized and awake animals. 29 distinct types have been identified within this area, many of which have various functions *in vivo* (Dudok, Klein, et al., 2021; Dudok, Szoboszlay, et al., 2021; Sanchez-Aguilera et al., 2021; Szabo et al., 2022; Tzilivaki et al., 2023; Varga et al., 2014).

Approximately ten years ago, researchers discovered that cortical interneurons can be categorised into three distinct classes, which have minimal overlap and originate from different developmental sources (Lee et al., 2010; Rudy et al., 2011). These classes include parvalbumin- (PV) and SOM-expressing interneurons, which are derived from the medial ganglionic eminence (MGE), as well as serotonin receptor 3a (5HT3aR)-expressing interneuronswhich originate from the caudal ganglionic eminence (CGE).

The distinction between SOM and PV cells in MGE-derived interneurons has been shown to be valuable, as numerous studies have demonstrated functional variations between "dendrite-targeting," slow-firing SOM interneurons and "soma-targeting," FS BC interneurons.The sequence of numbers is (De Filippo et al., 2021; Jouhanneau et al., 2018; Miao et al., 2017). Nevertheless, there exists significant variation in shape and firing parameters within these two types(Katona et al., 2014; Varga et al., 2014).

CGE-derived interneurons can be classified into three primary subclasses depending on the expression of Lamp5, Sncg, or vasoactive intestinal peptide-expressing (Wallin et al., 2012; Yao et al., 2021). Lamp5 interneurons comprise ivy and neurogliaform cells (NGFCs). The ivy cell is the predominant interneuron subtype in CA1 (Fuentealba et al., 2008). It exhibits a unique structure, with an axonal cloud that spans multiple layers of the hippocampus. Additionally, it co-express neuronal nitric oxide synthase (nNOS), indicating retrograde signalling mediated by nitric oxide (NO). NGFCs exhibit a similar structure to compact ivy cells, characterised by a distinctively "bushy" dendritic tree and a densely packed axonal cloud(Cajal, 1998). Sncg cells were shown to include many cholecystokinin (CCK)-positive cell types(Harris et al., 2018). However, CCK cells are a remarkably diverse group in the hippocampus (Lasztóczi et al., 2011) that extends beyond Sncg cells (Dudok, Klein, et al., 2021). The VIP subclass predominantly consists of interneurons that particularly target other interneurons, rather than principal cells (PCs)(Guet-McCreight et al., 2020; Tzilivaki et al., 2023).

In this thesis I will detail the 3 subclasses of interneurons, which parvalbumin (PV)-, Cholycystokinin (CCK)- and calretinin-expressing interneurons.These three subcalssses

have been chosen to be studied during AD progression as they have specific role in the micro-circuitry.

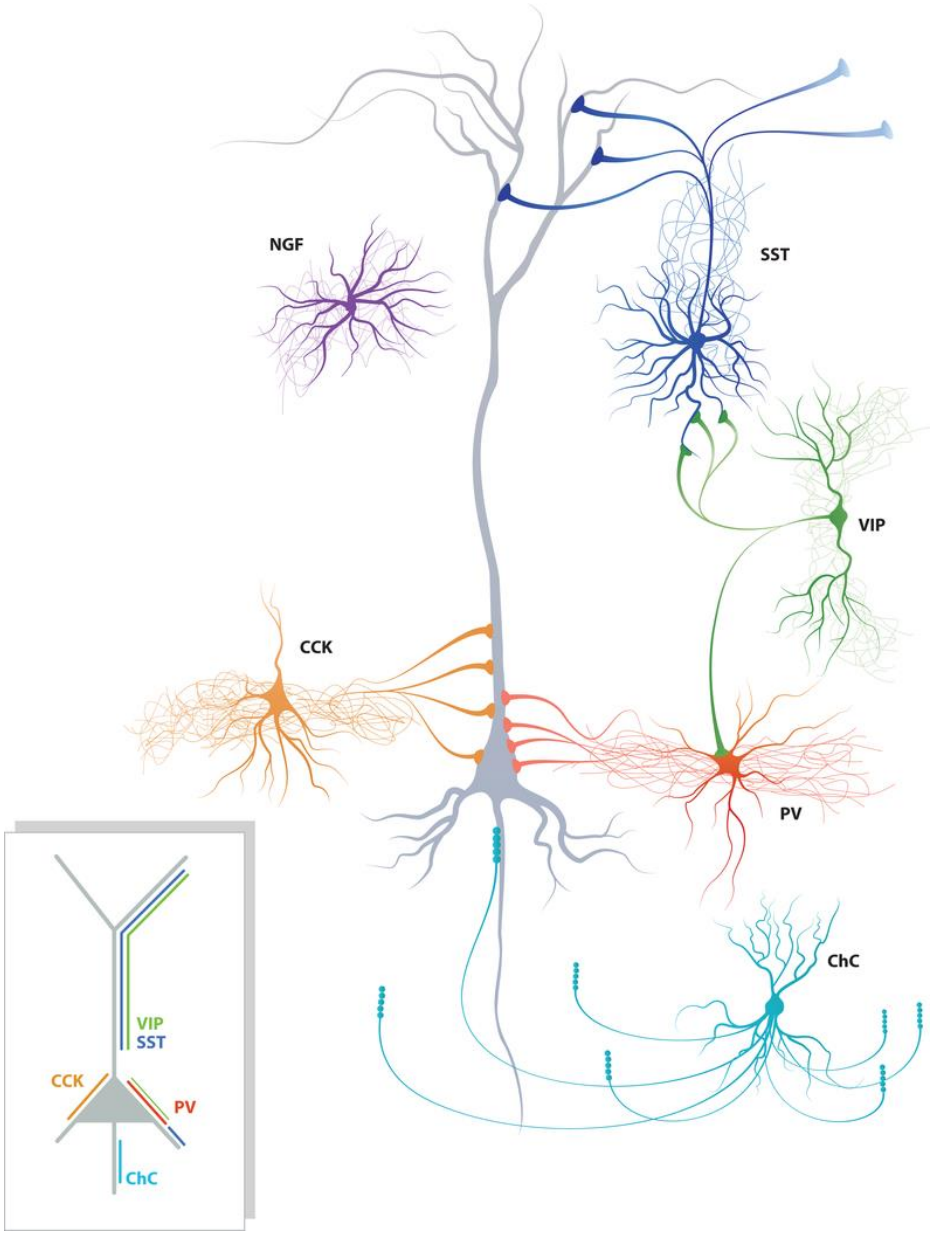


Figure 4. Various subtypes of interneurons exhibit distinct specialisation in selectively addressing specific domains of pyramidal cells as well as other interneurons. Various kinds of interneurons selectively innervate certain locations along the axo-somato-dendritic axes of pyramidal cells. that are targeted somatically can be categorised into two major types, namely parvalbumin (PV)-expressing basket cells and cholecystokinin (CCK)-expressing basket cells. Chandelier cells, also known as ChCs, exhibit a preferential innervation of the axon beginning segment. The dendrites are the primary target of somatostatin (SST) interneurons, whereas vasoactive intestinal peptide (Wallin et al., 2012) interneurons primarily target SST interneurons and, to a lesser extent, parvalbumin (PV) interneurons. Neurogliaform (Poon et al., 2020) cells employ volume transmission as a mechanism to provide gradual inhibitory signals to the surface layers. The inset presents an illustration of the interneuron's process of targeting pyramidal cells.(Fishell & Kepecs, 2020)

1.7.2.1 Parvalbumin positive interneurons alteration in AD

A major subpopulation of GABAergic interneurons which contain calcium binding protein parvalbumin (PV), has raised much scrutiny in the last few years (Deng et al., 2019; H. Hu et al., 2014), because they mediate neocortical-hippocampal interactions that are necessary for memory conslidation (Xia et al., 2017). PV-expressing cells display the fastest action potential in cortical regions and their synaptic events have the short latency (Estebanez et al., 2017). PV-positive interneurons mainly inhibit somatic membranes or initial axon segments of pyramidal cells and other inhibitory cells, thus having a fast and powerful inhibtory action (Tzilivaki et al., 2023). PV are the largest class of GABAergic interneurons in CNS (Bezaire & Soltesz, 2013) (Figure 4). Several studies have reported their vital functions in the modulation of principal cells (H. Hu et al., 2014; Petrache et al., 2019; Tremblay et al., 2016), as well as other inhibitory interneurons (Ferguson & Gao, 2018),synaptic plasticity (Donato et al., 2013) and the initiation of Gamma network oscillation (Guan et al., 2022; Cardin et al., 2009).

Post-mortem brain tissue of AD patients and AD mouse models demonstrated that PV neurons degenerate and exhibit dysfunction early in AD progress (Hijazi et al., 2023; Villette & Dutar, 2017). Previous studies have reported that PV interneurons were greatly sensitive to Aβ accumulation, resulting in PV losing functions and causing hyperexcitability (Palop & Mucke, 2016), Furthermore, our lab has shown that the impaired excitatory–inhibitory balance primarily originated from a decreased cellular distribution and hypoactivity of GABAergic function of PV interneurons(reduced in density, firing properties and the capacity to produce the neurotransmitter GABA) (Petrache et al., 2019)

1.7.2.2 CCK (Cholecystokinin) positive interneurons

CCK is one of the most abundant neuropeptides in the brain (Reisi et al., 2015). It was first discovered as a gastrointestinal peptide (Bliss & Whiteside, 2018; Edwards, 2019). In various cortical regions, including the hippocampus, major subclasses of interneurons express CCK, and these inhibitory cells are thought to be modulatory as they target dendrites of pyramidal cell and other inhibitory cells, fine tuning excitatory inputs (Ali, 2007). These cells are thought to be important in generation brain oscillation including theta oscillations (Fasano et al., 2017). These cells are also thought to play an important role in memory and learning, since CCK and its receptors CCK1 and CCK2 make

outstanding contributions in neuronal functions, *i.e.,* spatial memory. There is thus, a high concentration of CCK in the hippocampus region (Sadeghi et al., 2017).

Studies have reported that interneurons with calcium-binding proteins such as CR might overcome the excitotoxicity induced by increasing intracellular Ca2+ concentration(Petrache et al., 2019), whereas interneurons without calcium-binding proteins but expressing neurotransmitters like CCK is more likely to degenerate in AD (Ali et al., 2023)

Previous studies, including our own, have demonstrated that the CCK sub-class of interneurons are especially prone to neurodegeneration. This susceptibility arises from their inherent hyperactivity during the initial stages of AD, which in turn leads to increased toxicity. This toxicity is associated with the infiltration of Aβ peptides (Palop & Mucke, 2016; Shah et al., 2018; Anqi Shi et al., 2020).

The vulnerability of these cells to degeneration in AD may be associated with shared cleavage mechanisms of the peptides, together with the infiltration of toxic soluble Aβ. This infiltration drives the aggregation and breakdown of Aβ, leading to a significant accumulation of intracellular Aβ. Consequently, the regular operation of CCK cells is disrupted, leading to cellular death. Furthermore, it has been demonstrated that Aβ buildup specifically affects GABA-producing interneurons (Villette & Dutar, 2017). Given the significant significance of these interneurons in the process of learning and memory, as well as the recognised connection between Aβ plaque deposition and neuronal death, it is expected that there will be a decrease in the number of CCK-positive neurons in knock-in AD model mice compared to WT mice. The correlation between the CCK-driven system and the glutamatergic system, and its function in safeguarding neurons from the harmful effects of glutamate (Löfberg et al., 1996), implies that the decline of CCK-positive interneurons may contribute, to some extent, to the gradual neurodegeneration observed in AD by enhancing neuronal cell death through excitotoxicity.

1.7.2.3 Calretinin (CR) positive interneurons

CR is a calcium-binding protein (CBP) family with 29 kDa calbindin (Saffari et al., 2019). Like other CBP, CR has a high affinity over the 6 EF-hand domains for Ca^{2+} binding, helping in the regulation of the amplitude and duration of the Ca^{2+} signal (Baglietto-Vargas et al., 2010). CR exhibits cooperative binding when Ca^{2+} binds to different EF-hand domains, increasing Ca^{2+} affinity when Ca^{2+} concentration rises.

Interneuron that express CR are distributed throughout the entorhinal cortex, hippocampus (all strata), and neocortex and account for 10-30% of GABAergic interneurons (Cauli et al., 2014) and can be identified with immunohistochemistry by staining for calretinin, a calcium-binding protein. Their shape may vary, but it is usually bipolar. They are specialised in connecting with other interneurons, among which are SST cells (Cauli et al., 2014). An interesting characteristic of CR cells is forming connections with each other- a cell being in contact with several others at a time (Freund & Gulyás, 1997). They form dendro-dendritic and axo-dendritic contacts with each other and only dendro-dendritic contacts with other cells and show a preference for contacting calbindin (Pelkey et al.) D_{K28} cells and vasointestinal peptide (Wallin et al., 2012)- expressing cells. They avoid interneurons that express parvalbumin (PV) (Gulyás et al., 1996). According to gross morphology and spatial distribution, two types of CR cells have been identified: spiny CR cells which are found in parts of the dentate gyrus and the CA3, and aspiny CR cells, which are evenly found in the hippocampus (Gulyás et al., 1996). Interestingly, CR cells in the strata pyramidale (SP) and radiatum co-express VIP. These CR/VIP cells show high input resistance and irregular spiking and have been shown to make synapses on to OLM SST cells (Freund & Gulyás, 1997).

Immunohistochemical studies of post-mortem tissue from human AD patients show a preservation of CR density throughout the affected brain (Rsibois & Rogers, 1992; Hof et al., 1993; Soriano, 1995), suggesting that they are unaffected in AD. The discharge and pattern of synchronisation of inhibitory interneurons can be regulated by other interneurons specialised in making interneuron-interneuron connections, such as CR-expressing cells (Acsady et al., 1996). Although CR cells were first characterised many years ago (Freund & Gulyás, 1997), there is a missing gap in the understanding of these specialised disinhibitory cells. Interestingly, post-mortem human studies in tissue with heavy amyloid deposits show that CR cells are preserved in AD (Hof et al., 1993). Furthermore, previous study in our lab has suggested that the density, morphology, and function of CR-expressing interneurons studied in *App*$^{NL-F}$ KI mice was not altered and nor did they contain soluble Aβ, while CCK and SOM expressing interneurons showed degeneration and Aβ penetration(Ali et al., 2023; A. Shi et al., 2020).Therefore, it was of interest to investigate this further in our new *App* $^{NL-F}$ */MAPT* $^{htau/wt}$ mouse model to see whether the CR positive interneurons stay unchanged in the new AD mouse models.

1.8 Ionotropic receptors and their role in excitatory-inhibitory homeostasis

Ionotropic receptors are a type of ligand-gated ion channels that consist of three, four, or five protein subunits. The best known ionotropic receptor is the nicotinic receptor, which has served as the prototypical model for ionotropic receptors (Baudry & Bi, 2017). These subunits collectively create a pore within the receptor, which facilitates the conduction of ions. The classification of ionotropic receptors encompasses four distinct families, characterised by variations in their chemical composition and the specific ligands responsible for their activation. The consequences of activating ionotropic receptors can exhibit either excitatory or inhibitory properties, contingent upon the equilibrium potential for the ions they permit to pass and the influence of these ion movements on the membrane potential. Ionotropic receptors have the capacity to be influenced by a variety of naturally occurring substances within the body, and they frequently serve as objectives for drugs (Stephen D. Meriney, 2019).

Glutamate, an amino acid that is not necessary for survival, is found in many parts of the brain, but is most concentrated in synaptic vesicles. Glutamate plays a crucial role in the central nervous system (CNS) as a primary excitatory neurotransmitter. It serves as a crucial intermediary for transmitting excitatory signals and facilitating changes in the nervous system's adaptability (Zhou & Danbolt, 2014). Multiple variants of ionotropic glutamate receptors have been discovered. NMDA receptors, AMPA receptors, and kainate receptors are three of these ligand-gated ion channels. The glutamate receptors are called for the specific agonists that activate them: NMDA (N-methyl-d-aspartate), AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), and kainic acid. The ionotropic glutamate receptors are nonselective cation channels, permitting the flow of Na+ and K+ and occasionally modest quantities of Ca2+. Similar to nACh receptors, the postsynaptic currents generated have a reversal potential in close proximity to 0 mV. Therefore, the activation of AMPA, kainate, and NMDA receptors consistently leads to excitatory postsynaptic responses (Yadav et al., 2023). Currently, ‘REM0046127’ modulating neuronal ca2+ homeostasis is under clinical trials at phase 2 (Cummings et al., 2024).

The glutamate receptor plays a crucial role in learning and memory as it is involved in both long-term potentiation (LTP) and long-term depression (LTD) which is the long lasting increase and reduction of synaptic strength respectively (Castillo, 2012). Synaptic plasticity plays a crucial role in this process, as it involves modifying the number of receptors on the neuronal membrane and associated proteins that regulate many

downstream processes, such as receptor internalisation and subcellular trafficking (Chamberlain et al., 2012).

Conversely, synaptic inhibition is predominantly mediated by the inhibitory neurotransmitter γ-aminobutyric acid (GABA).GABA is synthesized from glutamate by the enzyme Glutamic Acid Decarboxylase and released by GABAergic interneurons (chapter 1.8.1) which are found in the hippocampus, thalamus, basal ganglia, hypothalamus, and brainstem (Perea et al., 2016). $GABA_AR$ allow rapid inhibitory synapses through ligand-gated channels, whereas $GABA_BR$ facilitate gradual inhibitory synapses through G-protein coupled receptors (Allen et al., 2018; Sharma et al., 2023).

The preservation of appropriate levels of glutamate-mediated synaptic excitation and GABA-mediated synaptic inhibition is of utmost significance for the. The malfunctioning of these mechanisms results in an imbalance between excitation and inhibition, which contributes to the development of diverse neuropathological conditions. These conditions range from acute dysfunction in neuronal networks,

The regulation of neuronal excitability is predominantly governed by the equilibrium between synaptic excitation and inhibition (E/I). This homeostasis between E/I is necessary for proper functioning of the brain in various physiological processes, such as learning and memory. any imbalance can manifest in to various neurological diseases such as epilepsy and cerebral ischemia, to neurodegenerative disorders like AD (Khan et al., 2018; Hines et al., 2012; Petrache et al., 2019; Sohal & Rubenstein, 2019; Wen et al., 2022).

Preclinical animal and cell models of AD demonstrate that the hyperexcited neurons show an increased frequency of action potential firing in neurons, together with a decreased firing threshold (Balez et al., 2016; Ghatak et al., 2019; Šišková et al., 2014). Crucially, observations of activity patterns in individual neurons, networks of neurons, or even entire brain areas consistently demonstrate hyperexcitability during the early stages of Alzheimer's disease (Targa Dias Anastacio et al., 2022). The cortex of post-mortem AD brain tissue was found to have a higher ratio of excitatory-to-inhibitory synapses utilising fluorescence deconvolution tomography and synaptic membrane microtransplantation (Lauterborn et al., 2021). AD post-mortem brain tissue in the middle temporal gyrus has shown a decrease in the expression of $GABA_A$ and $GABA_B$ receptors (Govindpani et al., 2020). The equilibrium between E/I neurotransmission is significantly disturbed in AD. The presence of this imbalance has been linked to the emergence and advancement of AD, and it may be the root cause of the cognitive impairments that are typical of the disorder (Govindpani et al., 2017). The neurotoxic effects caused by Aβ are facilitated through an excitotoxic mechanisms, which includes the buildup of glutamate

and excessive activation of NMDA receptors. However, drugs that target the GABA system can potentially decrease the susceptibility of neurons to excitotoxic harm by restoring the balance between excitatory and inhibitory signals (Gail Canter et al., 2019). Reducing the excessive activity of neurons helps restore the equilibrium and alleviate neuronal dysfunction, hence preventing cell death and enhancing cognitive impairments in mice with AD (Busche et al., 2012; Govindpani et al., 2017). Therefore, conducting additional research on $GABA_AR$ to govern the balance between neuronal excitation and inhibition could make a substantial contribution to our existing comprehension of brain physiology and pathophysiology.

1.8.1 $GABA_A$ receptor structure and function

$GABA_A$ Receptors ($GABA_AR$) are hetro-pentameric ligand-gated chloride channels which consists of eight members (α, β, γ, δ, ε, π, ρ, θ) that have 70-80% similarity in their sequence(Sharma et al., 2023). The $GABA_AR$ subunits possess a shared structure, which consists of around 450 amino acid residues. The structure of the protein includes an N-terminal, a large hydrophilic extracellular domain (ECD), four hydrophobic transmembrane domains (TMD: TM1-TM4), with TM2 being responsible for forming the chloride channel pore. Additionally, there is an intracellular domain (ICD) located between TM3 and TM4, which serves as the site for protein interactions and post-translational modifications that regulate receptor activity (Chen & Olsen, 2007; Jacob et al., 2008). The neurotransmitter GABA and psychotropic medicines like benzodiazepines (BZDs) attach to the N-terminal at binding sites α-β and α-γ interfaces, respectively. Neurosteroids and anaesthetics such as barbiturates are located in the transmembrane domain (TMD) of α and β subunits (Macdonald & Gallagher, 2014).

Different compositions of $GABA_ARs$ play distinct roles in physiological or pathophysiological conditions, such as the involvement of the α1 subunit in sedation and the α2 subunit in anxiety (McKernan et al., 2000; Wen et al., 2022). Nevertheless, it is generally accepted that the prevailing form of $GABA_AR$ in the central nervous system consists of α1, β2, and γ2 subunits, organised in an anticlockwise manner around a central pore when viewed from the cell exterior (Figure 5)(Baur et al., 2006; Brohan & Goudra, 2017). Within the central nervous system (CNS), certain subunits of the $GABA_AR$ receptor are widely expressed, while others have limited expression. The GABAA receptors found in postsynaptic locations in the brain consist primarily of the α1–3, β1–3,

and γ2 subunits(Amr Ghit et al., 2021). These receptors can connect with the GABA neurotransmitter and activate chloride channel which is termed as phasic inhibition.

In addition to GABA, a diverse range of ligands have been identified that bind to different sites on the $GABA_AR$ and modulate its activity. Specific receptor subtypes house binding sites that dictate the unique pharmacological characteristics of the receptors (Olsen, 2015). The GABA-binding site, referred to as the active site or orthosteric site, is the specific location where orthosteric agonists and antagonists attach. Orthosteric agonists, including GABA, gaboxadol, isoguvacine, muscimol, and progabide (Bartholini, 1984; Amr Ghit et al., 2021; Vashchinkina et al., 2012; Wahab et al., 2009), stimulate the receptor, leading to an elevation in Cl− conductance. In contrast, orthosteric antagonists, such as bicuculline and gabazine (Johnston, 2013), directly compete with GABA for binding, hence limiting its function and reducing the conductance of Cl− ions. Allosteric modulators, in contrast, attach to a different location on the receptor and produce their impact by inducing structural alterations in the receptor, either in a positive manner (Galeano et al., 2014) like barbiturates, benzodiazepines, z-drugs (nonbenzodiazepines), alcohol (ethanol), etomidate, glutethimide, anaesthetics, and specific neurosteroids, or in a negative manner (NAM) like pregnenolone sulphate and zinc (Olsen, 2018; Vega Alanis et al., 2020; Wang et al., 2006). Ligands like as picrotoxin, which are non-competitive chloride channel blockers, bind to the central pore of the $GABA_AR$ and inhibit the flow of Cl− ions(Alqazzaz et al., 2011). In addition, silent allosteric modulators (SAM) are a type of $GABA_AR$ modulators that can rival a positive allosteric modulator (Galeano et al., 2014) or a negative allosteric modulator (NAM) for occupancy of the binding site, such as flumazenil (Vega Alanis et al., 2020). Ligands possessing specific features are commonly employed as pharmacological agents for anxiolysis, anticonvulsion, sedation, and muscular relaxation. Conversely, ligands that hinder the functioning of receptors typically produce contrasting pharmacological outcomes, such as convulsions and anxiogenesis(Amr Ghit et al., 2021)..

Depending on the subunit composition, $GABA_A$ receptors can subdivided into synaptic and extrasynaptic receptors that have unique roles in the local neuronal circuitry.

Synaptic $GABA_A$ receptors

Studies have revealed that receptors comprising α2, α3, and β3 subunits are approximately 100 times more abundant at synapses compared to the extrasynaptic membrane (Kasugai et al., 2010). The $GABA_A$ receptors found in postsynaptic sites in the brain consist primarily of the α1–3, β1–3, and γ2 subunits. Undoubtedly, the signalling of GABA/$GABA_AR$s is the primary inhibitory mechanism in the central nervous system. The GABA that is produced at the synapse activates the post-synaptic $GABA_AR$s, causing

short-lived inhibitory post-synaptic currents (IPSCs) which leads to a decrease in excitability of the postsynaptic neuron and causing phasic inhibition (Farrant & Nusser, 2005; Li et al., 2016).

Extrasynaptic $GABA_A$ receptors

$GABA_A$ receptors that consist of the α4–6, β2/3, and δ subunits can be found in extrasynaptic locations. In addition, Some γ2-containing receptors are not exclusively located postsynaptically. For instance, α5βγ2 receptors can be found at extrasynaptic locations where they play a role in tonic inhibition. In these locations, the receptors can be activated by low levels of GABA for an extended duration, a phenomenon known as tonic inhibition (Maguire et al., 2005). The predominant isoforms of extrasynaptic $GABA_A$Rs that facilitate tonic inhibition are α4βδ receptors in the forebrain, α6βδ receptors in the cerebellum, and α1βδ receptors in the hippocampus (Bernhard Luscher et al., 2011). The phenomenon of "tonic inhibition" is not synchronised with the rapid synaptic events, resulting in a constant background inhibitory conductance. These conductances change the cell's input resistance, which in turn affects synaptic efficacy and integration. Tonic extrasynaptic conductances, by making the dendritic membrane more electrically leaky, significantly and indiscriminately reduce the magnitude of excitatory signals in dendrites. Tonic inhibition is a significant factor in synaptic plasticity, neurogenesis (Duveau et al., 2011), and cognitive functioning (Lee et al., 2016).

Disruptions in either phasic or tonic inhibition are linked to numerous neurological and mental disorders. Excessive levels of extrasynaptic GABA have been proposed as a potential cause of severe pathological alterations that result in the death of cells in the brain affected by AD (Govindpani et al., 2017; Marczynski, 1998). This aligns with the documented rise in GABA levels in the culture media after the treatment of Aβ1–42(Vinnakota et al., 2020). Measurements of extracellular GABA levels in human AD brains have not been conducted yet due to technical difficulties. Nevertheless, research conducted on rodents using microdialysis indicates that ambient levels of gamma-aminobutyric acid (GABA) can decrease to low micromolar concentrations, which can negatively impact the survival of neurons in a laboratory setting(Marczynski, 1998). Consistently having GABA in its surroundings has the counterintuitive effect of making a cell more susceptible to glutamate toxicity, leading to neuronal degeneration and cell death (Kwakowsky et al., 2018). Therefore, manipulating these signals has become the foundation of pharmacological therapy and anaesthesia (Olsen, 2018; Pedrón et al., 2019; Termsarasab et al., 2016).

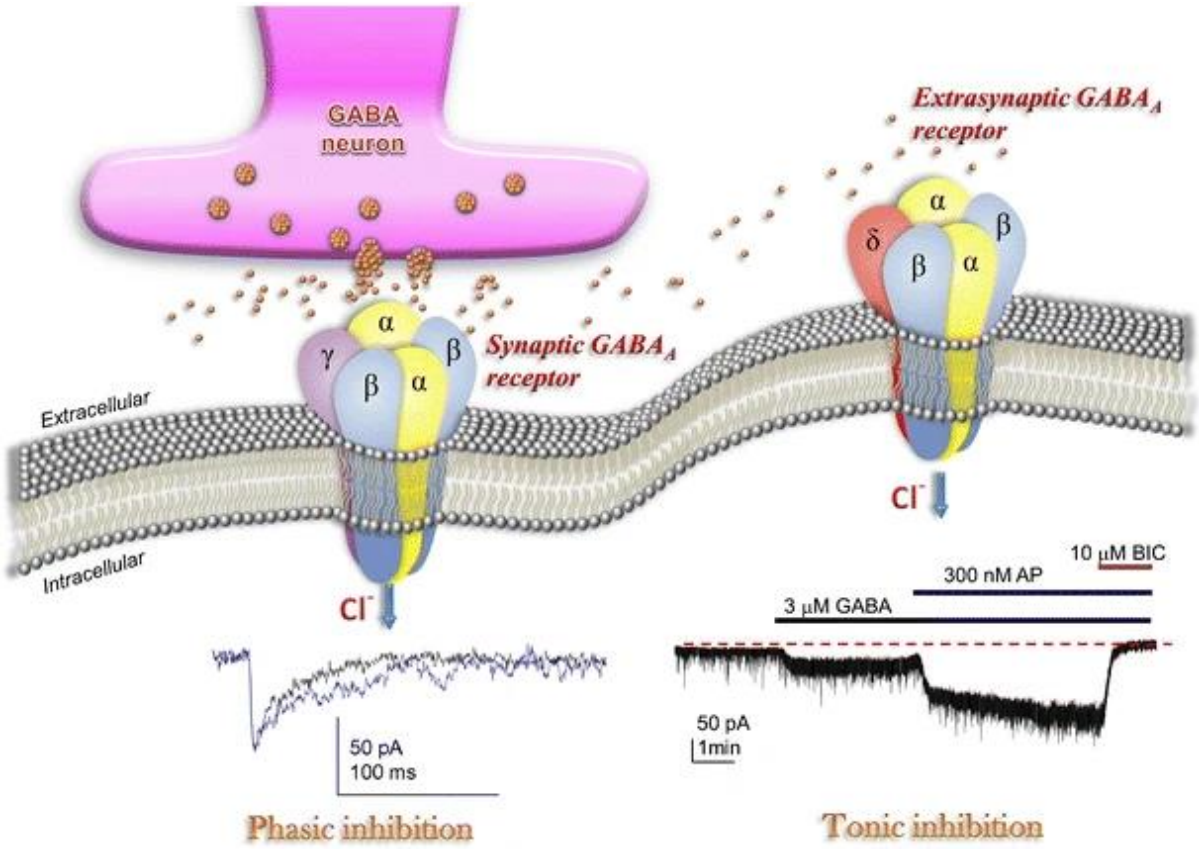


Figure 5. GABA, released from presynaptic vesicles, acts as the primary rapid inhibitory neurotransmitter in the brain by the activation of postsynaptic GABAa receptors. The structure of GABAa receptors consists of five subunits organised in a pentameric formation, with a central pore that selectively allows the passage of chloride ions. Postsynaptic $GABA_AR$, composed of 2α2βγ subunits, mediate the phasic inhibition while extrasynaptic $GABA_AR$, composed of 2α2βδ mediate tonic inhibition. The trace illustrating phasic inhibition shows an IPSC produced by endogenous GABA release (black) or in the presence of 300 nM allopregnanolone(AP) (blue). Neurosteroids enhance the IPSCs by prolonging the deactivation/decay kinetics. The trace illustrating tonic inhibition shows tonic conductance activated by GABA that was further enhanced by application of AP (C. M. Carver & D. S. Reddy, 2013).

1.9 Current status of AD therapy

Extrasynaptic $GABA_A$ receptors containing the pi (π) subunit mediate tonic inhibition in most brain regions, and the alpha (α)-5 and delta (δ) subunits are the major $GABA_A$ receptors mediating inhibition in the hippocampus (Glykys et al., 2008 2008). Hippocampal neurons receive inhibitory charges from tonic inhibition that account for ~75% of the total inhibitory charge received by hippocampal neurons (Xu et al., 2020 & Zhang, 2020). Early studies showed that GABA inhibitory interneurons are not vulnerable to Aβ attack (Pike & Cotman, 1993; Rossor et al., 1982). Therefore, research efforts have mostly focused on the effect of Aβ on excitatory neurons or excitatory synaptic (Mucke & Selkoe, 2012; Selkoe, 2019; Tackenberg & Brandt, 2009). Recent studies, however, have shown that AD patients suffer from memory and cognitive impairment that is obviously different to normal age-related decline and partly due to hippocampal neuron over-activity caused by GABA inhibitory interneuron dysfunction (Huang & Mucke, 2012). The investigation of the role played by GABA inhibitory interneurons in AD development may provide future drug targets for AD.

1.9.1 Therapeutic target for alleviation of cognitive deficits

Recently, two drugs, Lecanemab (Leqembi®) and Donanemab (Kisunla™), got approved by the FDA to treat early Alzheimer's disease, including people living with mild cognitive impairment (Meshkat et al.) or mild dementia due to Alzheimer's disease who have confirmation of elevated beta-amyloid in the brain (Espay, Kepp, et al., 2024; Terao & Kodama, 2024). These two drugs dramatically reduced brain Aβ-positron emission tomography (PET) burden and demonstrated a highly significant, albeit clinically modest, delay of cognitive decline. The Lecanemab treatment is administered every two weeks through an IV, lasting about one hour for each infusion while Donanemab treatment is administered every four weeks through an IV, lasting about 30 minutes for each infusion (Daly et al., 2024; Høilund-Carlsen et al., 2024). However, these drugs are still not the perfect cure for AD. Fo r example, Donanemab is claimed to remove up to 86% of cerebral amyloid and produce 36% delay in cognitive impairment relative to placebo. Actually, these are quite little modifications and perhaps less than what cholinesterase inhibitor/memantine treatment allows. Furthermore, the "removal" of amyloid, depending on the lowered accumulation of amyloid-PET tracer, most certainly represents tissue damage associated to therapy. This would also line up with the little clinical impact, higher frequency of amyloid-related imaging abnormalities, and faster brain volume decrease in treated rather than placebo patients seen with these antibodies (Espay, Herrup, et al., 2024; Jin & Noble, 2024). Therefore, we still have to identify a more advanced therapy for AD with better clinical impact and total cure.

Except the above two treatments, the rest existing pharmacological interventions for AD mostly focus on alleviating symptoms rather than providing a definitive cure (See Table 2 for current FDA-approved medications).

These medicines aim to mitigate the advancement of cognitive impairments as well as behavioural and psychosocial symptoms of dementia. Four medications, namely donepezil, memantine, galantamine, and rivastigmine, have received approval for market distribution. These drugs can be categorised into two categories: anticholinesterase inhibitors and anti-glutaminergic. The administration of these therapies occurs via either the oral or transdermal route (Atri, 2019; Briggs et al., 2016).

Anticholinesterase inhibitors are pharmacological agents that have been specifically developed to enhance the concentration of acetylcholine in the brain. Acetylcholine is a crucial neurotransmitter involved in facilitating communication between select neurons and is also implicated in cognitive processes such as memory. The purpose of these treatments is to address the reported shortage of acetylcholine in the CNS of individuals with AD. Anti-glutaminergic is employed for the purpose of modulating glutamate levels by means of a non-competitive antagonist mechanism targeting NMDAR. Glutamate serves as a neurotransmitter implicated in cognitive processes such as learning and memory.

Drug therapies are employed with the aim of retarding the progression of the disease, achieving stabilisation or temporary enhancement of cognitive abilities, and managing behavioural abnormalities. While these treatments do not provide a cure, they nonetheless contribute to the preservation of autonomy and enhancement of the quality of life for individuals with AD and their carers. Nevertheless, the efficacy of these treatments is limited and short-lived, primarily addressing the manifestations of AD rather than its underlying aetiology (Cummings et al., 2018; Cummings et al., 2019; Fish et al., 2019; Scheltens et al., 2016). Drug therapy may have greater efficacy during the initial asymptomatic phase prior to the onset of neurodegenerative processes. There are additional factors that contribute to the limited efficacy of these treatments, one of which is the challenge of directing drugs to the brain. This is mostly due to the restricted passage of drugs from the circulation to the CNS through the blood-brain barrier (BBB)(Zenaro et al., 2017). Numerous medication trials in the context of AD encounter significant challenges due to permeability difficulties at the BBB.

As a result of this, the administration of a higher dosage becomes imperative, hence potentially augmenting the likelihood of secondary adverse effects (Abbott et al., 2010; Banks, 2012; Chakraborty et al., 2017). The blood-brain barrier (BBB) poses a significant obstacle in the delivery of CNS drugs, prompting the development of numerous ways to

overcome this difficulty (Banks, 2012). The efficacy of drugs may be diminished as a result of age-related alterations in neuronal membranes and membrane receptors, a factor that is not often taken into account in pre-clinical research. A recent study has demonstrated alterations in the microdomains of synaptosomes obtained from aged mice. These alterations have been discovered to increase their vulnerability to amyloid stress and hinder the neuroprotective characteristics of the ciliary neurotrophic factor (Colin et al., 2017).

Another potential constraint in the efficacy of treatments could arise from their delivery during the advanced stages of AD. For instance, scientific investigations involving mice with genetic abnormalities in the ADAD gene, which leads to the early and rapid buildup of amyloid plaques, have facilitated the evaluation of anti-amyloid immunisation as a potential method for eliminating these plaques (Poon et al., 2020)). Nevertheless, several human clinical trials employing this methodology have observed a reduction in amyloid load, albeit without any substantial clinical amelioration or attenuation of disease progression (Huang et al., 2020). Hence, it is plausible that the administration of medications occurs during a period when AD has already progressed to its severe stages, thereby diminishing their efficacy. The significance of prompt intervention is underscored, highlighting the imperative for improved identification of the initial phases of AD through the incorporation of supplementary biomarkers (Cummings et al., 2018). Undoubtedly, the expeditious and precise diagnosis ought to consider specific demographic groups that exhibit risk factors, such as individuals with a family history of the condition (including genetic variables like the ε4 allele) and those experiencing solitary memory problems. The focus of drug development has been directed on the inhibition of amyloid plaque formation, while it is imperative to investigate alternative targets for further advancements (Briggs et al., 2016).

The lack of efficacious therapeutic interventions and challenges in accurately detecting AD in its initial stages serve as compelling evidence for the necessity of implementing preventive and neuroprotective approaches (Klimova & Kuca, 2015; Passeri et al., 2022). At present, researchers are exploring pharmaceutical compounds that directly stimulate δ-$GABA_A$R and those that function as positive allosteric modulators. These compounds have the potential to treat a vast array of disorders, such as depression (Christensen et al., 2012), insomnia (Wafford & Ebert, 2006) , pain (Bonin et al., 2011) and cognitive dysfunction (Whissell et al., 2013; Wang et al., 2007). For example, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), which is an agonist that primarily targets the δ-$GABA_A$R, has been proven to improve discrimination memory in a mouse model(Whissell et al., 2013). Exploring δ-$GABA_A$R could potentially lead to the identification of novel targets for treating cognitive impairments and NPS.

3

1 *Table 2 Current FDA approved drugs to treat Alzheimer's diseases (National Institute On*
2 *Aging, 2023)*

FDA-approved medications to manage symptoms	
Brexpiprazole	Atypical antipsychotic. Treats agitation resulting from Alzheimer's. Possible side effects include common cold symptoms, dizziness, high blood sugar, and stroke
Donepezil	Acetylcholinesterase inhibitor. Treats symptoms of mild, moderate, and severe Alzheimer's by preventing the breakdown of acetylcholine in the brain. Possible side effects include nausea, vomiting, diarrhea, insomnia, muscle cramps, fatigue, and weight loss.
Galantamine	Cholinesterase inhibitor. Treats symptoms of mild to moderate Alzheimer's by preventing the breakdown of acetylcholine and stimulates nicotinic receptors to release more acetylcholine in the brain. Possible side effects include nausea, vomiting, diarrhea, decreased appetite, weight loss, dizziness, and headache.
Memantine	NMDA antagonist. Treats symptoms of moderate to severe Alzheimer's by blocking the toxic effects associated with excess glutamate and regulates glutamate activation. Possible side effects include dizziness, headache, diarrhea, constipation, and confusion.
Memantine and Donepezil	NMDA antagonist. Treats symptoms of moderate to severe Alzheimer's by blocking the toxic effects associated with excess glutamate and prevents the breakdown of acetylcholine in the brain. Possible side effects include headache, nausea, vomiting, diarrhea, dizziness, anorexia, and ecchymosis (small bruising from leaking blood vessels).
Rivastigmine	Cholinesterase inhibitor. Treats symptoms of mild, moderate, and severe Alzheimer's by preventing the breakdown of acetylcholine and butyrylcholine (a chemical similar to acetylcholine) in the brain. Possible side effects include nausea, vomiting, diarrhea, weight loss, indigestion, decreased appetite, anorexia, and muscle weakness.

FDA-approved medications to treat Alzheimer's	
Lecanemab	Disease-modifying immunotherapy. Treats mild cognitive impairment or mild Alzheimer's by removing abnormal beta-amyloid to help reduce the number of plaques in the brain. Possible side effects include brain swelling and bleeding, headache, cough, diarrhea, nausea, vomiting, fever, chills, body aches, fatigue, high blood pressure, low blood pressure, and low oxygen.
FDA Accelerated Approval to treat Alzheimer's	
Aducanumab	Disease-modifying immunotherapy. Treats mild cognitive impairment or mild Alzheimer's by removing abnormal beta-amyloid to help reduce the number of plaques in the brain. Possible side effects include brain swelling and bleeding, headache, dizziness, falls, diarrhea, and confusion.

1.9.2 Extrasynaptic δ-$GABA_A$Rs subunit as a potential therapeutic target to alleviate anxiety in AD

As mentioned in section 1.3, NSP symptoms are common in AD, one such condition is anxiety. In this section, I will be introducing the rationale for targeting δ-$GABA_A$Rs in alleviating anxiety in AD.

In general, there are numerous studies linking $GABA_A$Rs with mood disorders (Fogaça & Duman, 2019; B. Luscher et al., 2011). However, the therapies that target widespread $GABA_A$Rs can be problematic because of the unwanted side effects such as sedation, dependency and toxicity. One example is the use of diazepam, which could possibly cause sedation, fatigue, respiratory depression and cardiovasucular collapse (Edwards & Preuss, 2024). However, if we could design an anxiolytic that targets discrete, selective synaptic pathways, these unwanted side effects would be eliminated. The extrasynaptic $GABA_A$Rs offer the later scenario of interests are the δ-$GABA_A$Rs, which are involved in diverse physiological and pathophysiological processes, such as learning and memory; anxiety; stress; sleep; pain; seizures; psychiatric and neurodevelopmental disorders (Hines et al., 2012; Whissell et al., 2015). δ-$GABA_A$Rs have, therefore, garnered significant interest as potential pharmacological targets for numerous disorders, including postpartum depression (Melón et al., 2018; Meltzer-Brody et al., 2018), epilepsy

(Petersen et al., 1983) trauma, panic and anxiety disorders (Rasmusson et al., 2017) and memory deficits (Arslan, 2021).

The distinct function of the δ subunit in extra-synaptic $GABA_AR$s, a set of receptors accountable for tonic GABAergic inhibition, has sparked significant therapeutic and scientific attention. Nevertheless, the intricate characteristics of the δ subunit assembly and the scarcity of δ-selective ligands are the primary factors impeding advancements in pharmacological investigations of these receptors. Variable substances have been purported to exhibit selectivity towards the δ subunit. The chemicals THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) and gaboxadol are recognised for directly activating αβδ receptors with more effectiveness and strength compared to αβγ receptors. However, they do not differentiate between αβ and αβδ receptors(Brown et al., 2002; Storustovu & Ebert, 2006). Like THIP, anaesthetics and neurosteroids also exhibit stronger effects on $GABA_AR$s that contain the δ subunit. However, their activity is not influenced by the specific combination of subunits, hence these drugs are not classified as δ-selective. On the other hand, 4-chloro- N-(2-thiophen-2-ylimidazo[1,2-a] pyridin-3-yl) benzamide, which was discovered to be a substance that enhances the activity of α4/6βδ receptors, has only a minimal effect on αβγ receptors and does not have any effect on αβ $GABA_AR$s (Amr Ghit et al., 2021; Jensen et al., 2013).

One the main reasons for the lack of progress in finding medication in alleviating cognitive deficits or NPS is probably due to the lack of physiological mouse models that accurately recapitulates human AD. The next section documents the status of the mouse AD models that are currently available to the field and the necessity in generating advanced models.

1.10 Animal models of AD

Presently, no mouse model has been successful in the accurate human recapitulation of AD pathology and tauopathy. I will review the latest developments in physiologically relevant preclinical AD models and explain why two knock-in mouse models were selected and crossbred in my study. Although familial AD models represent ~5% of people suffering from AD, they share similarities to idiopathic cases and are commonly used to predict idiopathic cases.

1.10.1 The first generation of mouse model

Based on the Aβ cascade theory, various transgenic mouse models have been established to overproduce APP, thereby pushing the research, and mimicking the human condition. Transgenic mouse models which overproduced APP formed the first generation of AD mouse models. They used various promoters*, e.g.,* Thy 1, prion protein (PrP) and platelet-derived growth factor-β(BDGF-β). The APP is constructed variously among APP695, APP770 and minigenes lines (Sasaguri et al., 2017). The most frequently used model is the Swedish mutation (K670N/M671L). Mice of the Swedish mutation showed extracellular amyloid deposits in the brain, differing from amyloid plaques in humans. Numerous studies revealed cognitive decline prior to the formation of amyloid plaques. Moreover, they did not exhibit neurofibrillary tangles or neuron loss. Limitations of the first-generation models also include that the overproduction of APP causes overproduction of other amyloid fragments besides Aβ. Whether the functional effect is from Aβ, or other overproduced APP fragments is hard to discern (Saito et al., 2014).

1.10.2 Second generation: APP knock-in mice

To obviate the disadvantage of the AD first-generation mouse models, the APP knock-in strategy was applied to produce pathogenic Aβ without other overproduced APP fragments. Saito introduced 3 amino acids that differ between mice and humans (G676R, F681Y and H684R) to humanise the mouse Aβ sequence. Also, two FAD mutations (KM670/671NL: Swedish and I716F: Beyreuther/Iberian mutations) were introduced into the endogenous mouse APP gene (Saito et al., 2014). Mice with the NL-F mutations (APP^{NL-F}) produced a higher level of $Aβ_{42}$ and a higher $Aβ_{42}/Aβ_{40}$ ratio without affecting

the production of other APP fragments, providing a better replication of the clinical features of AD. A high level of $A\beta_{42}$ resulted in Aβ accumulation and enhanced neuroinflammation in microglia and astrocytes in the cerebral cortex and hippocampus from 6 months-old *App*$^{NL-F}$ KI mice. They were also found to exhibit a further cognitive decline in spatial memory and flexible learning depending on age and pathology (Masuda et al., 2016). However, the *App*$^{NL-F}$ KI mouse model still has limitations that do not exhibit tau pathology or neuron loss. Aβ pathology accounts for parts of the cognitive dysfunction but not all symptoms of AD. AD patients also show irreversible tauopathy neurodegeneration even in FAD mutations (Bateman et al., 2012).

1.10.3 Third generation: *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ and *App* $^{NL-F}$*/MAPT dKI* mice

Several studies have reported that no neurofibrillary tangles (NFT) are observed in App knock-in mice in their lifetime (Saito et al., 2014; Sasaguri et al., 2017). This could potentially occur because murine tau is not fit to produce NFT. The longest brain isoforms of tau in humans and mice show 89% amino acid identity. However, the human has tau 3R and 4R isoforms while mice have only the tau 3R isoform (Hernández et al., 2020). Saito has created a new form of mutant mouse in which all murine tau, including exons and axons, are replaced by humanised tau (MAPT KI mice) to circumvent the drawbacks. The MAPT KI mice are crossbred with single *APP*$^{NL-G-F}$ mice to produce the double KI mice (*APP*$^{NL-G-F}$*/MAPT dKI* mice), showing similar pathological and cognitive properties to single APP^{NL-G-F} mice. Human tau exhibits similar pathological and physiological effects as murine tau, meaning that humanised tau will not add artificial phenotypes in the mice.

Six-month-old *APP*$^{NL-G-F}$/MAPT dKI mice showed higher tau phosphorylation than WT and single MAPT KI mice. Tau proteins are present in dystrophic neuritis around Aβ plaques while no NFTs are found. Humanised MAPT genes do not affect Aβ deposition, neuroinflammation or memory in *APP*$^{NL-G-F}$ mice.

Although *App*$^{NL-F}$ mice display a less aggressive pathology than APP^{NL-F-G} mice, they still showed higher tau phosphorylation than single MAPT KI mice (Saito et al., 2019). To avoid the occurrence of premature tau pathology in mouse brains, *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ and *App* $^{NL-F}$*/MAPT dKI* mice are used to investigate Aβ and tau in this study. *App*$^{NL-F}$ */MAPT*$^{htau/wt}$ and *App* $^{NL\ F}$ */MAPT* dKI mice are produced by crossbreeding App$^{NL-F/NL-F}$ with MAPT^{htau} mice which has both murine tau and humanised mapt gene to produce 6

isoforms of tau. *App*$^{NL-F}$ KI mice only have murine tau with only 4 isoforms. App^{NL-F} /MAPT$^{htau/wt}$ has both humanised and murine tau and App $^{NL\ F}$ /MAPT dKI has humanised tau only. *App*$^{NL-F}$ */MAPT*$^{htau/wt}$ and *App* $^{NL\ F}$ */MAPT* dKI mice have better human-like characteristics in comparison to other animal models of AD currently in use. Table 3 summarises the current mouse models of AD (Zhong et al., 2024). Most AD mouse models, e.g.Tg2576, 5xFAD etc, showed the formation of Amyloid plaque before cognitive decline. This is similar to AD patients whose abeta plaque occurs decades before the cognitive impairment shown in Figure 1. The advantage of Compared to other existing AD mouse models, *App*$^{NL-F}$ */MAPT*$^{htau/wt}$ and *App* $^{NL\ F}$ */MAPT* dKI mice have the benefit of having both human APP and Tau features, and these two human genes do not overexpress to accelerate the progression of the disease. More details could be found in table 3.

Although these mice show mainly familiar AD progression, they can predict the features of idiopathic cases as they share similar characteristics. Thus, the new models will mimic the human AD brain more accurately and yield more reliable data.

3

1

2 Table 3. Summary of 14 commonly used AD mouse models (Zhong et al., 2024).

Name	Mutation	Phenotype/Pathology	Behavior	Additional
Tg2576	hAPP 695	Dense plaque (7–8 mo); major plaque deposition (11–13 mo) on parenchyma and vascular structures	Impairment of spatial and working memory (9–12 mo). Electrically evoked seizure (12–14 mo). Decrease of frequency of burrowing prior amyloid plaques (3 mo).	High lethality in certain genetic background. Lack of tau pathology.
TgCRND8	Double mutant: hAPP 695 (KM670/671 NL and V717F)	Aβ40 levels stabilized and Aβ42 increased (4–10 weeks). Amyloid deposition in the cerebral cortex (2–3 mo). Dense-cored plaques and neuritic pathology in the hippocampus, midbrain, brainstem and cerebellum (4–5 mo). Metabolic impairment, reduced NAA levels in hippocampus and cortex (2–3 mo) and dysregulation of myo-inositol levels througout mice aging.	Disrupt in sleep cycle. Metabolic disturbance.	Aggressive model. Lack of tau pathology. Shortened life span.
PS19	hMAPT (P301S) - mixed background	Tau seeding (1.5 mo). NFTs (6 mo). Neurodegeneration begins in hippocampus and entorhinal cortex (9 mo).	Impairment in memory and learning, limb weakness, hyperactivity (3 mo). Paralysis (7 mo)	No amyloid pathology. Shortened life span.

	hMAPT (P301S) - congenic background	NFTs (6 mo). Median lifespan of 11–15 mo.	Hyperactivity (3 mo). Altered pain perception and startle response (3 mo).	Less variability in pathologenesis compared to mixed background
rTg4510	hMAPT P301L crossed with a tTA allele	Pre-tangles develop (2.5 mo). Argryophilic tangle-like inclusions (4–5.5 mo). Robust tau hyperphosphorylation, neuronal loss and tangle formation (5.5 mo)	Decline in spatial memory function (4 mo). Hyperactivity and increased anxiety (7 mo)	Pathology restricted to the cortex and hippocampus. Robust tau expression and neurodegeneration. endogenouse mouse gene disruption
P301L	Human 4R/2N introduced to P301L mutation	NFTs without axonal dilations in brainstem and spinal cord (6 mo). Lifespan of 8–12 mo.	Impairments in passive avoidance test (5 mo) and object recognition test (9 mo). Motor deficits (7 mo).	Of younger mice, P301L mice may have better cognitive abilities compared to wt controls.
APP/PS1	Double mutant: hAPP695 (KM670/671 NL) and PS1 (delEx9)	Aβ deposits, microglial and astrocytic activation (4 mo). Amyloid plaques in hippocampus and cortex (9 mo). Modest neuronal loss adjacent to amyloid plaques and synaptic dysfunction (8–10 mo). Increase in Aβ40 and Aβ42 in hippocampal regions of Nrf2 KO mice and increase in microglial activation and an accumulation of endosomes and lysosomes.	Memory deficits (6 mo); Deficits in spatial navigation and learning (12 mo). Nest-building and burrowing (8–14 mo).	Lack of tau pathology. LTP impairment (8–10 mo) but no PPF deficits (8–9 mo). Hippocampal neuronal circuit dysfunction.

5xFAD	Five mutations: Human Swedish, London, Florida APP mutations in APP and M146L and L286V in PS1 genes - Tg6799, Tg 7092, Tg 7031	Amyloid aggregates (1.5 mo). Amyloid plaques (2 mo) in hippocampus and cortex. Neuroinflammation phenotypes with atrogliosis and microgliosis (2 mo). Progressive neuonral loss (6 mo). Dystrophic neurites plateued (8–12 mo). High vs medium vs low expression	Impaired spatial memory (4 mo). Motor impairments (9 mo). Reduced anxiety, increased hyperactivity (12 mo)	LTP deficits (12 mo). Lack of tau pathology. Molecular signatures are well aligned with human AD brains.
	5xFAD (C57BL6)	Amyloid plaques in subiculum and layer V pyramidal neurons (16 days). Intraneuronal plaques (6 weeks). Plaques in cortex, hippocampus, thalamus (2 mo) and spinal cord (3 mo). Thinner myelin sheathes (1 mo) and shorter axon calibers (2–3 mo). Loss of 40% of layer V pyramidal neurons (12 mo).	Impaired memory in cross-maze test and reduced anxiety in elevated plus maze (3–6 mo).	Lack of tau pathology. Aggressive onset of amyloid pathology.
	5xFAD (AD-BXD)	Varying amyloid pathology and cognitive impairment, which did not correlate.	Varying behavior and cognitive function. Impaired function due to age and existence of transgene.	Used to model the genetic variation in humans and to identify transcriptional networks protective against AD-related cognitive decline.
3xTg-AD	Triple mutant: hPS1 (M146V), hAPP (KM670/671 NL), and MAPT P301L	Extraceullular amyloid deposits in frontal cortext (6 mo); Plaques in hippocampus (12 mo). Aggregates of conformationally-altered and hyper-phosphorylated tau in hippocampus (12–15 mo).	Impairment with spatial learning and memory deficits (6 mo). Age depedent cognitive decline noticed at 6, 12, and 20 mo.	Intraneuronal Aβ immunoreactivity (3–4 mo). Lack of neuronal loss. Genetic drift observed within this model.

APP KI	APP NL	N/A	Increase of anxiogenic-like behavior (15 mo).	Lack of tau pathology and no decline in spatial learning and memory.
	APP GF	Initial Aβ deposition (4 mo); Aβ deposition in a much larger brain area than in APP NLF or APP NLGF mice (12 mo).	N/A	N/A
	APP NLGF	Cortical Aβ amyloidosis (2 mo) and saturated by 7 mo. Consistent subcortical amyloidosis (4 mo). Greater microgliosis and astrocytosis than NL-F mice (9 mo).	Decline in spatial learning but retained memory (8 mo). Anxiolytic-like behavior (15 mo). Hyper-reactivity to pain stimuli (15–18 mo).	N/A
	APP SAA	Amyloid deposition detectable (4 mo). Increase of total brain density of Aβ plaques with highest burden in the cortex and hippocampus (8 mo).	Robust hyperactivity (18 mo).	Female: more pronounced hyperactivity (8 mo).
	APP NL-F	High production of Aβ42 with the highest ratio of Aβ42/Aβ40. Initial deposition of Aβ (6 mo). Cortical amyloidosis (24 mo). Accumulation of microglia and activated astrocytes, and neuroinflammation near Aβ plaques.	Memory impairment (18 mo).	Provide a better frame for upstream factors that affect Aβ amyloidosis than other mutations.
Tau KI	Exons 1 to 14 of mMAPT replaced with hMAPT	Normal axonal localization of tau.	N/A	Little difference compared to WT in phenotype. Often crossed with other models. MAPT P290S KI developed murine tau aggregates.

	MAPT KI x APP NLGF	Faster spread of pathological tau (19 mo). Tau humanization did not affect A beta or neuroinflammation.	Tau humanization did not affect memory	More tau accumulation in the presence of amyloidosis. APP NLGF x MAPT P290S dKI mice demonstrated more tau inclusions than age matched MAPT P290S KI mice. dKI mice also demonstrated tau seeding abilities.
	5xFAD x MAPT KI	Tau humanization suggested to have a protective effect against AD. Seemed to offset LTP impairment compared to WT.	Decrease of anxiogenic-like behavior and better spatial learning compared to 5xFAD	Enrichment in lysosome, phagocytosis,and ocidative phosphorylation by GSEA compared to 5xFAD and human co-expression modules.
APOE KI	Target replacement ApoE KI	E4FAD accumulation of Aβ42, tau hyperphosphorylation (1–4 mo), neuronal loss, deterioration of BBB, and reduced cerebral blood flow compared to E3FAD mice.	Female APOE4 KI mice have significant deficits in learning and memory. E4FAD mice developed hippocampal-associated memory deficits and had a substantial drop in nest construction scores compared to E3FAD mice.	Female: E3FAD and E4FAD have significantly higher Aβ42 and Aβ40 levels than male counterparts. Female E4FAD more deficits in learning and memory. Other deficits such as phospholipid and cholesterol dysregulation, microglial dysfunction, neuroinflammation, and taupathy-related neurodegeneration.

	Floxed APOE KI	APOE protein found in astrocytes but not in reactive Iba-1 positive microglia.	N/A	Typically used to cross with other transgenic models like APP/PS1 mice or PS19 mice. Overall cerebral accumulation of amyloid plaques in APOE4 KI mice crossed with APP/PS1 mice was not affected. PS19-E4 crossed mice demonstrated higher degree of neurodegeneration.
	APOE KI: JAX	Female APOE4 KI JAX mice had lower plasma Aβ42 and a decreased Aβ42/40 ratio. However, there were no differences between APOE4 and APOE3 KI mice. Aβ40 levels did not differ regardless of APOE genotype or sex.	Locomotor activity, motor coordination, and working memory tested by open field, rotarod, and Y-maze tests, respectively were similar between APOE4 KI and control mice with an age-depedent decline (2 mo and 12 mo).	It is suggested that there are higher levels of aggregate-prone Aβ42 in the brain in female APOE4 KI mice compared to their male counterparts.

TREM2 KO	Del exons 3 and 4	Reduced microglial numbers and size, decreased myelin repair. Prolonged microgliosis, impaired cholesterol transport.	Decreased performance on motor coordination tests (12 mo) when fed with CPZ	No apparent neurological phenotypes except for impaired immune response and altered transcriptome. TREM2 deficiency increased early-stage plaque growth, but not overall plaque deposition in an APP/PS1 dE9 mouse model with human APOE3 or APOE4.
	TREM2 KO x 5xFAD; TREM2 R47H x 5xFAD	High levels of amyloid in hippocampus and reduced IBA1 expression near plaques (8 mo).	N/A	Microglia are less viable than TREM2 + / + 5xFAD mice with reduced levels of CSF-1. Decreased TREM2 shedding with imparied downstream signaling in TREM2 R47H x5xFAD mice.
	TREM2 KO x PS19	Less neurodegeneration and microgliosis compared to PS19 mice. No differences in p-tau levels and tau solubility.	N/A	Decreased inflammatory markers. Suggests that severe microglia response can contribute to neurodegeneration.
hTREM2 KI	TREM2 CV and TREM2 R47H by Song et al.	Impaired lipid sensing and DAM responses to amyloid. Impaired soluble TREM2 cell-surface interactions with decreased TREM2 shedding noted on neurons.	N/A	Mice developed less brain atrophy and synaptic loss with diminished microglial reactivity and phagocytosis when compared to PS19-TREM2(CV) mice.

	TREM2 R47H and APPPS21-TREM2 + /R47H by Cheng-Hathaway et al.	Attenuated microglial response to amyloid with reduced amounts of dense-core plaques. TREM2 R47H mice with cuprizone-induced neuro-inflammation demonstrated age-depedent impairments in microglial interaction with plaques (4 mo), and LTP and synaptic loss (12 mo).	N/A	Attenuated microglial response to amyloid with increased neurite dystrophy.
	LOAD1, LOAD2 and others by JAX	No amyloid plaques or other AD hallmark changes observed in LOAD1 mouse models even at 24 months of age. After high fat diet (HFD) treatment, LOAD2 mice demonstrated neuronal loss and elevated brain Aβ42 (16 mo).	No cognitive deficits observed in LOAD1 mouse models even at 24 months of age. LOAD2 mice on HFD exhibited behavioral deficits.	Reduction in brain TREM2 protein levels and changes in circulating cytokine levels. Regional changes in glycolysis and vascular perfusion. Female LOAD1 mice showed increased risks of mortality and glycolysis was significantly altered (4 mo-12 mo).
hTREM2 Tg	BAC TREM2 Tg	Reduced mamyloid plaques with associated gene signature changes including dampened damage-associated microglial gene expression and up-regulated neuronal gene expression.	Cognitive performance improved in BAC hTREM2 Tg x 5xFAD mice compared to 5xFAD mice with increased phagocytic microglia and reduced neurite dystrophy seen.	N/A

1.11 Aims and hypothesises

The overall aim of this study is to investigate the impact tauopathy when introduced to an App mouse model of AD by cross breeding two different mouse models of AD, the following details the key aims and objectives:

1) Investigate whether the humanised MAPT tau gene would influence the rate of cognitive decline and anxiety levels by performing four different behaviour experiments, including novel object recognition (NOR), T-maze tests, open field test and light dark chamber.

Hypothesis*:* Heterozygous mouse models of AD containing the App and tau genes (*App* $^{NL-F}$*/MAPT* $^{htau/wt}$ and *App* $^{NL-F}$ */MAPT* dKI) will show worse cognitive deficits and anxiety levels compared with WT counterparts.

2) Investigate whether the humanised MAPT tau combined with App in a mouse model of AD would exert higher levels of pathophysiology by investigating the levels of A β, tau correlated with changes in CD68 and GFAP, markers of neuroinflammation.

Hypothesis: The humanised MAPT tau gene would exaggerate neuroinflammation, Aβ accumulation and tau hyperphosphorylation in *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ and *App* $^{NL-F}$ */MAPT* dKI compared to *App* $^{NL-F}$ KI mice.

3) Investigate the alteration of 3 sub-classes of interneurons that have specific roles in regulating brain activity in these 3 AD models and compare this in post-mortem human brain tissue in healthy human and those with confirmed cases of AD. We chose to study PV and two interneuron sub-types for study because they all have unique roles in their local circuits as described above. PV- expressing interneurons are important for fast control of their postsynaptic partners, while CCK- and CR-expressing interneurons have been shown to be major modulators of inhibition in hippocampal regions of the brain in which we are interested, however, CCK-expressing interneurons can modulate both excitatory and inhibitory cells, but CR expressing interneurons only contact each other and other inhibitory cells, preferably somatostatin containing and CCK expressing interneurons.

Hypothesis: *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ and *App* $^{NL-F}$ */MAPT* dKI mice will have reduced amounts of GABAergic interneurons expressing cholecystokinin and parvalbumin and calretinin compared to WT mice.

4) This study focuses on testing a novel compound (delta-specific compound 2; DS2, 4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide) targeting the extrasynaptic delta-subunit of the $GABA_A$ receptor (δ-$GABA_AR$) via classical

benzodiazepine-based anxiolytic distinct mechanisms, and thereby providing an alternative potential therapeutic target for anxiety disorders in patients with AD.

Hypothesis: The δ-$GABA_AR$-specific PAM, DS2 would lower levels of anxiety and normalised the AD hallmarks e.g. astrocyte, glial cells, Aβ and hyperphosphorylated tau in App^{NL-F} KI mice.

2 General Methods

2.1 Animal model of AD

The study was based on procedures carried out as per the British Home Office regulations of the Animal Scientific Procedure Act 1986, under the project licence PPL : 7007558, PPL: P1ADA633A (awarded in March 2018) and PPL: PP7588442(awarded 2023) held by the principal investigator, Dr. Afia B. Ali. All procedures were approved by both internal and external UCL ethics committees, and in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010).

We are committed to the principles of the three "R's" (Replace, Reduce, Refine) and have performed all experiments in this project according to these principles. Where possible, we have implemented randomisation, blinded experiments and analysis, including statistical power calculations once experimental variability was verified to estimate the appropriate numbers of animals.

This study was conducted over ~5 years and used 200 male mice (disease models and WT mice). Specifically, ~40 mice were used at the following time windows (6-9 months, 12-16 months, and 18-22 months). The authors acknowledge that females are disproportionately affected at a higher rate by AD and that future studies should include experimental designs including female mice.

App^{NL-F} KI mice and *MAPT*$^{htau/htau}$ mice obtained from RIKEN were crossed with WT C57BL/6J mice at the School of Pharmacy, ordered from Charles River, and the heterozygous animals that resulted were further used for breeding to yield homozygous WT, *App*$^{NL-F}$ KI mice, *App* $^{NL-F}$ */MAPT* dKI mice and heterozygous *App* $^{NL-F}$ */MAPT* $^{htau/wt}$. The animals were genotyped centrally at UCL, using tissue from the ear. The following primers were used for genotyping via polymerase chain reaction, as per the original publication (Hashimoto et al., 2019; Saito et al., 2014) :

5-ATCTCGGAAGTGAAGATG-3, 5-TGTAGATGAGAACTTAAC-3, 5-ATCTCGGAAGTGAATCTA-3, 5-CGTATAATGTATGCTATACGAAG-3 and Fwt5: 5‘’GTCAGATCACTAGACTCAGC-3′, Rwt5: 5′-CTGTGCTCCACTGTGACTGG-3′ and Rhm5: 5′-CTGCTTGAGTTATCTTGGCC-3′.

The animals were housed in cages of up to 5 inhabitants and given ad-libitum access to food and water. The day: night cycle was 12 hours: 12 hours. 200 adult male animals were used in this study, the youngest age being 6 months and the oldest 22 months. No females were used in this study. AD mice and WT mice were grouped into the following

age brackets: 6-9 months, 12-16 months and 18-22 months. The grouping was decided based on previous observations in the laboratory which showed that no differences were observed within the respective age groups, as well as on comparable studies from speciality literature.

All rodents that went through experimental procedures were thoroughly monitored for signs of discomfort. Their weight and general health were inspected and recorded twice daily before starting the experiments, during experiments, as well as up to a week afterwards to ensure there was no lasting harm to their health.

Animals which underwent one type of cognitive test were generally not utilized in another test. If it was required, a break of minimum one month was taken between tests to allow for the animals to recover their naive state. The animal welfare signs checked included: maintenance of weight levels, general grooming or mouse grimace scale (Langford et al., 2010; Wolfensohn & Lloyd, 2013).

2.2 Tissue preparation

Animals were anaesthetised with 60 mg/kg pentobarbitone, which was administered intraperitoneally prior to each transcardial perfusion. Pedal and tail pinch reflexes were monitored, as well as depth and pattern of respiration. The level of anaesthesia was determined to be adequate when there was no response to the pedal pinch reflex and when the breathing became shallow. Then, an incision was made through the abdomen of the animal, the skin pulled back to expose the thorax, the diaphragm cut and the rib cage removed to allow access to the heart for perfusion. The animals were perfused transcardially with ice-cold artificial cerebrospinal fluid (ACSF) with sucrose, containing the following in mM: 248 sucrose, 3.3 KCl,1.4 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25.5 $NaHCO_3$, and 15 glucose, which was bubbled with 95% O_2 and 5% CO_2. This step in the procedure helped to preserve the structural integrity of the brain tissue and it ensured exsanguination to eliminate peroxidase-containing red blood cells, which could interfere with histochemical experiments. To perfuse an animal, a 23G butterfly needle (Greiner Bio-One) was inserted into the left ventricle of the heart and the peristalic pump (Waton-Marlow, 502s, Cornwall,UK) circulating blood at 5 mL/minute was turned on. The right atrium was cut,and the ice-cold sucrose solution was allowed to perfuse for approximately 10-15 seconds, until the fluid coming out of the animal showed no traces of blood. After perfusion, the animal was decapitated, an incision was made on the head along the anterior-posterior axis to reveal the skull and snips were made with fine scissors in the

skull plates to allow for pulling of the plates away from the brain without causing any damage to the soft tissue. The brain was collected and briefly placed in an ice-cold solution of ACSF containing the following (in mM):121 NaCl, 2.5 KCl, 1.3 NaH2PO4, 2 CaCl2, 1 MgCl2, 20 glucose, and 26 NaHCO3, bubbled with with 95% O2 and 5% CO2. After the brief immersion, the tissue was distributed among experiments and experimenters accordingly, so as to be mindful of the ”Reduction” principle of the ”3Rs”.

2.3 Mouse brain fixation

50 ml 0.1M phosphate buffer was heated with a magnetic stirrer in a beaker to 60 degrees centigrade. 2 g paraformaldehyde was added into the beaker and dissolved with the temperature kept around 60 °C. 50 µl glutaraldehyde was added into the beaker after the solution cooled down to room temperature. The fixative solution was stored in a fridge once made. Brains were kept in the fixative solution overnight after perfusion. The fixative solution was replaced by phosphate buffer before sectioning.

2.4 Mouse brain slice sectioning

Hippocampal coronal slices were sectioned at 70 _m thickness using a vibratome (Leica, Munich, Germany) from the same region of CA1 in reference to mouse brain atlases (Allen mouse brain atlas). Figure 6 illustrates example representative whole brain sections that have been sectioned and imaged for analysis for CA1.The brain was sliced coronally using a vibratome (Vibroslice, Camden Instruments, Loughborough, UK). The thickness of the mouse brain sections was 70 µm. Each section contained the hippocampal formation (figure 6). After collecting the sections, they were placed in 24-well plates containing 0.1 M PB.

A B

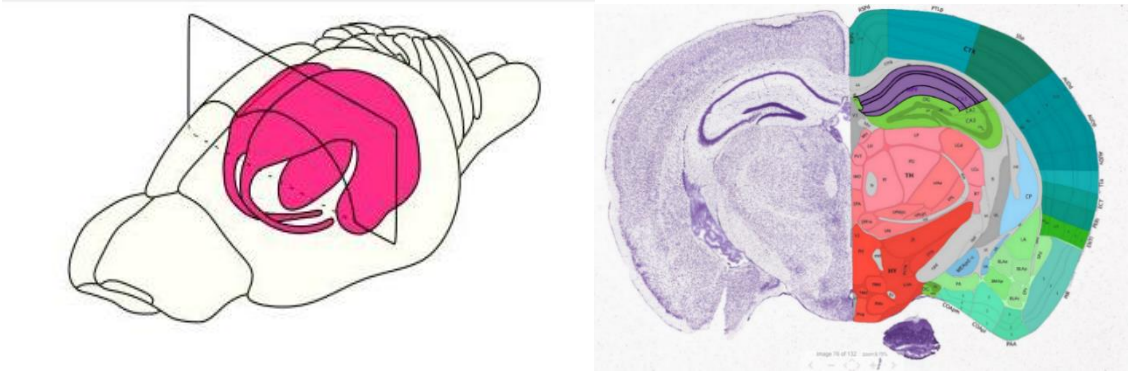


Figure 6. A) Coronal dissection of mouse brain (Temido-Ferreira et al., 2019) B) CA1 region highlighted in the coronal mice brain section from http://atlas.brain-map.org. Mouse brains were cut in the coronal plane. The dark purple area is CA1 which is the central area of interest in this study.

2.5 Human Brain Tissue

A total of 14 hippocampal post-mortem brain tissue sections from 7 AD patients and 7 age-matched control individuals were obtained from Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology according to the Human Tissue Act (HTA) 2004 and under the HTA license (see Table 4 for details). Ethical approval was obtained from the local research ethics committee for the National Hospital for Neurology and Neurosurgery.

3

1 Table 4. List of the human case studies used for tissue samples along with relevant details.

Cases ID	Group	Regions Used	Age (years)	Sex	Post-mortem Delay (hours)	Brain Weight (g)	Braak Staging	CERAD Score	Thal Staging
1	AD	Hippocampal region	67	Male	35.27	1,223	Braak 6	CERAD 3	Thal 5
2	AD	Hippocampal region	55	Female	47.50	1,100	Braak 6	Frequent	---------
3	AD	Hippocampal region	90	Male	89	1,200	Braak 4	CERAD 0	Thal 1
4	AD	Hippocampal region	86	Male	96.1	1,203	Braak 6	CERAD 3	Thal 5
5	AD	Hippocampal region	68	Male	70.05	1,522	Braak 6	---------	Thal 5
6	AD	Hippocampal region	69	Male	35.04	891	Braak 6	Frequent	Thal 5
7	AD	Hippocampal region	88	Male	58.1	1,084	Braak 6	---------	Thal 5
8	Control	Hippocampal region	101	Male	60.35	1,450	Braak 1	CERAD 0	---------
9	Control	Hippocampal region	79	Male	105.5	1,355	Braak 2	---------	---------
10	Control	Hippocampal region	88	Male	96	1,240	Braak 2	CERAD 1	Thal 3
11	Control	Hippocampal region	71	Female	76	1,214	Braak 3	CERAD 2	Thal 2
12	Control	Hippocampal region	86	Female	120	1,234	Braak 2	---------	---------
13	Control	Hippocampal region	80	Female	49.10	1,242	Braak 2	---------	---------
14	Control	Hippocampal region	83	Male	105.00	1,244	Braak 4	CERAD 2	Thal 3

2

2.6 Immunoperoxidase(IP) protocol and analysis

Free-floating sections in 24-well plates were permeabilised with 0.3% TBS-T. They were subsequently incubated in 1% hydrogen peroxide aqueous solution for 30 minutes. The slices were then washed with 0.3% TBS-T before being blocked with blocking solution for 1 hour at room temperature. Sections were then incubated with the diluted primary antibodies (listed in Table 5) at 4°C. After 24-hour incubation, the sections were washed with 0.3% TBS-T. They were then incubated in appropriate secondary antibody dilutions (listed in Table 5) in a blocking solution for 2 hours at room temperature. After being washed three times with 0.3% TBS-T and three washes with PBS, the sections were incubated in ABC solution for 2 hours at room temperature. The sections were then washed three times with PBS and twice with a Tris buffer. After incubating in DAB for 20 min, sections were put together with 0.2% H_2O_2. Sections were washed with a Tris buffer immediately at the appearance of a dark colour. The sections were subsequently washed three times with a Tris Buffer. The sections were then mounted on glass slides and left to dry. The sections were then washed with graded alcohol followed by histoclear washes. The slices were ultimately covered by coverslips with D.P.X.

Sections treated for immunoperoxidase were imaged at x10 and x20 objectives using a light microscope (Leica, Munich, Germany). The sub-brain regions of interest that were imaged include CA1 and DG. Quantification from immunoperoxidase-stained images aimed to quantify the number of markers by measuring the density/mm3 using ImageJ software (1.5.3 version). DAB-stained pictures were taken under x20 magnification using a light microscope. Pictures were processed by colour deconvolution and “H-DAB” to prevent staining artifacts and improve quantification. ROIs were carefully selected and were located using the manual joystick through the x20 objective lens by systematically searching the slice and consistently evaluating the location with reference to an appropriate mouse atlas for all the brain regions studied including CA1 (stratum oriens, pyramidal layer, and stratum radiatum). The numbers of stained markers within each ROI were counted using the cell counter function in ImageJ, and the total number obtained per region was divided by the volume of the section to determine cell density in cells/mm^3.

When intensity levels of images processed with immunoperoxidase protocols were measured (e.g. to obtain levels of expression in a whole section, rather than an ROI), the ‘Measure’ function from Fiji was used on the 8-bit images so as to obtain the mean of Integrated Density, which was then normalized by the slice volume. An overall average was obtained per animal and then a group average for cohort and age bracket, respectively.

Furthermore, primary antibodies for Aβ were raised in rabbits, and PV-expressing interneurons were raised in mice and used in mouse tissue samples. Therefore, the control experiments are shown in S1, evidencing specific binding related to these antibodies.

Table 5 List of Antibodies used in IP and IF experiments.

Primary Antibody				
Antibody name	**Antibody target**	**Manufacturer**	**Host**	**Dilution**
Immunofluorescence				
Anti-$GABA_A$ Delta	N-terminus of δ-$GABA_A$ Receptor	Novus Biologicals	Rabbit	1:500
Anti-Parvalbumin	PV+ Interneurons	Thermo Fisher Scientific	Mouse	1:1000
Monoclonal anti-calretinin	CR	Swant	Goat	1:1000
Neuropeptide Y	NPY	Novus	Goat	1:1000
Wisteria floribunda agglutinin (WFA)	N-acetylgalactosamine	Vector Laboratories	-	1:400
Anti-GAD67	Gutamic acid decarboxylase 67kDa	Merck Millipore	Mouse	1:1000
Anti-GABA transporter3	GAT3	Abcam	Rabbit	1:100
Immunoperoxidase				
Anti-$GABA_A$ Delta	N-terminus of δ-$GABA_A$ Receptor	Novus Biologicals	Rabbit	1:500
Anti-Parvalbumin	PV+ Interneurons	Thermo Fisher Scientific	Mouse	1:5000
Beta amyloid Polyclonal	C-terminal region of APP695	Thermo Fisher Scientific	Rabbit	1:2000
CD68 Monoclonal	Mouse Macrosialin	Bio-Rad	Rat	1:3000
GFAP Monoclonal	Glial fibrillary acidic protein	Agilent	Mouse	1:3000
Phospho-Tau Monoclonal (AT8)	Human PHF Tau (Ser202/Thr205)	Thermo Fisher Scientific	Mouse	1:3000
Anti-CCK	Cholecystokinin	UCLA	Rabbit	1:1000
Secondary Antibody				
Immunofluorescence				

Alexa 488		Abcam	Goat	1:500
Alexa 488		Abcam	Rabbit	1:500
Alexa 555, streptavidin		Thermo Fisher Scientific	Various	1:500
Alexa 568		Abcam	Rat	1:500
Alexa 568		Abcam	Rabbit	1:500
Alexa 647		Abcam	Mouse	1:500
Alexa 647		Abcam	Rabbit	1:500
Texas Red		Thermo Fisher Scientific	Rabbit	1:750
DAPI		Sigma-Aldrich	Multiple	1:1000
FITC		Sigma-Aldrich	Mouse	1:200
Immunoperoxidase				
Biotinylated Anti-Rabbit		Vector Laboratories	Goat	1:500
Biotinylated Anti-Goat		Vector Laboratories	House	1:500
Biotinylated Anti-Mice		Vector Laboratories	Goat	1:500

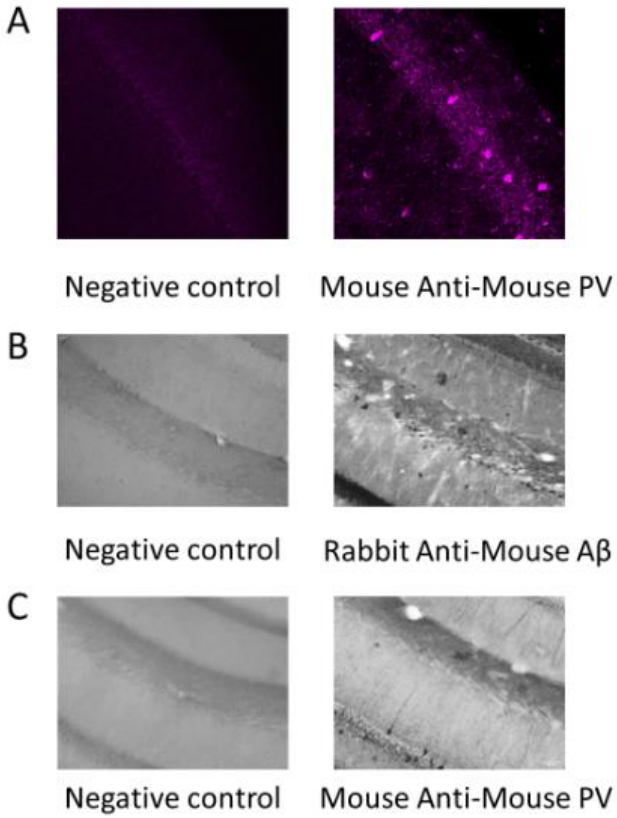
2.7 Immunofluorescence (IF) protocol and analysis

Brain slices obtained as per section 2.5 were placed into 24-well plates divided by age and genotype. To make the tissue readily permeable by the antibody solution, the slices were washed in 0.3% Triton X-100 detergent diluted in Tris Buffer Saline, (TBS-T 0.3%) in three 10-minute changes. The slices were incubated in 0.3% H_2O_2 at room temperature for 30 minutes as a blocking step to eliminate residual blood traces. After the H_2O_2 incubation, the three washing steps with TBS-T 0.3% were repeated, then slices were placed for one hour at room temperature in blocking serum (20%). This was followed by incubation in a TBS-T 0.5% solution containing primary antibody. For primary antibodies used in each experiment, please see Table 5. After 72 hours in primary antibody at 4°C, the slices were washed in TBS-T 0.3% twice for 10 minutes and once in TBS-T 0.5% for 10 minutes, then incubated for 3 hours at room temperature in TBS-T 0.5% containing secondary fluorophore antibodies (see Table 5) and 0.05% blocking serum. The plates were wrapped in aluminium foil to avoid light exposure and potential bleaching of the fluorophores. Next, the slices were washed in TBS-T 0.5% three times for 10 minutes. If staining of the nuclei was required, an 8-minute incubation with 4',6-diamidino-2-phenylindole (DAPI) was added in between the second and third wash. After washes, the slices were mounted on plain glass slides using a paintbrush and in dim light to minimise light exposure that could cause bleaching of the fluorophore. The excess fluid surrounding the slices was absorbed using filter paper, then the slides were placed in a dark drawer for a short period to dry. Antifade mouting medium Vectashield (Vector Laboratories) was applied on top of the slices and a cover slip gently lowered over it. Excess medium was removed using filter paper. After a brief drying time, the sides of the coverslip were sealed with transparent nailpolish to secure it in place. The control experiments are shown in S1, evidencing specific binding related to these antibodies. The sections were incubated in primary antibody (Table 5) for 48 hours.

From each brain section, an average of two Z-stacks at ×20 and ×63 objective were taken using the Zeiss LSM880 confocal microscope in unison with the Zeiss Zen Black imaging software from the DG and CA1. Regions of interest (ROI), CA1 (including stratum oriens, stratum pyramidale and stratum lacunosum) and DG (including the molecular layer, granule cell layer and polymorphic layer), were located using the manual joystick through the ×20 objective lens by systematically searching the slice and consistent evaluation of location in reference to appropriate mouse and human atlases. Z stack images were taken at a resolution of 1024x1024 pixels with 12–14 Z steps through the depth of the slice and with application of appropriate filters to complement secondary antibody fluorescence: DAPI (405λm), FITC/Alexa 488 (488λm), Texas Red/Alexa 568 (561/594λm) and Alexa

647 (640λm).

Single-blinded image analysis was undertaken using the ImageJ software using an automated macro. The Z-stack images were split into their constituent colour channels. Following this, all markers in a given image were selected through the Huang auto thresholding method in the ImageJ software, to demarcate signal from background and produce the ROI (Huang & Wang, 1995). The Coloc2 plugin was then used to obtain Pearson's correlation coefficient R as a measure of colocalization between the channels corresponding to the ROIs and to the biomarker of interest. Integrated Density (mean intensity of fluorescence multiplied by area) was calculated for each ROI in the x20 Z-stack images same as mentioned in 2.6.



S1. Immunofluorescence and DAB staining without primary antibodies were employed as the negative control for mouse antibodies used on mouse tissue. Negative control did not show positive staining. Scale bar: 200 µm, x20 objective lens used for immunofluorescence and imunoperoxidase staining examples shown here for anti-mouse PV (A), anti-phospho-tau (B) and anti-mouse PV (C).

2.8 Animal behaviour experiments

The animals were chosen randomly, and all experiments were performed and analysed double blinded, where mice IDs were randomly allocated by our animal house staff before the experimenter continued with the dosing and/or behavioural experiments. Therefore, the experimenters were unaware of the genotype or the treatment of each individual mouse.

All experimental animals in this study were taken care of by the same biological service unit (BSU) personnel as well as the experimenter. To minimise the impact of the environmental cues or the experimenter when conducting behavioural experiments, the mice were regularly handled by the same experimenter from 4 months onwards in the same BSU. The experimenter would routinely weigh the mice as well as perform health checks using the Grimace Scale (Langford et al., 2010).

2.8.1 Novel location and object recognition tests to investigate cognition deficits

The novel object and location recognition tests are standard behaviour tests to investigate learning and memory functions in mice. A novel objective recognition test was first used on rats by Ennaceur and Delacour in 1988 (Ennaceur & Delacour, 1988). These two tests include 3 processes: habituation, training, and testing. In our experiment, the training session involved the exploration of two visually identical objects (shown in figure 7). One object was then changed to a new location, accounting for the novel location recognition test. The last session included novel object recognition, in which a new object replaced one of the two identical objects.

To obtain the endpoint measurements from NOR and NOL, the mean discrimination index (%) was calculated using the formula:

Discrimination index = (time spent in novel location or object – time spent in the old location of object)/total time in the arena

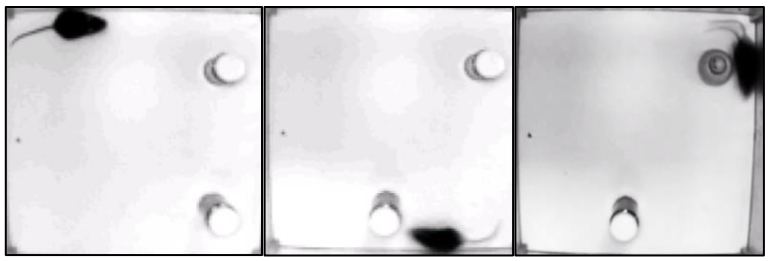


Figure 7. Apparatus of novel object and location recognition test (open field box: 40 cm X 40 cm X 40 cm).

2.8.2 T-maze experimental paradigms to investigate cognitive deficits

A T-arm maze was designed using Tinkercad and Inkscape and laser-cut from acrylic sheets (Figure 8). The design was based on the diagram and dimensions from (Denninger et al., 2018), but was adapted to create a maze easy to disassemble and store. The maze was brown and the inner walls were sanded to diminish reflection from the acrylic sheets. The maze had an end arm and two" goal" arms that spread left and right. Each of the goal arms had a small white well made of plastic at the end filled with a drop of condensed milk (Essential Waitrose and Partners Condensed Milk), diluted 1:1 in water.

Before the experiment, the animals had a habituation process with the experimenter for several days, until they reached a state of relaxation and readily engaged in interaction. In general, the mice were effectively acclimated and were running within a matter of seconds. In addition, the mice were given tiny amounts of condensed milk in their cage over a period of several days in order to familiarise them with it and prevent hyponeophagia. Prior to the experiment, the maze was sterilised using ethanol, and this process was repeated after each animal run. Paper cues were positioned on the walls of the room, equally distant from the walls of the maze. A modest quantity of approximately 2 mL of condensed milk was deposited into the food wells located at the terminus of the two target arms. Newly cut wood chips were spread on the floor of the maze. Once each animal completed its runs, the wood chipping and food reward were replenished, and the maze was cleaned.

The mice were placed in the starting area with the door shut. The removal of the door gave the mice a choice to go either right or left. The door was shut again after the mice fully entered one of the mazes and were allowed to rest for 20 s. Then, the mice were put back at the starting position to make second choices. Baiting the arms with milk is not

necessary to carry out the experiment but acts as another incentive for the mouse to follow its natural instincts and alternate its choices. Similar protocols were performed using the same apparatus with delays of 30 s and 60 s between each trial.

Each cohort of mice was aged and re-tested for the 3 different age groups studied in the NOR/NOL and T-maze tests.

Endpoint measurements for the T-maze test were obtained as:

An alternation rate (%)=correct pairs of choices/total pairs of choices

In a given experiment, the correct pair of choices would be one left and one right turn in the two runs of each trial.

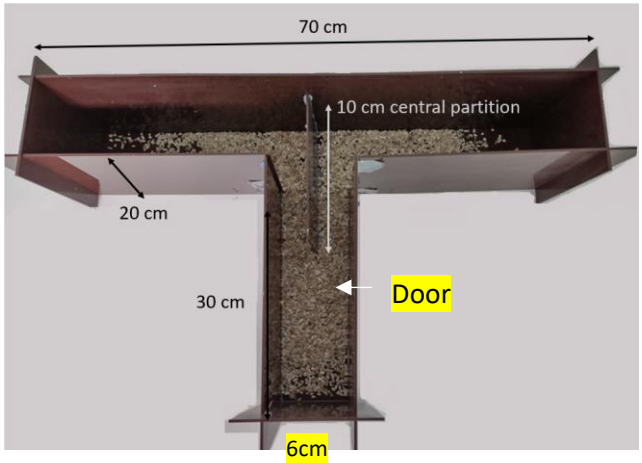


Figure 8. Illustration of T-arm maze apparatus. It is composed of two chambers and one starting position filled with woodchips. The mice were put at the starting position and allowed to choose one of the chambers to explore. Doors were shut after the mice chose one direction.

2.8.3 Open field test to test anxiety level

Open field test was a widely used test to investigate anxiety in mice. Mice was placed inside the 40cmx40cmx40cm box for 10min (shown in figure 9). A 20cmx20cm square was marked as centre zone and rest is marked as peripheral zone. Experiments were recorded by camera with ANYMAZE. Higher percentage of time spent in centre zone shows less anxiety level in mice.

For open arena tests, the data endpoint was taken as: time spent in the peripherial zone / total time.

We aim to minimise chances of bias, hence the central area is significantly smaller than the periphery area. The camera may not accurately identify which zone the mice are in when the central zone's limits are too near to the outer wall.

Open arena exploration

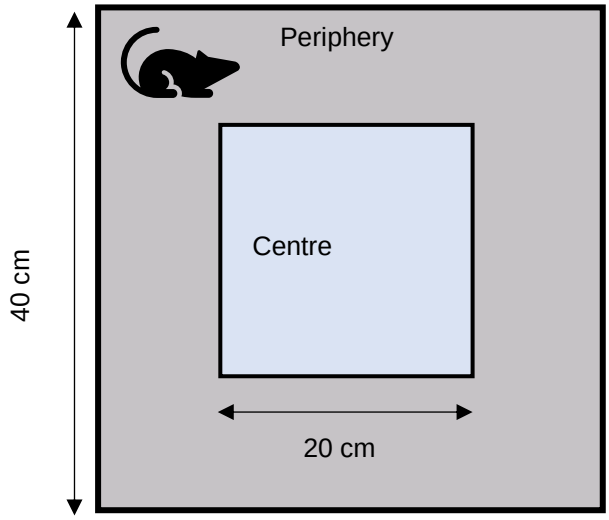


Figure 9 Illustration of open field test. It is composed with 40cmx40cmx40cm box. Mice was put inside the box for 10min.

2.8.4 Light dark chamber to investigate the anxiety level in WT and AD mouse models

The dark chamber consisted of a 40cmx40cmx40cm box, which was equally separated into two equal-sized zones: light and dark zone (shown in Figure 10). The dark zone was covered by a non-transparent board to block light entering the zone. Mice was placed at the light zone when the experiments started. Mice are allowed to move freely between the two chambers for 10 min. The camera would record 10 minutes of the experiments and analyse (ANYMAZE) the time percentage mice spent in the light zone. Mice with lower anxiety levels should spend more time in exploring light zone(Takao & Miyakawa, 2006).

The endpoint measurement for light dark chamber tests, was taken as: time spent in the dark zone/total time

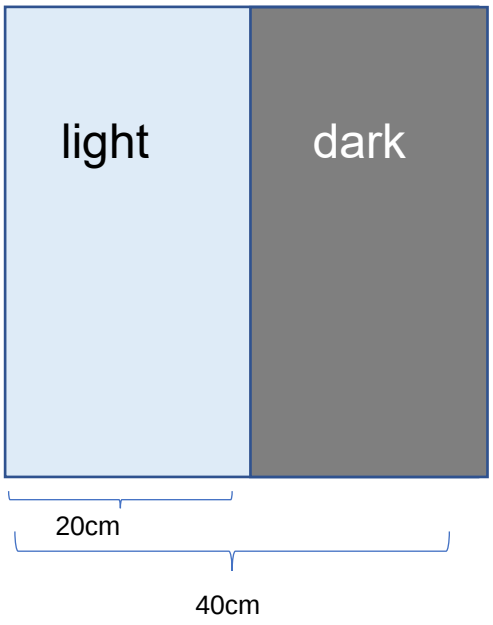


Figure 10 Illustration of Light dark chamber. It is consisted with a 40cmx40cmx40cm box which is equally separated to two area: light zone and dark zone. Dark zone is covered by non-transparent board to block the lights entering the zone.

2.9 Mice dosage procedure

DS2 are highly selective for α4β3δ receptors (EC50 = 142 nM, in vitro). Evidence from computational modelling, site-directed mutagenesis studies show that the functional selectivity of DS2 is on specific binding pockets on the transmembrane domain of α4β1δ (Falk-Petersen et al., 2021), providing extrasynaptic $GABA_AR$s with an advantage of low affinity to benzodiazepine in the extracellular domain α(+)γ(-) interface. Therefore, in vivo dosing experiments mice were treated with DS2 drugs (Tocris, UK), dissolved in DMSO (ThermoFisher scientific, USA), 1, 2, and 4 mg/kg, Intraperitoneal injection (ip) or vehicle. The stock solution of DS2 was prepared in a solution consisting of 10% DMSO (ThermoFisher scientific, USA) and 90% saline (G-BIOSCIENCES, USA). The vechile solution was made of using 10% DMSO (ThermoFisher scientific, USA) and 90% saline (G-BIOSCIENCES, USA).

In each experiment, animals were injected with vehicle or drug 60 mins prior to testing. The animals were allocated randomly, and all experiments were performed and analysed double blinded, where mice ID were randomly allocated by our animal house staff before the experimenter continued with the dosing and/or behavioural experiments. Therefore, the experimenters were unaware of the genotype or the treatment of each individual mouse. Doses and pre-treatment times were based on previous studies of drug-induced anxiety in mice (Neumann et al., 2019; Nickolls et al., 2018). Preliminary experiments were performed to test whether the DS2 injection caused a behavioural change, here the mice were injected once with DS2 or vehicle once and tested after 1h. The following doses of DS2 was used to determine a suitable dosage without harming the animal that would elicit a behavioural change, 1 mg/kg, 2 mg/kg, and 4 mg/kg. 5 mice per group for either drug or vehicle treated.

Following, these preliminary experiments, 2 mg/kg dose was chosen to give the optimal experimental condition and the treatment with DS2 (or vehicle) was given by ip for 5 consecutive days at the same time of the day. On the 5th day, behavioural tests were conducted after 60 mins of ip injection. Each mouse within a cohort received 1 dose/injection per day followed by subsequent behavioural tests performed on the same day for both experimental conditions mentioned above. This was repeated for 5 days for each animal.

2.10 Power Calculations and Statistics

All experiments were designed to generate groups of equal size using, analysed using randomisation and blinded analysis.

Power calculations were based on the differences observed between control and AD data sets obtained from preliminary studies. The power calculations were performed using the online tool ClinCalc (Kane). For example, we expected to obtain a 60-70 % change in the discrimination index (calculated from object location tests which assess learning and memory) with a sample size of $n \geq 10$ animals per cohort; this would reveal a statistical difference of >80% power assuming a 5% significance level. For neurochemistry parameters (e.g., colocalisation of expression of δ -GABAAR in interneurons), a sample size of n=5 animals would be necessary to meet an expected change of 80-90 % between the AD mice age-matched to the WT controls.

For neuroanatomical and biochemical analysis, Student t-test, one-way ANOVA and two-way ANOVA were performed to determine statistical significance using GraphPad Prism version 9.0 for Windows. Before performing any statistical test, the normality of the raw data was verified using the Shapiro-Wilk test and a ROUT test to identify potential outliers. In almost all the cases no outliers were identified. Where an outlier existed, it was removed from the data pool via the software. When comparing two data sets, t -test was used. The t-test produces two values as its output: t-value and degrees of freedom. The t-value, expressed as t (Dickerson & Atri, 2014), is a ratio of the difference between the mean of the two sample sets and the variation that exists within the sample sets. A large t-value indicates that the groups are different while a small t-score indicates that the groups are similar. When comparing three or more data sets from two genotypes, a one-way analysis of variance (Benitez et al., 2021) was performed. When a second factor, age, was taken into account, a two-way ANOVA was used. After any ANOVA, a post-hoc test for multiple comparisons was applied. Direct comparisons between only two data sets were performed using an unpaired two-tailed Student's t-test. To assess the significance of the differences between groups, F-values were also taken into consideration. They are displayed as (F values (degrees of freedom numerator (dfn), degrees of freedom denominator (dfd)); P value). The numerator value is the difference between the mean of the two sample sets. The denominator is the variation that exists within the sample sets and is a measurement of the dispersion or variability. As for the interpretation, larger F values were considered significant, whereas smaller F values were considered non-significant. Pearson's correlation analysis was used to assess the colocalization between

receptors and cell types using GraphPad Prism. Fisher's Transformation was applied to Pearson's R value to normalise the data.

Data are represented as mean ± SEM. Statistical analysis was undertaken only for studies where each group size was at least n=5, and for all statistical tests performed, a 95% confidence interval was used ($P < 0.05$) and tests were one-tailed. The "n" is given as the number of observations and the number of animals used, unless otherwise stated. Specific statistical methods and the significance of each analysis are described in the legend of each figure.

A power calculation was conducted utilising preliminary experimental data or preexisting data in the laboratory. The power calculations were conducted with the online programme ClinCalc (Kane), employing the subsequent equation:

$$N_1 = \{z_1 - \alpha/2 * \sqrt{\bar{p} - \bar{q} * (1 + \frac{1}{k})} + z - \beta * \sqrt{p_1 * q_1 + \frac{p_2 * q_2}{k}}\}/\Delta^2$$

- q_1=1-p_1
- q_2=1-p_2
- $\bar{p}=\frac{p_1+kp_2}{1+K}$
- $\bar{q} = 1 - \bar{p}$
- p_1, p_2 = proportion (incidence) of groups 1 and 2
- $\Delta = |p_2 - p_1|$ = absolute difference between two proportions
- n_1 = sample size for group 1
- n_2 = sample size for group 2
- α = probability of type I error (usually 0.05)
- β = probability of type II error (usually 0.2)
- z = critical Z value for a given α or β
- K = ratio of sample size for group 2 to group 1

The power (1-β) was set to 0.80. Before performing any statistical test, the normality of the raw data was verified using the test Shapiro-Wilk and a ROUT test

to identify potential outliers. In almost all of the cases no outliers were identified. Where an outlier existed, it was removed from the data pool via the software. When comparing between three or more data sets from two genotypes, a one-way analysis of variance (Benitez et al., 2021) was performed. When a second factor was taken into account, for example age, a two-way ANOVA was used. After any ANOVA, a post-hoc test for multiple comparisons was applied. Direct comparisons between only two data sets were performed using an unpaired two-tailed Student's t-test. All *P*-values below 0.05 were considered significant and asterisks added to the presentation of the data as follows: * $P<0.05$ ** $P<0.01$ *** $P<0.001$ **** $P<0.0001$.

3 Results

3.1 Results I: Investigation of cognitive deficits in *App* and tau mouse models of AD

The following chapters will detail the results obtained from four types of behavioural experiments performed using WT, App^{NL-F} KI, $App^{NL-F}/MAPT^{htau/wt}$ and $App^{NL-F}/MAPT$ dKI mouse models to investigate the alteration of cognition during the progression of AD, and whether there is a difference in the cognition abilities between the different mouse models of AD. These behavioural tests included novel object recognition (NOR), novel object location recognition (NOL) tests and T- maze tests at the following age windows: 6-9, 12-16, and 18-22 months. These time points were determined to be significant testing points when investigating cognitive decline shown by previous studies (Masuda et al., 2016; Saito et al., 2014; Saito et al., 2019).

The sample size was determined from preliminary studies, which involved four animals from each genotype to assess the study's statistical power.

Based on the differences observed between control and diseased data sets obtained in our preliminary studies, we expect to obtain 60-70 % changes in memory tests; a sample size of n=18 animals per cohort will reveal a statistical difference of >80% power, assuming a 5% significance level.

3.1.1 12-16 months' old App^{NL-F} KI, $App^{NL-F}/MAPT^{htau/wt}$ showed a cognitive decline compared to WT in novel location/object recognition tests

NOR and NOL are time-efficient and effective methods for assessing various stages of learning and memory in mice (Lindsay M Lueptow, 2017). The technique was initially documented by Ennaceur and Delacour in 1988 and predominantly employed in rat models (Ennaceur & Meliani, 1992). Nevertheless, subsequent modifications have enabled its effective application in mouse models (Leger et al., 2013; Lueptow et al., 2016). The assessment is dependent on a minimal number of three sessions, encompassing a habituation session, a training session, and a test session. The training phase consists of visually

examining two similar things, but the test phase entails substituting one of the previously examined objects with a new location and a new object. Due to the inherent inclination of rodents towards novelty, it may be observed that a mouse with the ability to recall a familiar thing will allocate a greater amount of time towards exploring the unfamiliar object (Lindsay M Lueptow, 2017).

These tests were one-trial tasks, as they did not involve learning rules. Moreover, the test did not require reinforcers and was purely based on the rodent's innate preference to explore the novel object rather than the familiar one. Thus, a rodent that remembered the familiar object would spend more time exploring the novel object.

Below show the results of *WT, App^{NL-F}* KI, *and App $^{NL-F}$ /MAPT$^{htau/wt}$* mice that performed 3 different behaviour tests at the following age windows: 6-9 months, 12-16 months and 18-21 months. To investigate cognition function, especially working memory in the mouse brain, a novel location test and novel objective recognition test was performed in three different age groups: 7-9 months, 12-16 months, and 18-22 months (in Figure 11). The *App $^{NL-F}$/MAPT* dKI mouse models of AD were not available to us during this experimental period as it was during the COVID-19 lockdown period. However, another cognitive experimental paradigm was used to validate the results from the above test, the T-arm maze test, which was used for all the genotypes studied shown in Figure 12.

Overall, a reduced discrimination rate in NOL at 12-16 months *for the App^{NL-F}* KI and *App $^{NL-F}$/MAPT $^{htau/wt}$* mouse models *were* observed compared to WT mice, which was in the magnitude of, 129% and 150% respectively (t(29)=2.434, *P*<0.05, WT n=21, *App^{NL-F}* KI n=11, App^{NL-F}/*MAPT*$^{htau/wt}$ n=11, Student's t-test). In the 6–9 months age groups, WT mice showed a relatively higher discrimination rate than the *App^{NL-F}/MAPT$^{htau/wt}$* mice, which denoted a pronounced interest in the exploration of a new location. WT mice expressed a relatively higher cognition ability than *App^{NL-F}* KI and *App $^{NL-F}$/MAPT$^{htau/wt}$* mice. However, due to the high variation in the results, the difference was not significant (*P*>0.05, WT n=17, *App^{NL-F}* KI n=9, App^{NL-F}/*MAPT*$^{htau/wt}$ n=6, Student's t-test). In the oldest age group, 18-22m, the WT mice showed a relatively higher discrimination rate than *App^{NL-F}/MAPT$^{htau/wt}$* mice. However, due to the large variation, the difference was also not significant (*P*>0.05, WT n=17, *App^{NL-F}* KI n=8, App^{NL-F}/*MAPT*$^{htau/wt}$ n=8, Student's t-test)..

In all 3 age groups studied, WT mice showed slightly improved, but not significantly different, recognition function compared to the *App^{NL-F}/MAPT$^{htau/wt}$* mice in recognition of the same object in new and old locations. To further investigate the cognition function, the NOR test was conducted in WT*, App^{NL-F}* KI and *App $^{NL-F/}$/MAPT ^{htaKI,wt}* mice in three age groups: 6-9 months,

12-16 months and 18-22 months, as illustrated in Figure 11. The main advantage of NOR was the dependence on the natural tendency to explore new things. Avoiding massive training sessions could reduce mouse stress levels to some extent and reduce the time for each experiment (Denninger et al., 2018; L. M. Lueptow, 2017).

Overall, the significant reduction in discrimination rate in NOR was 78% and 126% for 12-16m *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT* $^{htau/wt}$ mouse models compared to WT mice (*P<0.05, WT n=18, *App*$^{NL-F}$ KI n=9, *App*$^{NL-F}$/*MAPT*$^{htau/wt}$ n=8, Student's t-test). In the 6-9m age group, the mean of the discrimination rate displayed by WT mice showed no significant difference compared to the *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT* $^{htau/wt}$ mice models. The increasing discrimination rate denoted the higher interest of mice towards the novel object rather than the old object. WT mice at 12-16m showed better cognitive function compared to the AD mouse models, however, in the oldest age group, 18-22 m, there was no significant difference between WT, *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT* $^{htau/wt}$ mice (WT n=17, *App*$^{NL-F}$ KI n=14, *App* $^{NL-F}$/*MAPT* $^{htau/wt}$ n= 8, *P*>0.05, Student's t-test). This variance could be due to *App*$^{NL-F}$ KI animals that show attention deficiencies, as well as significant impulsivity, which can affect their performance in specific tests (Masuda et al., 2016).

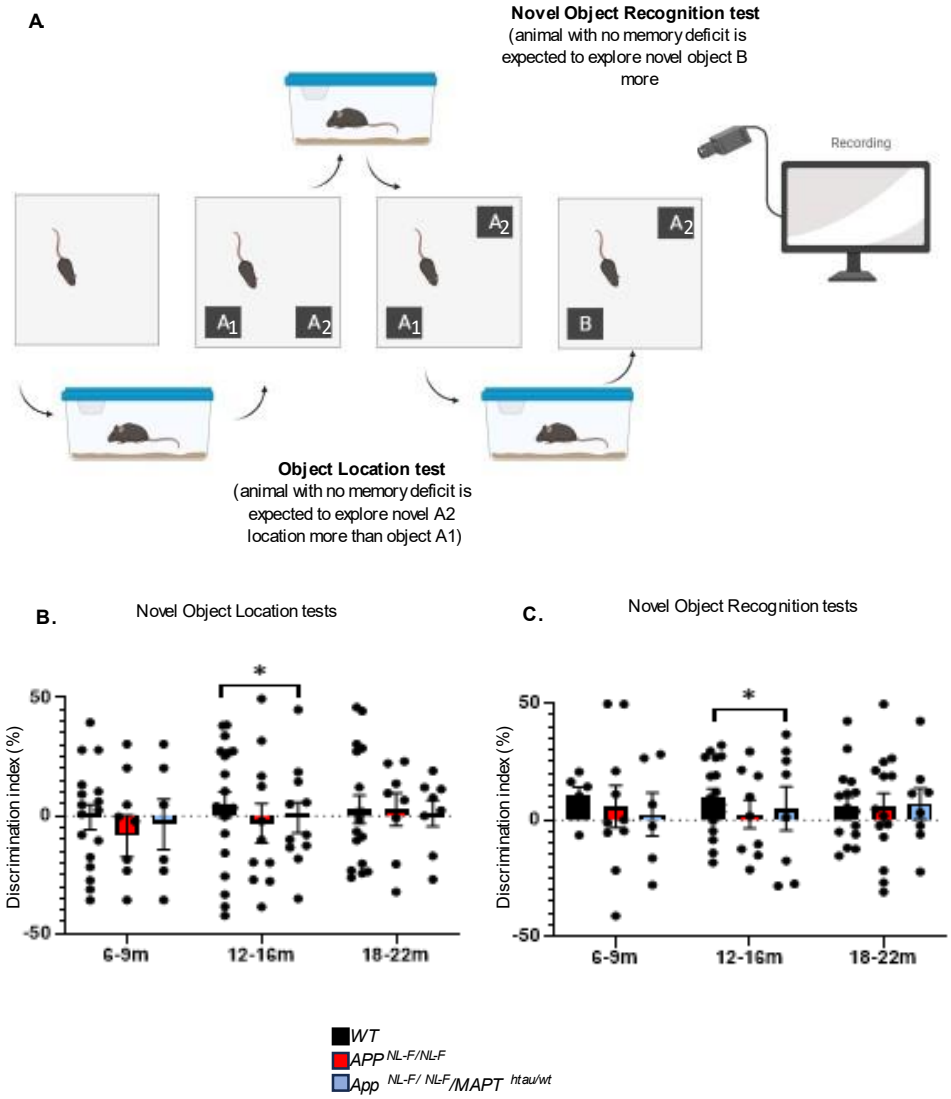


Figure 11. **Aged knock-in mouse models of AD display decline in working memory. A)** Schematic diagram of novel object recognition (NOR) and location (NOL) test. **B-C)** 12-16m *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ showed significantly worse working memory in NOR and NOL compared with age-matched WT. Graph showed the novel location recognition test of WT, *App*$^{NL-F}$ KI, *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ mice at 3 different age groups: 6-9m (wt n=17, *App*$^{NL-F}$ KI n=9, *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ n= 6), 12-16m (wt n=21, *App*$^{NL-F}$ KI n=11, *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ n=11) and 18-22m (wt n=18, *App*$^{NL-F}$ KI n=8, *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ n= 8) (*P<0.05, Student's t-test).

3.1.2 T-maze tests reveal time-dependent cognitive decline in App and tau mouse models of AD

To validate our result from the NOR/NOL and to further test the time-dependent memory performance of our AD mouse models, we used spontaneous alternation experimental paradigms measured using the T-arm maze tests. The schematic of this setup is shown in Figure 12D. The T-maze test is one of the various ways to test the animal's cognitive ability. The T-maze experiment is based on the rodent's natural interest to explore new directions without returning to the same directions. They are using 'working memory', *i.e*., the response on each trial varies according to their previous immediate actions. Alternation reflects the motivation of the animal to explore its environment and locate the presence of resources such as food, water, mates or shelter. Animals do not require the deprivation of such resources to show alternation behaviour; in this case, it is called 'spontaneous alternation'. It has been highlighted in the literature as the most appropriate methodology for mice to be tested for their cognitive impairment (Stewart et al., 2011). This test is based on the innate inclination of the mice to alternate left-right when given the option (Richman et al., 1986) and is a task that assesses working hippocampal memory (Olton et al., 1979). The protocol was adapted from (Deacon & Rawlins, 2006) a maze made in-house.

Each mouse was given two runs to assess the alternation. Mice were tested three times, first with a 0-second delay between the two runs and then with 30-second and 60-second delays introduced. The three trials were pooled, and a two-way ANOVA with genotype and age as factors was used for statistical analysis. Each trial was conducted after 2 minutes of rest in the home cage.

Figure 12B-D illustrates the overall T-maze performance at 6-9, 12-16 and18-22 months, the average of the alternations scores from the three trials: no delay, 30-second delay, and 60-second delay, ± SEM are shown. A two-way ANOVA with genotype and alternation time as factors identified genotype as making a significant contribution to the variance ($F_{(3,6)}=9.857$, $P<0.05$, $n=16$, for each cohort). All AD mouse models showed a significant decline in memory performance, with the *App* $^{NL-F}$ */MAPT* dKI mice showing the most cognitive impairment compared with WT at 0s 30s and 60s at age 6-9m. In addition, 12- 16m *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ mice also showed significantly worse memory at 60s alternations when compared with age-matched WT. There was a significant reduction in memory during 18-22 months for all AD models studied as expected. For example, in *App*$^{NL-F}$ KI mice, there was a reduced alternation, which was of 23.8 %, and for *App* $^{NL-F}$ */MAPT* dKI mice, there was a reduced alternation of 58.4% compared to age-matched WT controls ($F_{(3,6)}=9.857$, n=10, *P<0.05. Two-way

ANOVA). The results for *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ were similar to the *App* $^{NL-F}$ */MAPT* dKI (Figure 12).

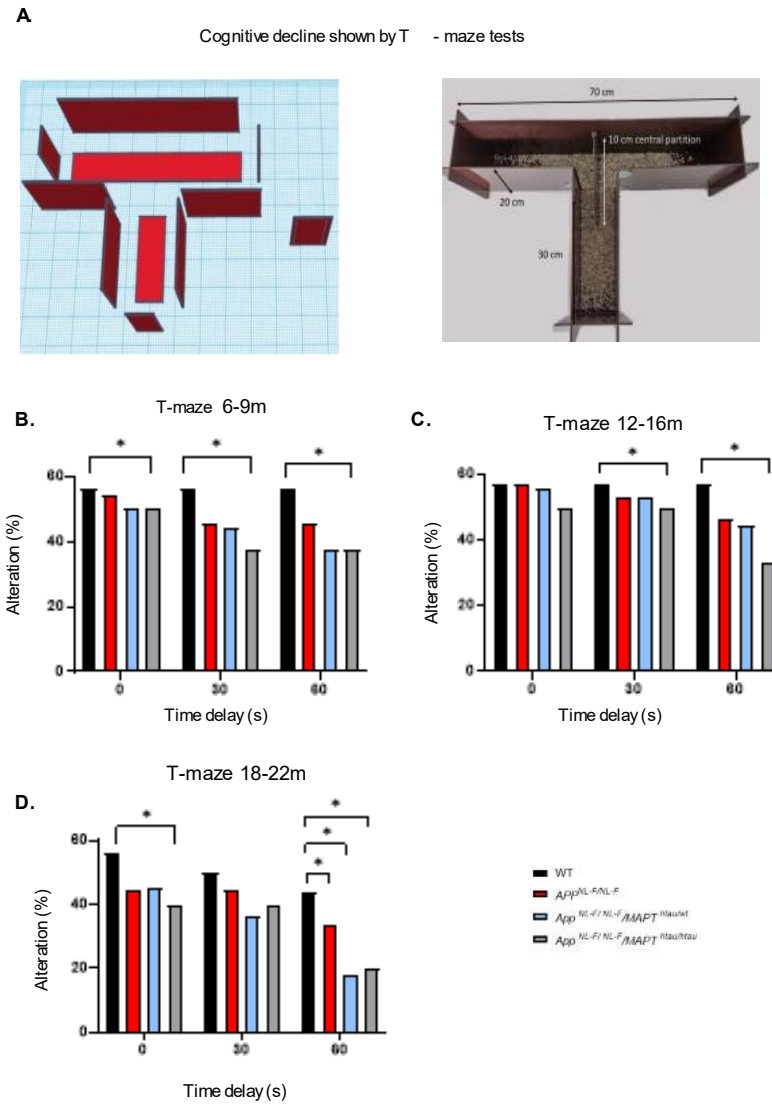


Figure 12. **Cognitive deficits in 6-9 months. 12-16 months and 18-22m AD mouse models shown by T-maze experiments.** Alternation rate of WT, *App*$^{NL-F}$ KI, *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ and *App* $^{NL-F}$*/MAPT* dKI mice in 3 age groups, 7-9 months, 12-16 months and 18-21 months. The alternation rate was the percentage of mice making alternative choices in two choices of one trial. 3 experiments were conducted on all age groups with no delay, 30 s delay and 60 s delay. **A)** Schematic diagram of T-maze experiments. **B)** 6-9 months *App* $^{NL-F}$ */MAPT* $^{htau/wt}$ and *App* $^{NL-F}$*/MAPT* dKI showed significantly worse working memory compared to age-matched WT in 0s, 30 s and 60 s delay. **C)** 12-16 months *App* $^{NL-F}$*/MAPT* dKI showed significantly lower alternation rate compared to age-matched WT at 30s and 60s delay tests. **D)** 18-22m months old WT mice showed a significantly higher alternation rate compared to age-matched *App* $^{NL-F}$*/MAPT* dKI at 0 and 60s delay tests while compared to *App*$^{NL-F}$ KI and *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ at 60s delay tests (n=9, *P<0.05, two-way ANOVA).

3.1.3 Discussion: time-dependent decline in memory performance in AD mouse models compared to age-matched WT

The aim of this chapter was to investigate whether there was a significant difference in memory performance using two experimental paradigms of working memory, NOR/NOL, and the T-maze test using two genetically different mouse models of AD age-matched to the WT. The key findings here include:

i. *App*$^{NL-F}$/*MAPT*$^{htau/wt}$ mice showed a discrimination rate decrease than WT in the age group 12-16m, which leads to a considerably worse spatial memory. In comparison, there was no significant difference observed between the control WT and AD mouse models using NOR/NOL at 6-9 and 18-21 months. A higher discrimination rate indicates more time spent exploring new objects or new locations, highlighting the better working memory of the mice.
ii. All AD mouse models showed a significant decline in memory performance, with *App* $^{NL-F}$ */MAPT* dKI mice showing the most cognitive impairment compared with WT at 0 s, 30 s and 60 s across all age groups.
iii. The humanised MAPT gene did not exacerbate the memory function throughout AD progression, as observed in the results of *App*$^{NL-F}$ KI, *App* $^{NL-F}$ */MAPT* $^{htau/wt}$ and *App* $^{NL-F}$ */MAPT* dKI

Our NOR/NOL results showed cognition deficits started at 12-16 months of AD mouse models, which is similar to previous studies using *App*$^{NL-F}$ KI. The *App*$^{NL-F}$ showed cognition deficits at 12 months (Masuda et al., 2016). However, there showed no significant difference in memory function of WT and AD mouse models in the 18-22 months age group.

The unexpected results from my NOR/NOL are consistent with previous research by Masuda and colleagues (Masuda et al., 2016), who suggest that the abnormalities of *App*$^{NL-F}$ KI mice, such as increased compulsivity, decreased attention control and increased impulsivity, contribute to greater changes in working memory at 12 months compared to 18 months AD mouse performance when directly compared with age-matched WT mice.

We used these experimental paradigms because it is thought that during the NOR/NOL tasks, the animals' ability of memory consolidation, as well as spatial or contextual characteristics can be examined, which are associated with different brain regions and pathways in the limbic system (Antunes & Biala, 2012; Denninger et al., 2018). When memory is recovered through novelty, it enters a labile phase that requires stabilisation to survive. This memory processing in the hippocampus is called reconsolidation and is engaged in rearranging previously created

memories, allowing the incorporation of new information. (Clarke et al., 2010; Lieberwirth et al., 2016; Nader, 2015; Sirichoat et al., 2020). The hippocampus receives inputs from the perirhinal cortex, which is the site of entrance of various information such as visual, olfactory, and somatosensory stimuli. All of them are involved in object and location recognition (Chao et al., 2022).

Interestingly, the results from the T-maze test (Figure 12) confirm the findings from prior research on cognitive impairments in *App*$^{NL-F}$ and MAPT AD models (Masuda et al., 2016; Saito et al., 2014; Saito et al., 2019) using many behavioural tests, enhancing our comprehension of the model. The T-maze test is based on mice's natural desire to investigate unknown areas and change their goal arm selection on each attempt. Mice make choices using working memory, suggesting that each response is influenced by prior selections, making the T-maze test appropriate for cognition assessment (d'Isa et al., 2021).

All our AD mouse models showed significantly worsening of working memory overall between; 6-9 months, 12-16m and 18-22 months in the T-maze in figure 12. This supports the findings of previous studies regarding the App^{NL-F} KI mouse (Castillo et al., 2017). However, it contradicts the results of previous studies that used the *App*$^{NL-G-F}$/$^{NL-G-F}$ mouse. This mouse model contains the Swedish (Seignourel et al., 2008), Iberian (F), and Arctic (G) App mutations from the original App^{NL-F} KI model (Saito et al., 2019). *App*$^{NL-F-G/NL-G-F}$ showed reduced memory function at 6 months old, which was 6 months earlier than our AD mouse models.

Two important investigations using the *App*$^{NL-F}$ KI mouse model showed decreased cognitive function at distinct ages: between 8-12 months in the IntelliCage study, according to Masuda et al. (2016), and at 18 months in Y-maze tests, according to Saito et al. (2014). The IntelliCage study examined various characteristics, including memory problems and obsessive conduct. These two studies found cognitive differences at varying ages and utilised various behavioural assessments. The current study attempted to fill the gap by utilising two memory assessments among three distinct age categories. Two different tests, NOL/NOR and T-maze tests were utilised to determine the most suitable one for these animals by experimenting with tests generally found in the literature (Denninger et al., 2018; Saito et al., 2019; Stewart et al., 2011). In conclusion, this investigation detected cognitive impairment from 6-9 months to 18-22 months of age, aligning with the results of the two previous studies.

The difference in these tests could be due to the fact that the AD mouse models had a tendency to favour the section of the maze they initially entered. The measurement of preference may have been used instead of alternation. However, neither of the four genotypes displayed any preference towards either the "left" or "right" arm of the maze. Additionally, there

was no preference for entering one arm over the other. To tackle this issue, in the future experiments, the experiment might be reproduced by first completing a trial without any incentives, then introducing a reward within the maze. This would encourage the mouse to seek the reward and may trigger the mouse's natural tendency to switch between the two arms of the T-maze.

Could a larger deficit have gone unnoticed in the t maze due to low performance in the control cohort? Yes, this is feasible and has been examined in a 2011 study (Stewart et al., 2011). The study discovered instances where control animals exhibited subpar performance in the Tarm (Deacon et al., 2008; Zhuo et al., 2007). It is typically anticipated that they would consistently execute accurately and alternate in over 50% of all attempts. In the current investigation, WT mice had an overall correct alternation rate of 56.25% at 6-9 months, 57.14% at 12-16 months, and 50% at 18-22 months across the three trials. The control performance may be associated with factors such as age, anxiety, or apathy, which could be present in both WT and AD mouse models. If the mice exhibit low levels of alternation that are similar to random chance, it is possible that they did not develop a memory of the maze and the task did not go as intended. If WT mice continuously achieve an accuracy rate of only 50% or less, modifications, such as adjustments to animal housing, handling, or maze and testing circumstances, should be implemented.

Our results showed that MAPT didn't make cognitive deficits worse which is similar to study done by Saito (Saito et al., 2019). Saito and his colleagues used *App*$^{NL\text{-}G\text{-}F}$*/MAPT* dKI mice to show that MAPT humanisation didn't affect memory in *App*$^{NL\text{-}G\text{-}F}$ mice. Other studies also showed similar results. Matthew and his colleagues used 6, 12, 18 and 24 months MAPT KI and WT mice for NOR/NOL tests. No memory performance difference was found at all ages (Benskey et al., 2023).

Conclusion

Overall, the memory impairment using two different experimental paradigms showed that AD mouse models *App*$^{NL\text{-}F}$ KI, *App* $^{NL\text{-}F}$ */MAPT*$^{\text{htau/wt}}$ and *App*$^{NL\text{-}F}$*/MAPT* dKI showed significantly reduced memory function compared to age-matched WT. Furthermore, our results showed that the insertion of the MAPT gene did not accelerate the decline of cognitive function. Our findings could contribute to developing a valuable mouse model for investigating the mechanism of Alzheimer's disease (AD).

3.2 Results II: Investigation of anxiety in App and tau mouse models of AD

Anxiety, as one of the NPS, is prevalent in approximately 40% of patients with AD (R. Botto et al., 2022; Mendez, 2021). Anxiety frequently manifests in the initial stages of AD, particularly in patients with mild cognitive impairment (Meshkat et al., 2023), mild dementia, or early-onset variants of the condition. This anxiety can contribute to the advancement and transition from MCI to dementia (Escher et al., 2019; Tchekalarova & Tzoneva, 2023). Identifying patients diagnosed with anxiety disorders such as posttraumatic stress disorder (PTSD) and generalised anxiety disorder (GAD) could lead to the implementation of early-intervention treatments for AD.

Therefore, it was interesting to investigate age-dependent anxiety-like behaviours in our AD mouse models and compare these findings to the age-matched WT.

The anxiety of mice was measured using two different experimental paradigms, open field and light-dark chamber tests. These tests were based on mice's natural preference of dark space and central area (Hefner & Holmes, 2007; Kraeuter et al., 2019). The experimental design is on the premise that mice with lower anxiety level would spend more time in the centre area and light chamber. Below were results of WT, App^{NL-F} *KI,* $App^{NL-F}/MAPT^{htau/wt}$, $App^{NL-F}/MAPT$ dKI tested at 12-16m age group.

3.2.1 Elevated anxiety level observed in AD mouse models in open field tests compared to the WT control mice

The Open Field Maze (OFM) was originally created in 1934 as an assessment tool to quantify emotional responses in rodents (Hall, 1934). It has achieved the distinction of being one of the most often utilised indicators of behaviour in animal psychology (Seibenhener & Wooten, 2015; Walsh & Cummins, 1976). The assessment offers a simple and relatively quick evaluation of clearly specified behaviours without requiring any training for the test participant and just minimal preparation for the person conducting the test.

The tests are popular because the psychological and physiological ideas they are based on are often easy to understand and well-known. For instance, it has been hypothesised that evolutionary pressures have favoured a shared reaction in animals, resulting in the majority of species exhibiting fear or flight responses to certain stimuli that are mediated by anxiety.

Rodents, such as mice and rats, have clear aversions to expansive, well-illuminated, unobstructed, and unfamiliar surroundings (Choleris et al., 2001). It can be inferred that they have been biologically adapted to perceive specific environments as hazardous. The open field maze incorporates all of these features and serves as the foundation for its utilisation in behavioural paradigm testing.

Animals with higher anxiety levels would be predicted to spend more time near the peripheral arena. This behaviour is likely due to their instinct to seek protection from potential threats, as opposed to the more vulnerable central area (Seibenhener & Wooten, 2015; Wable et al., 2015).

Control anxiety levels measured in all genotypes studied (WT, *App*$^{NL-F}$ KI, *App* $^{NL-F}$*/MAPT* $^{htau/wt}$, and *App* $^{NL-F}$*/MAPT* dKI mice) using the two experiment paradigms for anxiety, the open field (schematic shown in Figure 13A). Mice were placed in a box measuring 40cm x 40cm x 40cm for a duration of 10 minutes. The duration spent in the centre area and periphery area, total travel distance and latency to enter the peripheral zone are recorded for analysis. The baseline anxiety levels in the AD mouse models were significantly greater compared to the age-matched WT (12-16m). For example, with the open field test, the level of anxiety expressed in *App*$^{NL-F}$ KI mice was 15.78 ± 3.98 % higher than the control WT (t(10) = 3.492, *P<0.01, n=6, Student's t-test)*.

Similarly, the anxiety level in *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ and *App* $^{NL-F}$*/MAPT* dKI mice was higher by, 16.8 ± 5.55%(*t(10) = 4.028*), and 19.56 ± 5.05%(*t(10) = 5.489*), respectively, compared to their WT counterparts (*P<0.01, n=6* for both genotypes), shown in Figure 13C. In Graph 15D, the total distance travelled was similar among all 4 mice groups, indicating that the locomotor function factor is removed concerning their time spent in different zones. In Figure 13E, there was a trend of shorter latency to enter the peripheral zone showed in AD models compared to the WT; figure 13B showed mice tracement in an open field test that the WT crossed the centre more than AD models.

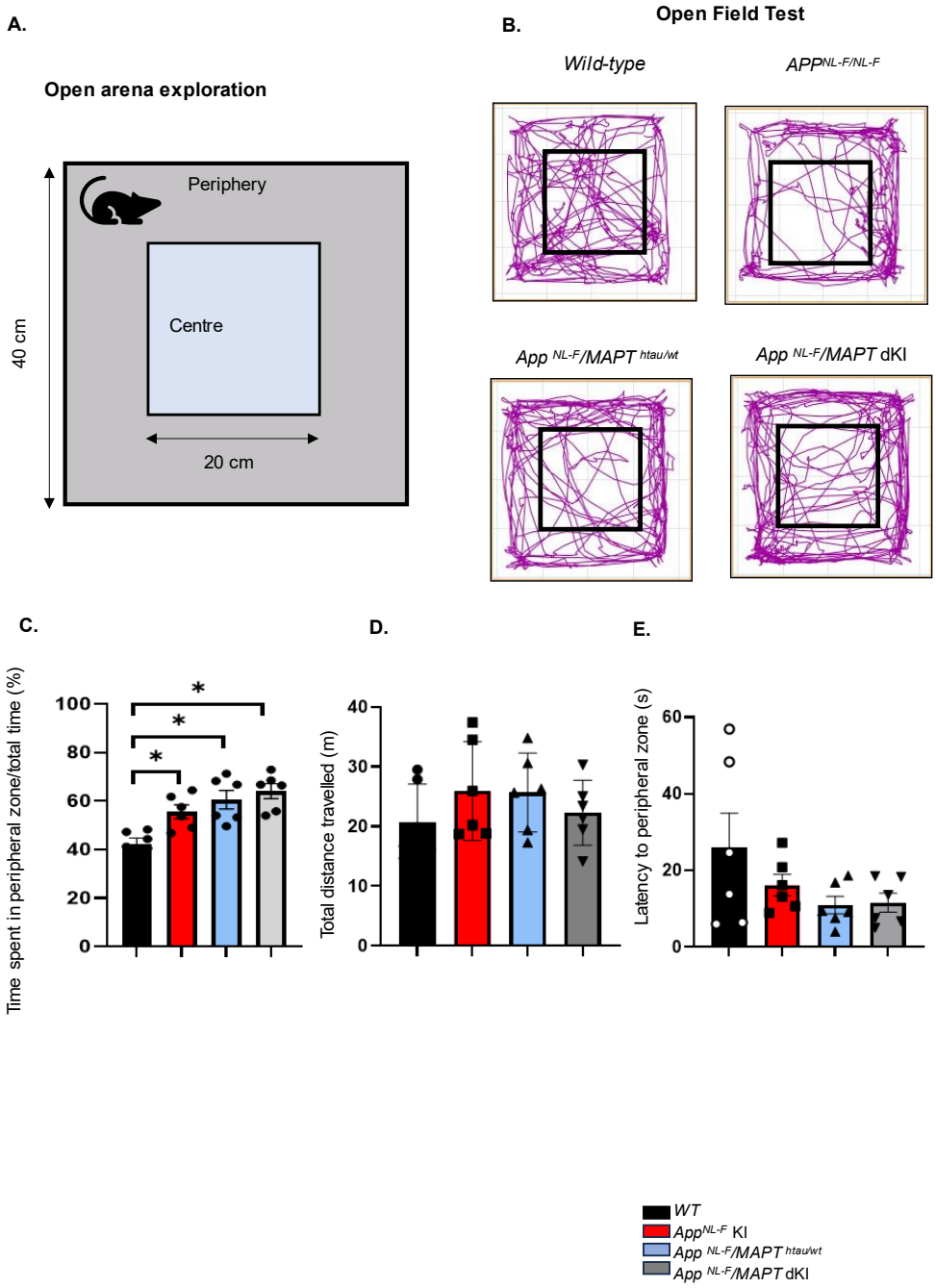


Figure 13. Elevated baseline anxiety exhibited by *App*$^{NL-F}$ KI, *App* $^{NL-F}$ */MAPT* $^{htau/wt}$ *and App* $^{NL-F}$ */MAPT* dKI mice shown in open field test. A) Schematic of open arena test. The experimental design consisted of a 40cmx40cmx40cm box. B) Panel shows traces of tracks recorded for the different experimental mice. The WT mice showed movement in both the centre and edges of the arena as compared the diseased AD mice which mostly kept to the edges which is quantified in panel C) AD mouse models showed significantly higher anxiety level compared to WT. D, E) No difference was observed in the mice locomotor abilities and reaction time between the AD mouse models and WT mice as seen in panels (n=6, *P<0.05, Student's t-test).

3.2.2 Light-dark chamber experimental paradigm validates higher anxiety level in AD mouse models

The light/dark box was initially proposed by researchers Crawley and Goodwin in 1980. It consists of two chambers: a small, dark chamber that makes up one-third of the overall space and a larger, lit chamber that makes up the remaining two-thirds of the box. The two chambers are interconnected by a narrow tunnel that allows the subject animal to transition between the two areas (Bourin & Hascoët, 2003; Pentkowski et al., 2021). The utilisation of the light/dark box is based on the premise that rats see brightly lit open spaces as unpleasant because they signify potential threats, whilst dark regions suggest a certain level of security (refer to: "Safety in the Shadows"). The light/dark box is a frequently employed tool for evaluating stress, anxiety, and depression levels in mice and rats. Mice were placed inside the paradigm for 10 minutes, recorded, and analysed by Any-maze software. Animals with higher anxiety levels are believed to spend more time in the dark area than in the light area.

The magnitude of the anxiety in each genotype observed using the light-dark chamber test (Figure 14) was similar to the data obtained from the open field test. For example, *App*$^{NL-F}$ KI mice showed a higher control level anxiety of, 13.12 ± 6.58 % compared to the control WT *(*P<0.05, n=6, Student's t-test).* This was also consistently similar to *App* $^{NL-F}$*/MAPT* $^{htau/wt}$ and *App* $^{NL-F}$ */MAPT* dKI mice, as shown in Figure 14C. In Figure 14D, the total distance travelled was similar among all 4 mice groups, indicating that the locomotor function factor is removed concerning their time spent in different zones. In Figure 14E, there was a trend of shorter latency to enter the dark zone shown in AD models compared to WT, which was similar to the shorter latency observed in the AD models when entering the peripheral zone. Figure 14B showed mice retracement in light-dark chamber test that WT explored the light zone more than AD models.

The fact the significantly higher anxiety level in AD models was corroborated with working memory deficits of AD models in NOL/LOR and T-maze in the same age group.

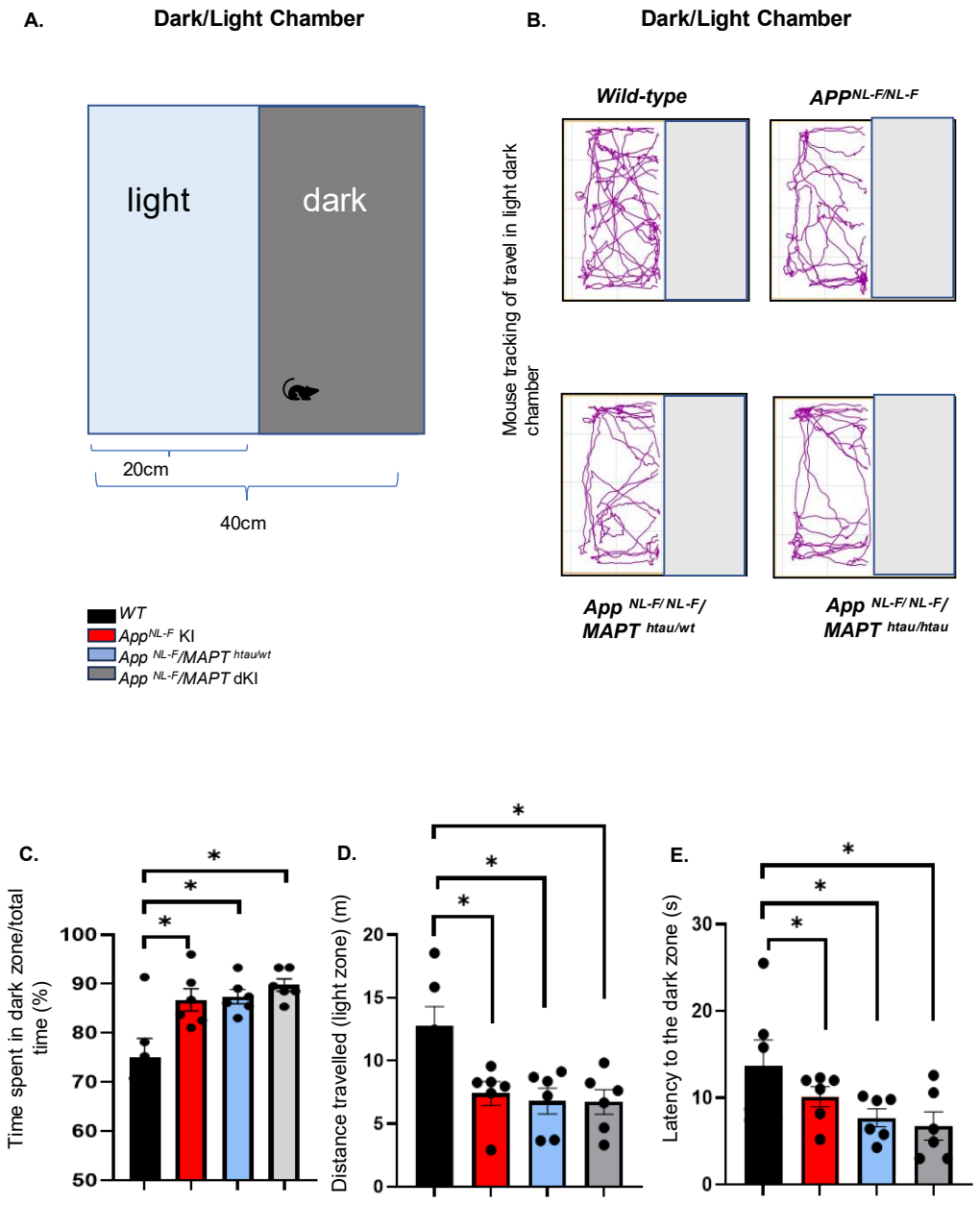


Figure 14 **Elevated baseline anxiety exhibited by *App*$^{NL-F}$ KI, *App* $^{NL-F}$ */MAPT* $^{htau/wt}$ and *App* $^{NL-F}$ /MAPT dKI mice in light dark chamber test. A)** Schematic of light dark chamber used for evaluating anxiety-like behaviour. **B)** Shows representative traces of AD model and WT mice recorded in the light dark chamber test. **C)** Baseline anxiety was significantly higher in the AD mouse models than age-matched WT in light dark chamber test. **D)** AD mouse models travelled significantly less distance in the light zone compared to age matched control mice. **E)** *App* $^{NL-F}$ /MAPT dKI mice took less time to enter the dark zone at the first time compared to age-matched WT mice. (n=6, **p≤0.05, one-way ANOVA with post-hoc Tukey test*)

3.2.3 Discussion: AD mouse models exhibit higher anxiety levels compared to WT

This study focused on examining anxiety by observing the subjects' tendency to choose sheltered areas (arena periphery) and avoid spending time in exposed areas (centre of arena). The anxiety signs were far more noticeable in the 12-16 months age group, where they exhibited decreased cognition.

In our study, we used an open field test and a light-dark chamber to investigate the anxiety of WT and AD mouse models along the progression of AD. 12-16 months AD mouse models showed significantly higher anxiety levels compared to WT. Similar total distance travelled in open field tests indicates the similar locomotor ability of WT and AD mouse models. The significantly higher anxiety level in AD mouse models matched their significantly lower cognition abilities compared to WT at 12-16m. Our results match those of previous studies in which the *App*$^{NL-F}$ KI model has been shown to exhibit anxiolytic behaviour from 8 to 13 months (Benskey et al., 2023; Masuda et al., 2016).

Interestingly, no significant difference in anxiety levels between *App*$^{NL-F}$ KI, *App* $^{NL-F}$*/MAPT* dKI mice and *App* $^{NL-F}$ */MAPT* $^{htau/wt}$ was observed in our two experiment paradigms. Within the new MAPT mouse model, the entire murine Mapt gene was replaced with the human MAPT gene and is expressed under the control of the endogenous murine Mapt promoter. MAPT KI mice express all six isoforms of tau in a ratio roughly equal to that of the adult human brain and show a normal subcellular localization of tau (Hashimoto et al., 2019; Saito et al., 2019)No significant difference in anxiety levels among these three AD models indicates that MAPT gene KI does not affect general anxiety-like behaviour or decrease locomotor activity as indexed by the open field task and light-dark chamber.

Was the less time spent by AD mouse models in the light zone affected by the lower distance travelled compared to WT in the experiment?

Our open-field study showed no significant difference in total distance travelled between WT and AD mouse models. Thus, the locomotor ability of AD models was not affected, which validated their different percentages of time spent in peripheral zones as due to genotypes. In light-dark chamber tests, the total distance was only recorded for the light area, so the significant difference between WT and AD mouse models did not represent their significant difference in locomotor abilities.

A recent study showed MAPT KI mice prevented the age-associated decrease in locomotor activity compared to WT, which is also reported in other tau transgenic mice (Benskey et al., 2023; Jul et al., 2016; Scattoni et al., 2010). For instance, hTau mice exhibit increases in total distance travelled in the open field task (Cho et al., 2021; Geiszler et al., 2016). Conversely, shRNA mediated knockdown of endogenous murine tau causes impaired performance on the rotarod task (Velazquez et al., 2018). Late in life, several tau transgenic mice exhibit reduced motor ability, including those lines that display early-life locomotor hyperactivity. For example, PS19 mice that are 9-10 months old have limb weakness that gradually leads to paralysis (Yoshiyama et al., 2007). On the other hand, JNPL3 mice that are 10 months old experience significant limb weakness and dystonic posture (Lewis et al., 2000). In contrast to tau transgenics, MAPT gene KI appears to maintain a consistent locomotor phenotype indefinitely without augmenting or diminishing locomotion. The available findings and those given here indicate that altering tau can affect motor function, while the underlying mechanisms are poorly comprehended. The motor impairment observed in most tau transgenic mice is a late-stage characteristic that occurs due to the degradation of motor neurons caused by the buildup of abnormal tau protein in the spinal cord (Benskey et al., 2023; Lewis et al., 2000; Yoshiyama et al., 2007). On the other hand, the specific processes that cause an increase in locomotor activity related to tau are not well understood. The presence of human tau or the absence of murine tau could potentially affect the movement patterns of rodents. Alternatively, these effects may arise via neurodevelopmental compensations resulting from the alteration of tau in the germline. This function of the tau protein is intriguing and has not received much attention. Further research is required to clarify these findings and provide insight into the possible role of tau.

There is considerable debate on whether the AD mouse models exhibit an anxiety phenotype. Certain researchers have found that the AD mouse model does not exhibit anxiety according to certain studies (Emre et al., 2022; Kundu et al., 2021; Maezono et al., 2020; Sakakibara et al., 2018), while others have reported an anxiety phenotype in certain but not all (Auta et al., 2022; Sakakibara et al., 2018).

Other studies suggest that the evaluation of anxiety-like behaviour has shown significant inconsistency among several transgenic lines (Pentkowski et al., 2021). The observations emphasise the constraints of current methods utilising transgenic animals to effectively replicate preclinical AD-related behavioural issues. Several reasons are likely responsible for these conflicting outcomes. Studies are frequently carried out at different stages of disease development, such as the preclinical phase and dementia stages, leading to variations in the severity of pathology in neural networks associated with anxiety-related protective behaviours,

like the hippocampus, frontal cortex, and amygdala. Studies frequently do not accurately describe anxious behaviours in genetically modified mice at the “pre-amyloid stages” of AD development (Boon et al., 2010; Galeano et al., 2014). Furthermore, the inconsistency in behavioural outcomes may be due to an insufficient evaluation of anxiety, as many studies rely on a single behavioural paradigm. Hence, we recommend that upcoming research should include a series of tests that describe anxiety-related traits in transgenic mice. A test battery may include traditional assessments like an elevated plus maze, open field test, and light-dark chamber, which focus on conflict as the primary threat source, along with additional models like social situation, predator threat, and shock-probe burying test.

Preclinical research investigating the neuropsychology of Alzheimer's disease (AD) has predominantly concentrated on memory deficits, namely the deterioration of spatial learning and memory. aside from cognitive impairment(Coughlan et al., 2019; Coughlan et al., 2018; Howett et al., 2019; Manuel et al., 2020), several data suggest that the initial phases of the disease are characterised by neuropsychiatric symptoms such as psychological distress and changes in mood (Dickerson & Atri, 2014; Geda et al., 2013). Individuals with AD often experience neuropsychiatric symptoms such as anxiety and depression before or at the same time as they start to have memory problems during the early stages of cognitive decline (Donovan et al., 2018; Gabryelewicz et al., 2004; Lyketsos et al., 2011). Significantly, findings from a recent meta-analysis suggest that anxiety raises the likelihood of developing dementia in people with mild cognitive impairment (Meshkat et al., 2023; Li & Li, 2018). In addition, persons who experience elevated levels of anxiety have a 48% higher likelihood of getting Alzheimer's disease (AD) (Petkus et al., 2016) and a more rapid progression from mild cognitive impairment (Meshkat et al., 2023) to AD (Gallagher et al., 2011).

To conclude, our study showed AD mouse models showed significantly higher anxiety levels at 12-16 months old compared with WT. Identifying anxiety as a symptom in the AD models improves its use as a preclinical model and could prove helpful in testing anxiolytic drugs, which could be potential drug targets to treat AD.

3.3 Results III Neuropathological hallmarks of AD

After seeing decreased cognitive abilities in AD mouse models, we examined pathological alterations at the cellular level. The study specifically examined CA1 regions which is among the first to be impacted in AD (Masurkar, 2018).The changes examined pertained to characteristic features of AD, such as neuroinflammation, buildup of Aβ, hyperphosphorylated tau, and their detrimental impact on neurotransmitters, transporter systems and cellular abundance. The modifications were evaluated in age-matched WT, *App*$^{NL-F}$ KI, *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ and *App* $^{NL-F}$*/MAPT* dKI at 6-9 months, 12-16 months and 18-22 months.

The assessment of gliosis levels (proliferation of microglia and astrocytes) was conducted to provide insight on neuroinflammation. GFAP, a glial fibrillary acidic protein, is the marker for activated astrocytes. CD68, named clusters of differentiation 68, is highly expressed by microglia in the brain. The CD68 level is an indicator of microglia density. Immunoperoxidase labelling was employed to examine the amounts of astrocytes, microglia, Aβ, and tau in the CA1 region located in the brains of age-matched mice from four different genotypes.

Overall, there is a general increase of GFAP and Aβ of all age groups as well as CD68 and tau at 12-16m and 18-22m in CA1 regions of AD mice brains.

3.3.1 Aβ and Phosphorylated tau immunoperoxidase results

We observed an age-dependent increase in the formation of Aβ plaques in the CA1 region of *App*$^{NL-F}$ KI*, App* $^{NL-F}$*/MAPT*$^{htau/wt}$ and *App* $^{NL-F}$*/MAPT* dKI mouse models of AD compared to age matched WT mice. The presence of Aβ aggregates in CA1 was age-dependent in the AD models, which was expressed as a significantly greater magnitude in the CA1 at 12-16 months and 18-22 months age (Figure 15, table 6) (*genotype*($F_{(3,16)}$*=31.89, P <0.05, age*($F_{(1.968,31.49)}$*=68.92),* *P <0.05, n=5, Student's t-test) in comparison to age-matched WT mice.

In Figure 16, Phosphorylated tau levels was similar at 6-9m of 4 genotypes(P>0.05, Student's t-test, n=5). At 12-16m age, *App* $^{NL-F}$*/MAPT* dKI showed a significantly increase 11.2%±4.6% of phosphorylated tau levels than WT mice (*P<0.05, n=5, student's t-test). Within 18-22m age group, there was no significant difference of phosphorylated tau levels between WT and *App*$^{NL-F}$ KI (P>0.05, Student's t-test, n=5) while two AD models (*App*$^{NL-F}$*/MAPT*$^{htau/wt}$ *and App* $^{NL-F}$

/MAPT dKI) showed significantly 22.6% and 19.5% increase respectively compared to age match WT mice (*P<0.05, n=5, Student's t-test).

3.3.2 GFAP and CD68 immunoperoxidase results

Neuroinflammation, indicated by microgliosis and astrocytosis was observed in all three AD models in an age-dependent manner.

Data from immunoperoxidase staining (Figure 17) showed that the activated astrocyte level was significantly increased in 6-8m *3* AD mice Models compared with WT (**P*<0.05, Student's t-test, n=5). For example, there was a significant 60.6%±6% increase of GFAP levels in *App* $^{NL\text{-}F}$*/MAPT*$^{htau/wt}$ than age-matched WT mice. Between the ages of 12 and 16 months, the level of activated astrocytes was significantly higher in three models of AD compared to WT mice of the same age (*t(8) = 1.628,* *P<0.05, n=5, Student's t-test). The difference in the number of activated astrocytes between AD models and age-matched WT mice was even larger at 18-22m (*P<0.05, n=5, Student's t-test)

CD68 levels didn't differ between AD models and WT mice, indicating a similar level of microglia at 6-9m in Figure 18 (P>0.05, n=5, Student's t-test). However, there is a significant increase of 12.5± 5.11% of CD68 levels in *App* $^{NL\text{-}F}$ */MAPT* dKI compared with WT mice at 12-16m (*t(8) = 4.454*, *P<0.05, n=5, Student's t-test) (Figure 18). At 18-22m, there was a significant increase (66.1 ± 2.62%) of CD68 levels in AD models compared with age-matched WT mice (**t(8) = 12.53*, *P<0.05, n=5, student's t-test)

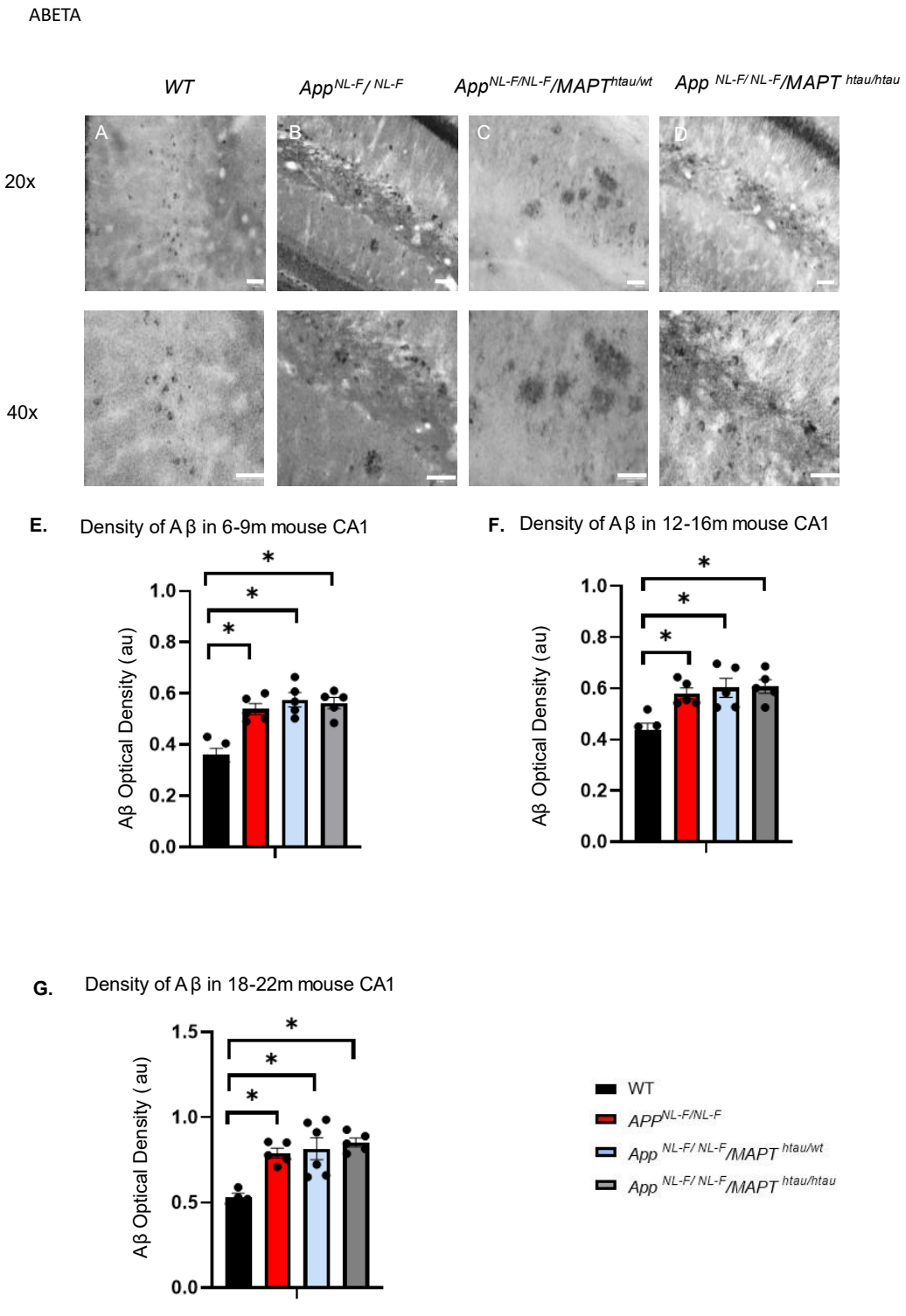


Figure 15. Immunoperoxidase staining showed the expression of abeta amyloid plaque changes in the CA1 region of the hippocampus in 6-9m, 12-16m, 18-22m WT, App $^{NL-F}$ *KI,* App $^{NL-F}$/MAPT$^{htau/wt}$ and *App* $^{NL-F}$ */MAPT dKI* mouse models. Images A-D illustrated the mean density changes of 4 markers under 20x and 40x lenses of a light microscope. there was no significant difference in Aβ expression in WT and *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mouse models. Results are expressed as mean ± SEM (*P<0.05, n=6, Student's-test).

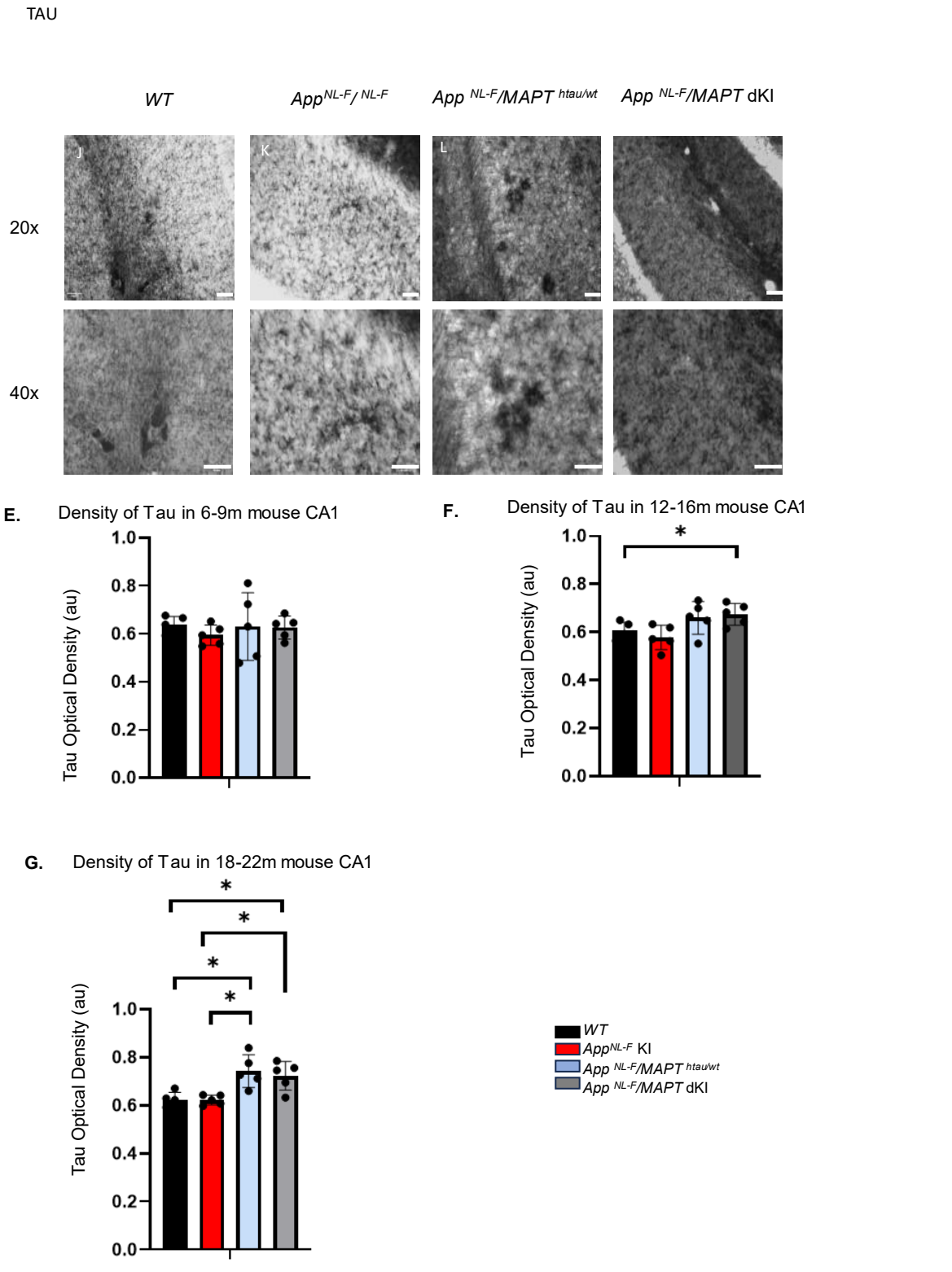


Figure 16. Immunoperoxidase staining showed the expression of hyperphosphorylated tau changes in the CA1 region of the hippocampus in 6-9m, 12-16m, 18-22m WT, *App* $^{NL-F}$ KI, *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ and *App* $^{NL-F}$*/MAPT* dKI mouse models. A-D) There illustrated the mean density changes of 4 markers under 20x and 40x lenses of a light microscope. E-G) Hyperphosphorylated Tau protein showed a significantly larger amount in *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ compared to WT mice. Results are expressed as mean ± SEM (*P<0.05, n=5Student's t-test).

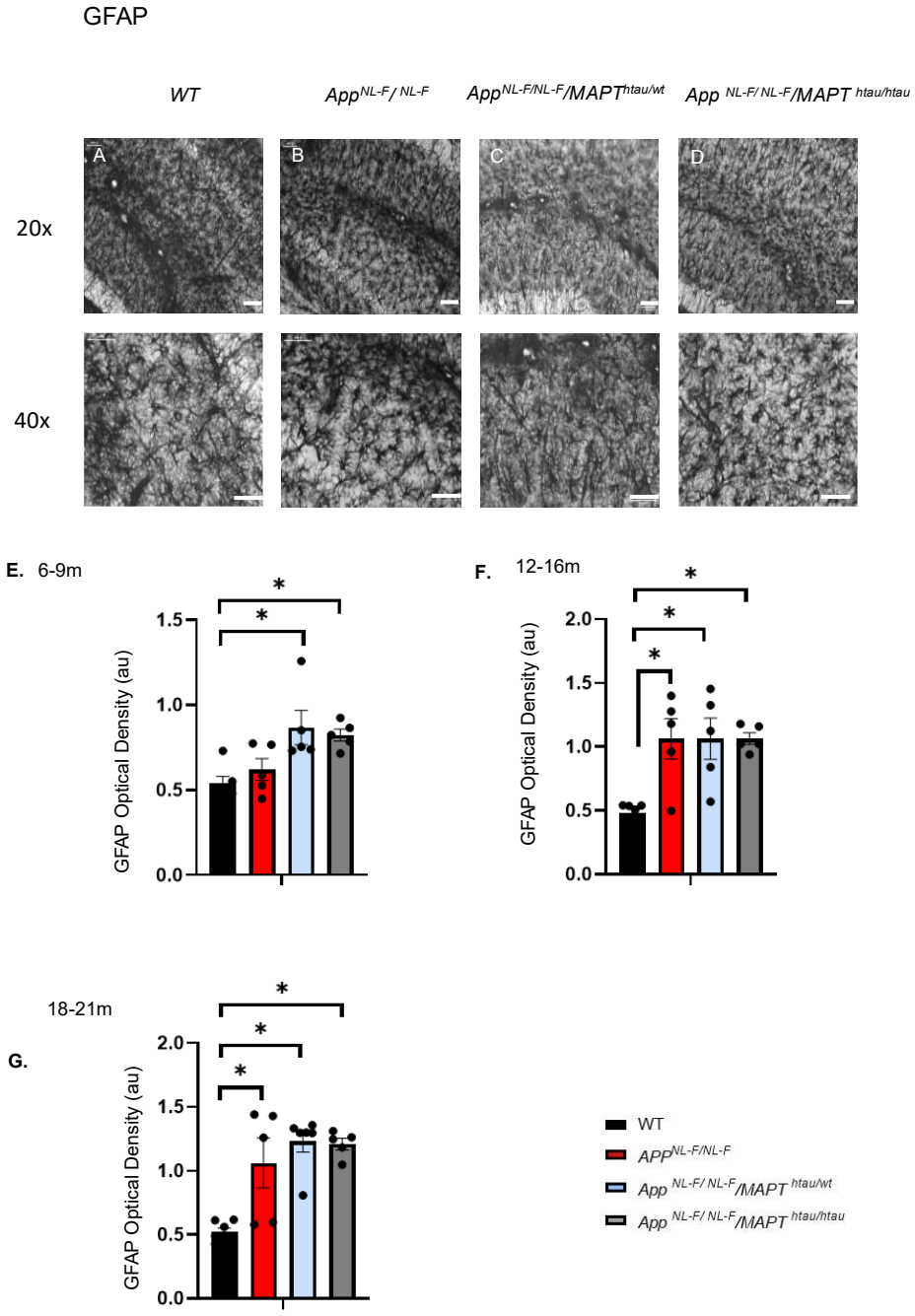


Figure 17. Immunoperoxidase staining showed the expression of astrocytes changes in the CA1 region of the hippocampus in 6-9m, 12-16m, 18-22m WT, *App* $^{NL-F}$ KI, *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ and *App* $^{NL-F}$*/MAPT* dKI mouse models. Images A-D illustrated the mean density changes of 4 markers under 20x and 40x lens of a light microscope. Astrocytes were marked by GFAP, of which the level showed the expression of astrocytes. The CA1 GFAP levels in *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mice (17E) was significantly higher than in WT mice (17F) (n=5, *P<0.05, Student's t-test). Results are expressed as mean ± SEM.

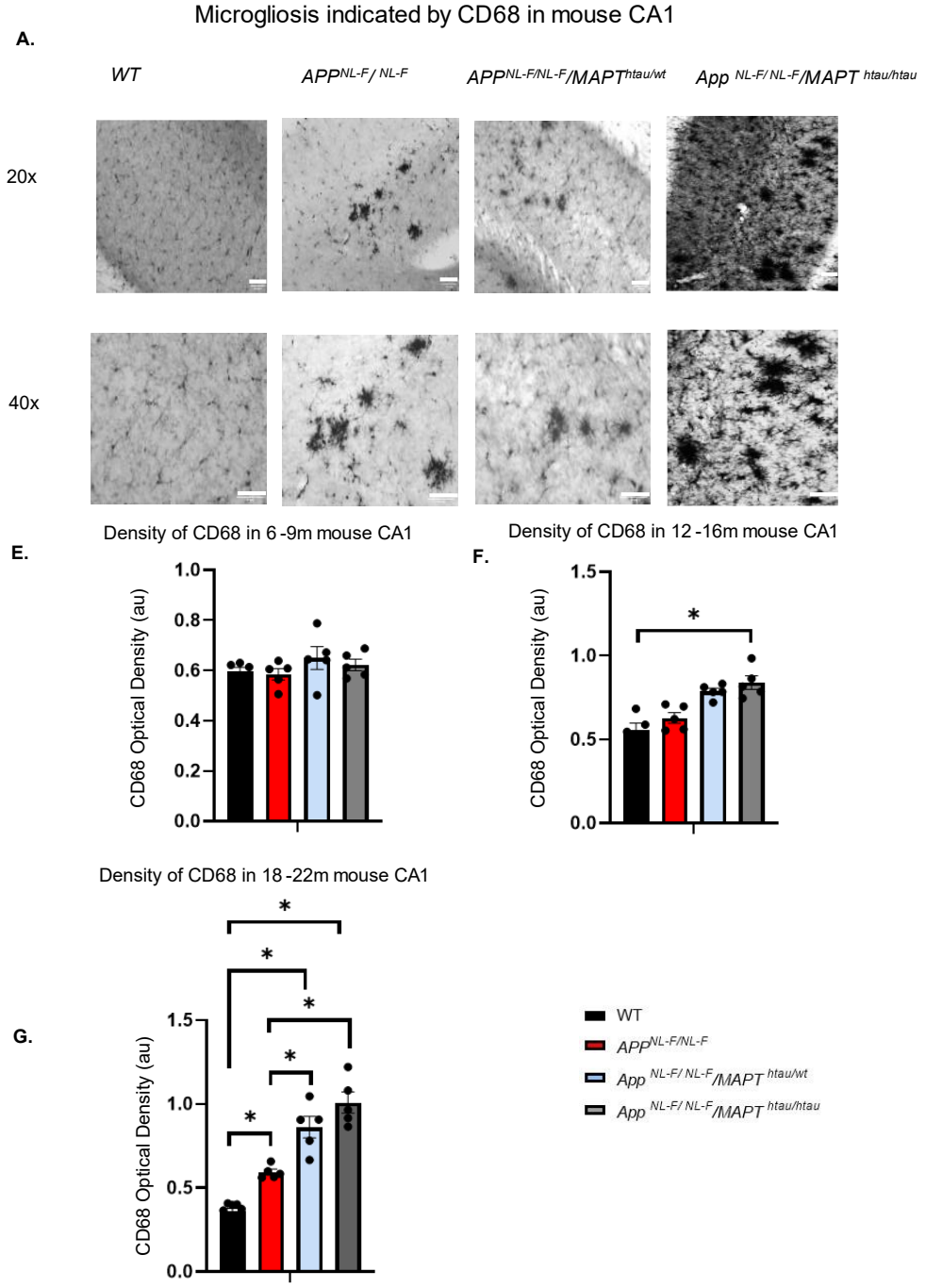


Figure 18. Immunoperoxidase staining showed the expression of microglia in the CA1 region of the hippocampus in 6-9m, 12-16m, 18-22m WT, *App* $^{NL-F}$ KI, *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ and *App* $^{NL-F}$ */MAPT* dKI mouse mouse models. Images A-D illustrated the mean density changes of 4 markers under 20x and 40x lens of a light microscope. Microglia cells were marked by CD68. *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mice showed relatively higher CD68 levels than the WT. However, the difference was not significant. In 12-16 months age group, there was a significantly larger amount of CD68 in *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ than WT mice. At 18-22 months age, 3 AD mouse models showed significantly higher amount of CD68 in CA1 area compared to age-matched WT mice. Results are expressed as mean ± SEM (n=5, *P<0.05, Student's t-test).

Table 6 Immunoperoxidase data of GFAP, CD68, Aβ, Tau expression in CA1 region of brains of WT, *App^{NL-F} KI, App $^{NL-F}$/MAPT$^{htau/wt}$ and App $^{NL-F}$ /MAPT dKI* mice at 6-9m, 12-16m and 18-22m old.

CA1	GFAP							
		Wild Type		*App^{NL-F}* KI		*App $^{NL-F}$/MAPT$^{htau/wt}$*		*App $^{NL-F}$ /MAPT dKI*	
		Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
	6-9M	0.540	0.098	0.621	0.145	0.867	0.224	0.823	0.079
	12-16M	0.484	0.106	1.063	0.355	1.063	0.360	1.064	0.102
	18-22M	0.522	0.075	1.061	0.438	1.232	0.409	1.21	0.102
	CD68								
		Wild Type		*App^{NL-F}* KI		*App $^{NL-F}$/MAPT$^{htau/wt}$*		*App $^{NL-F}$ /MAPT dKI*	
		Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
	6-9M	0.596	0.038	0.585	0.051	0.650	0.102	0.622	0.050
	12-16M	0.558	0.088	0.628	0.074	0.786	0.042	0.839	0.092
	18-22M	0.357	0.053	0.593	0.039	1.022	0.320	1.009	0.143
	ABETA								
		Wild Type		*App^{NL-F}* KI		*App $^{NL-F}$/MAPT$^{htau/wt}$*		*App $^{NL-F}$ /MAPT dKI*	
		Mean	±SEM	Mean	SEM	Mean	±SEM	Mean	±SEM
	6-9M	0.359	0.026	0.539	0.022	0.575	0.028	0.562	0.022
	12-16M	0.436	0.027	0.581	0.021	0.603	0.037	0.607	0.027
	18-22M	0.533	0.020	0.790	0.028	0.816	0.064	0.852	0.025
	TAU								
		Wild Type		*App^{NL-F}* KI		*App $^{NL-F}$/MAPT$^{htau/wt}$*		*App $^{NL-F}$ /MAPT dKI*	
		Mean	±SEM	Mean	SEM	Mean	±SEM	Mean	±SEM
	6-9M	0.597	0.099	0.595	0.049	0.63	0.163	0.626	0.048
	12-16M	0.606	0.034	0.578	0.051	0.659	0.068	0.674	0.046
	18-22M	0.605	0.052	0.759	0.023	0.742	0.101	0.723	0.060

3.3.3 Discussion: Contributions of glial cells and astrocytes to AD pathogenesis

This chapter aims to determine if there is a substantial difference in AD hallmarks such as reactive astrocytes, microgliosis, Aβ levels, and hyperphosphorylated tau levels between AD mouse models and WT at three age windows (6-9m, 12-16m, and 18-22m).

The result described in this chapter links these behavioural alterations to the traditional markers of Alzheimer's disease, namely neuronal cells responsible for neuroinflammation, astrocytes, and microglia.

Our study demonstrated significant increases in reactive astrocytes, microgliosis, Aβ, and hyperphosphorylated levels, which are consistent with the progression of AD in humans. (Liu et al., 2024; Self & Holtzman, 2023; Zhang, 2023). Both mouse models showed increased Aβ aggregation; however, only *App* $^{NL-F}$ */MAPT* $^{htau/wt}$ and *App* $^{NL-F}$ */MAPT dKI* animals showed an increase in tau hyperphosphorylation, consistent with earlier findings. The buildup of hyperphosphorylated tau in the CA1 region of *App*$^{NL-F}$ KI mice remained unchanged, comparable with earlier research utilising these models (Saito et al., 2014).

The relationship between astrocytes and microglia, as well as their significance in Alzheimer's disease, is even deeper, demonstrating that malfunctioning glial cells are more than just diseased byproducts. Indeed, both types of glial cells secrete apolipoprotein E (APOE) and TREM2, which are two important risk factors for Alzheimer's disease (Deczkowska et al., 2018; Guo et al., 2020).

Microglia play a crucial role in tissue homeostasis by engulfing and clearing debris. TREM2 is necessary for microglial phagocytosis of many substrates, such as apoptotic neurons, bacteria, LDL, lipoproteins, and Aβ (Atagi et al., 2015; Hansen et al., 2018; Kleinberger et al., 2014; Yeh et al., 2016). Aβ aggregates are more effectively absorbed by microglia when complexed with lipoproteins such LDL, APOE, and CLU/apoJ (Terwel et al., 2011; Yeh et al., 2016). . TREM2-deficient microglia exhibited decreased absorption of Aβ-lipoprotein complexes in vitro (Yeh et al., 2016) and less evidence of Aβ internalisation in vivo (Wang et al., 2016; Yuan et al., 2016). Currently there is one drug named ‘AL002’ targeting TREM2 receptors at phase 2 stage under clinical trials (Cummings et al., 2024).

Glial cells' ability to phagocytize Aβ also depends on the APOE isoform. APOE has three predominant alleles in humans; the ε2 (APOE2), ε3 (APOE3), and ε4 (APOE4) alleles (Raulin et al., 2022). APOE4 is a key genetic risk factor for AD in a gene dose-dependent manner,

increasing risk by up to 12 times in homozygotes, but APOE2 reduces AD risk by nearly half while also contributing to longevity (Corder et al., 1994; Serrano-Pozo et al., 2021). On the one hand, increased APOE4 synthesis affects astrocytic function, hence disrupting its immunological role in the CNS. For instance, carrying at least one copy of the APOE ε4 allele may impede the astroglial response to Aβ plaques, resulting in cognitive deterioration (Mahan et al., 2022; Mathur et al., 2015; Wang et al., 2021). APOE4 competes with LDLR and LRP1 on glial cells and neuronal surfaces, inhibiting Aβ clearance and leading to its accumulation, resulting in oligomerization and senile plaque formation (Litvinchuk et al., 2024; Theendakara et al., 2018). On the other hand, APOE4 inhibits Aβ breakdown by blocking astrocytic NEP and MMP-9, as well as the extracellular insulin-degrading enzyme (IDE). Some investigators have revealed that LOAD patients bearing the APOE4 allele, as opposed to APOE3 carriers, show a drop of roughly 50% in hippocampus IDE protein levels, which may explain the Aβ accumulation in this brain location (Abe et al., 2022; Anderson et al., 2022; Pires & Rego, 2023). Currently, there is a drug named ‘Masitinib’ targeting on the inhibition of mast cell and microglia/macrophage activities at phase 3 stage under clinical trials (Cummings et al., 2024).

APOE4 potentiates inflammatory cascades, modifies microglial phenotype towards a proinflammatory profile and promotes APP-mediated activation of microglia which induces neuroinflammation. APOE isoforms have been linked to elevated amounts of neurotoxic and inflammatory cytokines, including TNFα, IL-6, IL-1β, and nitric oxide, generated by microglia and astrocytes in various AD models. (Guo et al., 2020; Kloske & Wilcock, 2020; Pires & Rego, 2023). Currently, two AD drugs ‘LX1001’ and ‘Bumetanide’ target APOE4 at phase 2 stage under clinical trials (Cummings et al., 2024).

Furthermore, in people with AD and in AD mice, there is a distinct alteration in microglia transcriptome, indicating immunological activation (Gerrits et al., 2021; Holtman et al., 2015; Kamphuis et al., 2016; Orre et al., 2014). Reactive microglia display enhanced reducing of synapses through complement-dependent pathways, leading to excessive synapse loss early in AD and eventually cognitive impairment (Hammond et al., 2018; Kater et al., 2023; Kettenmann et al., 2013).

To conclude, abnormal activation of microglia and astrocytes during AD development is thought to be detrimental. Inhibiting the malignant glial response to aberrant Aβ and tau, as well as blocking pro-inflammatory cytokine release, may slow AD progression.

3.4 Results IV Alterations of GABA transporters in AD mice and humans.

To quantify the change in GABA content inside astrocytes, we labelled GAD67, an enzyme that catalyses the conversion of glutamate to GABA (see methods). In addition, we stained for the astrocyte-specific GABA transporter, GAT3/4, to explore the morphological alterations of astrocytes in AD. Figure 19 depicts the findings of the examination of immunofluorescence labelling (GFAP, GAD67, and GAT3/4) from mouse and human brain slices, including the CA1 and DG areas of the hippocampus.

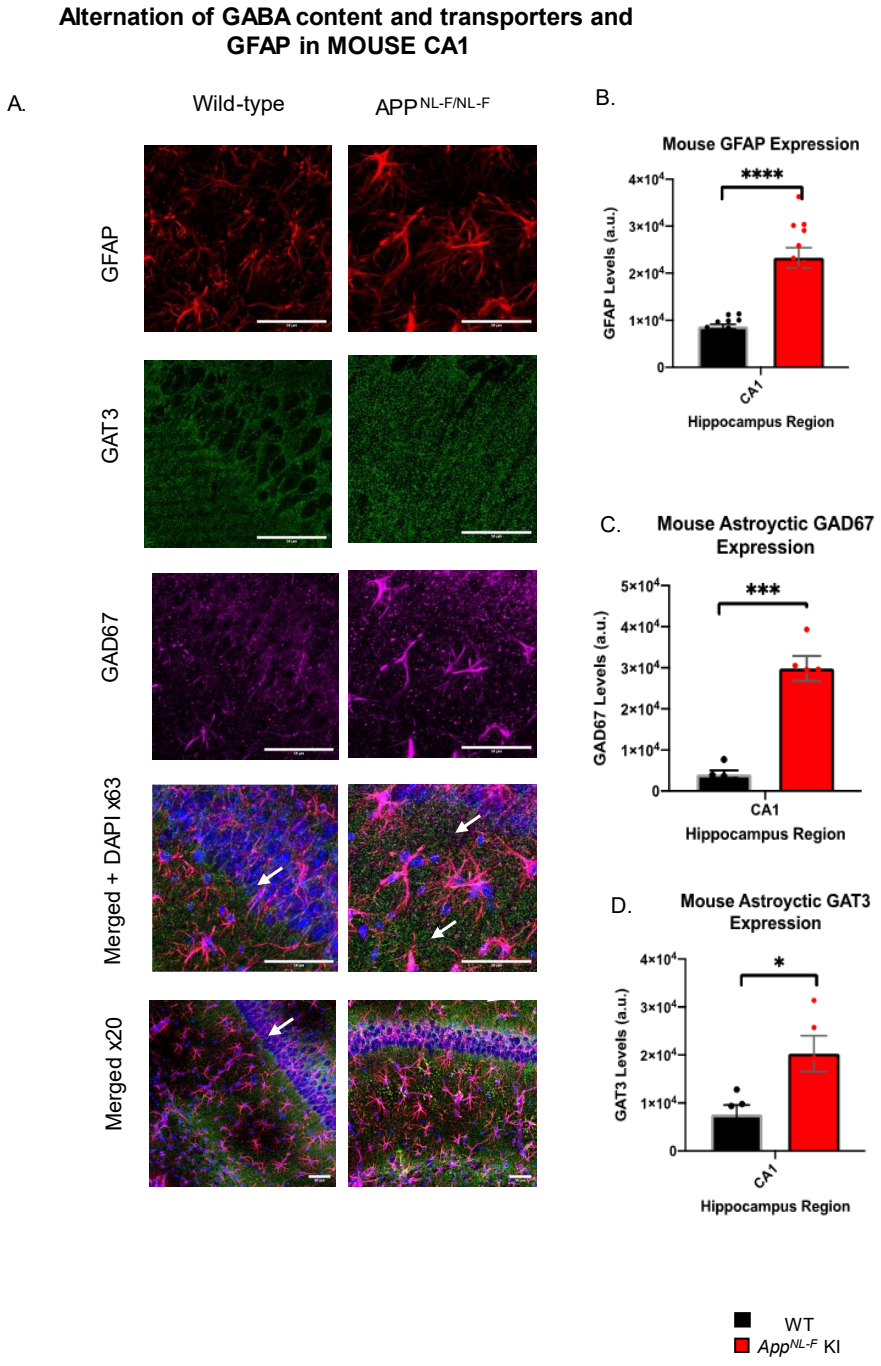
3.4.1 Increased GFAP, GAD67 and GAT3/4 GABA transporter in *App*$^{NL-F}$ KI

The results demonstrated a notable rise in GFAP levels in the *App*$^{NL-F}$ KI AD mouse model compared to age-matched WT mice, as shown in Figure 19. The average integrated density of GFAP considerably increased by 168.21 ± 15.40% in the CA1 region and by 157.34 ± 19.98% in the DG region (Figure 20). The same outcomes were observed in the CA1 and DG regions of human patients with AD, in comparison to the control group of human patients. GFAP levels in humans showed a significant increase of 338.50 ± 119.46% in CA1 and 368.90 ± 70.64% in the DG. The two-way ANOVA results showed a significant influence of genotype, but not of brain area, and no interaction between the two components. The Šidák's post-hoc multiple comparisons test revealed a significant statistical difference in the expression of GFAP in AD tissue. The difference was very significant in the mouse study (n = 12, ****p ≤ .0001, student's t-test) and significant in the human study (n = 10, **p ≤ 0.01, student's t-test).

Neurons and astrocytes both express GAD67, and the levels of GAD67 are elevated in AD tissue. We analysed GAD67 levels in reactive astrocytes. Šidák's post-hoc multiple comparisons test revealed a statistically significant difference between the genotypes, with no significant regional variation within or between them. An elevation of GAD67 was observed in astrocytes in AD tissue, suggesting higher GABA levels in reactive astrocytes in the CA1 and DG regions of the *App*$^{NL-F}$ KI mice compared to WT (Figure 19), by 638.04 ± 64.80% in CA1 and by 400.26 ± 44.24% in DG (n = 5, ****p < 0.0001, student's t-test). Average levels of GAD67 in astrocytes significantly increased in post-mortem brains of AD patients by 111.89 ±

17.76% in the CA1 region and 106.80 ± 7.86% in the DG region, compared to age-matched control human patients (n = 5, **p ≤ .01, student's t-test).

We investigated the levels of the GAT3/4 GABA transporter within astrocytes to understand how astrocytes regulate GABA and its impact on GABA homeostasis. Šidák's post-hoc multiple comparisons test revealed a significant difference in GAT3/4 expression between genotypes. The expression was significantly higher in App^{NL-F} KI mice compared to age-matched WT control mice by 165 ± 30.49% and 196.44 ± 17.91% in CA1 and DG, respectively (n = 5, **p ≤ 0.01, Student's t-test), as shown in Figure 19D. GAT3/4 levels were significantly higher in AD patients compared to control human tissue, with increases of 226.13 ± 58.09% in CA1 and 630.86 ± 79.33% in DG (n = 5, **p < 0.01, student's t-test), as shown in Figure 20. The two-way ANOVA findings showed no interaction between genotype and brain region.



1

2 Figure 19. The immunofluorescence image (63x) illustrated levels of GFAP, GAT3 and GAD67 in WT
3 and *App*$^{NL-F}$ KI mice and with stains of different colours: red (GFAP), green (GAT3) and purple (GAD67).
4 *App*$^{NL-F}$ KI mice showed levels of GFAP and GAD67 three times higher than the levels of WT mice.
5 Besides, WT mice showed approximately 50% of GAT3 levels than *App*$^{NL-F}$ KI mice (n=7, *P<0.05, ****
6 P<0.0001, *** P<0.001, Student's t-test).

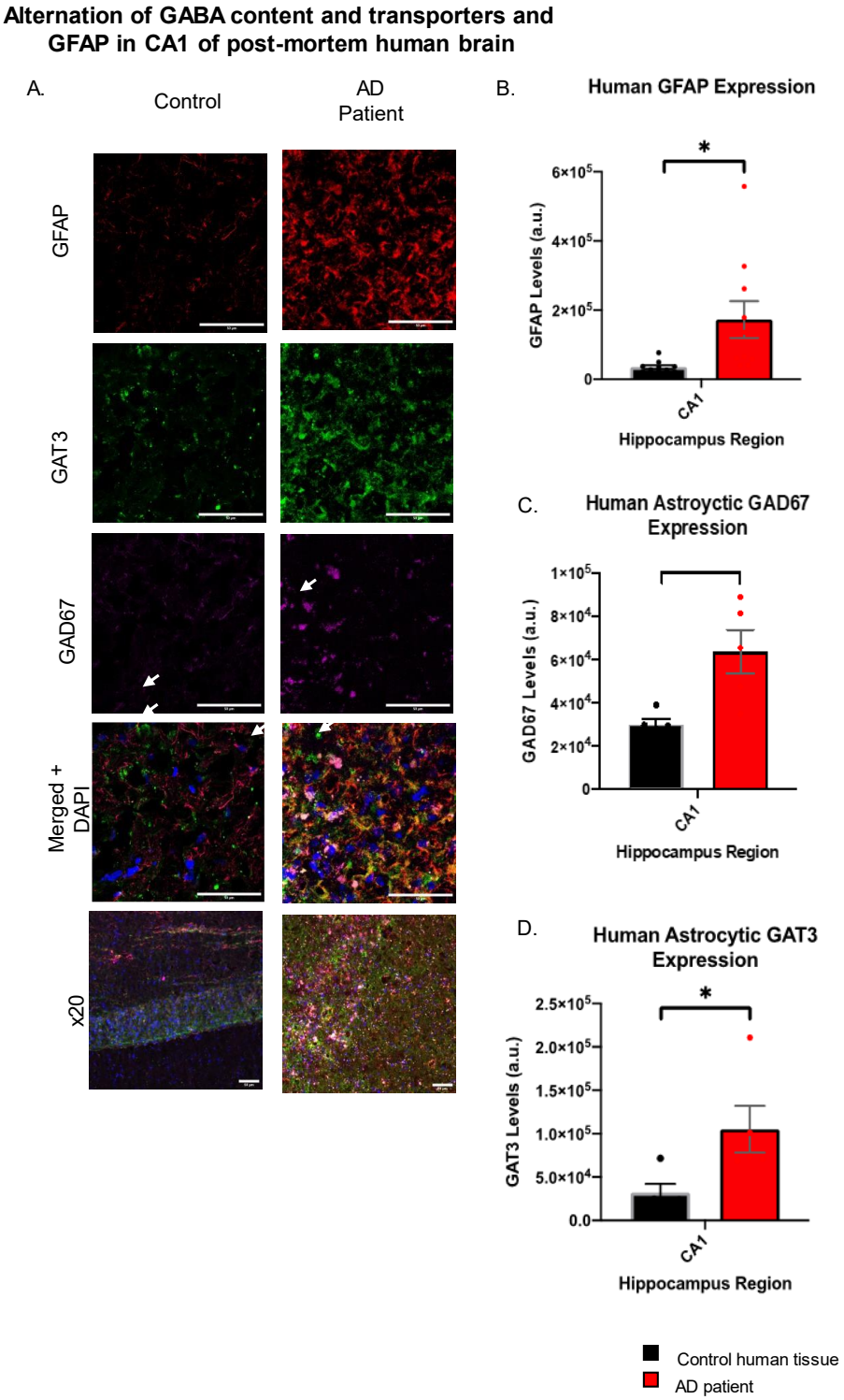


Figure 20. Immunofluorescence images data showing GFAP GAD67 and GAT3 levels in human brain tissue. A similar pattern was observed in human AD patient brain tissue, which showed a significantly higher level of GFAP, GAD67 and GAT3 than in control human brain tissue AD patients: No.4 and control patient No.14 (n=10, *P<0.05, ***P<0.001, Student's t-test).

3.4.2 Discussion: GABA dysregulations in AD

This study reveals new information about the possible role of the GABA transporter GAT3/4 in AD and provides mechanistic insight into the pathophysiology of AD in terms of synaptic imbalance caused by astrocyte-specific mechanisms that contribute to altered background tonic inhibition in the first knock-in *App*$^{NL\text{-}F/NL\text{-}F}$ mouse model of AD. Our key findings are consistent with other studies in the field, which show that reactive astrocytes cause an enhanced inflammatory response, which correlates with increased expression of GAD67 and GAT3/4 in hippocampal regions in AD models (Kiljan et al., 2019; Salcedo et al., 2021; Tang et al., 2020). Nonetheless, it should be highlighted that while GFAP is a fundamental component of most reactive astrocytes, its growth is not necessarily proportional to the severity of inflammation, particularly due to variances in basal GFAP levels and physiological responses (Aldabbagh et al., 2022; Escartin et al., 2021; Giovannoni & Quintana, 2020).

Our main findings show morphological changes, including an increased amount of the GAD67 enzyme expressed by astrocytes. This shows that astrocytes produce more GABA, which is connected with elevated levels of the astrocyte-specific GAT3/4 in both the CA1 and DG regions of the hippocampus in our AD animal model. This is consistent with our comparative investigations on postmortem human brain tissue from AD patients. Although some research implies that GAT3/4 is only expressed in astrocytes (Lee et al., 2006; Minelli et al., 1996), it is possible that these receptors are largely, if not solely, expressed in astrocytes. Therefore, in the present investigation, we assessed the expression of GAT3/4 solely from the astrocyte region as stained by GFAP. Under normal physiological settings, astrocyte-specific GAT3/4 maintains the 'proper' extracellular environment for neuronal activity and tonic inhibition, modifying network behaviour by removing excess GABA from the synaptic environment. Furthermore, the activity of GAT3 has also been shown to inhibit neuronal glutamate release via the activation of presynaptic adenosine A1 receptors due to an increase in intracellular astrocytic Na+ and Ca2+ through the Na/Ca exchange, leading to the subsequent release of ATP/Adenosine from the astrocyte (Salcedo et al., 2021; Scimemi, 2014). Taken together, these findings add credence to the theory of a general inhibitory deficiency in the AD brain, affecting many molecular processes within the GABA signalling system.

Excessive neuronal activity, which has been proven in ageing animal models to reflect GABAergic dysfunction, is also observed in ageing humans. Numerous task-based functional MRI investigations have found obvious neurological abnormalities in persons at risk for AD, in the early stages of AD, and with MCI, including increased brain activity in the hippocampus and medial temporal lobe areas (Dickerson et al., 2005; Jiménez-Balado & Eich, 2021).

Notably, neuronal dysfunction precedes anatomical atrophy in Alzheimer's disease, and this includes increased activity in the hippocampus (A. Ghit et al., 2021; Xu et al., 2020; Yassa et al., 2010). Furthermore, MCI patients exhibit greater activation of the hippocampus formation during episodic memory tasks than both healthy older adults and AD patients, implying that hippocampal hyperactivity may be stage-specific (Dickerson et al., 2005; Kircher et al., 2007; Klink et al., 2021). Furthermore, MCI patients with increased task-related medial temporal lobe activity had a higher risk of clinical decline following a two-year follow-up (Dickerson et al., 2004; Jiménez-Balado & Eich, 2021). Similarly, greater hippocampus hyperactivation has been demonstrated to correlate with cortical thinning in AD-signature regions in both cognitively intact and MCI patients, implying that hippocampal hyperactivity is related with other hallmarks of AD (Dickerson et al., 2009; Putcha et al., 2011). Animal studies confirm that neuronal activity leads to higher levels of Aβ and tau pathology (Li et al., 2021; Sosulina et al., 2021). The PiB distribution of Aβ accumulation correlates with enhanced network activity in AD patients (Snellman et al., 2023; Tian et al., 2022; Tian et al., 2023). As a result, hippocampus hyperactivity may be contributing to the emergence of the hallmark AD-related pathology in the early stages of AD, rather than simply being a correlate.

3.5 Results V: GABAergic Interneurons changes in AD

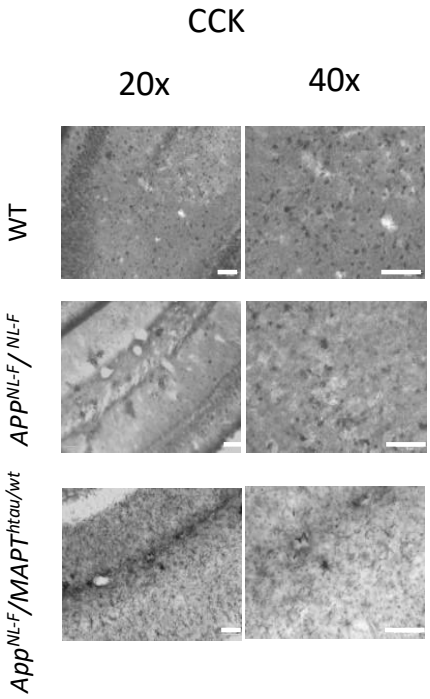
This chapter details the alteration of 3 major subclasses of interneurons during AD pathogenesis. The following three interneurons wereinvestigated: PV-, Cholycystokinin (CCK)- and CR- expressing interneurons. These interneurons have been chosen because they have different roles in the circuitry (see introduction 1.7), and our aim was to revealwhether these cells were more vulnerable to death in the *App*$^{NL-F}$ KI and/or *App* $^{NL-F}$/*MAPT*$^{htau/wt}$AD model compared to WT mice.

3.5.1 CCK-expressing cells decline in AD progression

CCK-expressing GABAergic neurons are perisomatic inhibitory cells that control the output and synchrony of principal cell populations. Studies have reported that significant loss of CCK-expressing neurons was revealed in AD human brains (Rehfeld, 2019). Decreased long-term potential is also accompanied by the loss of CCK-expressing interneurons and memory and learning ability decline (Plagman et al., 2019).

One recent study found no correlation between Aβ and CCK, while there was a strong connection between high tau level and high CCK level (Plagman et al., 2019). The hippocampus has a high concentration of CCK-specific binding in the brain. Hence it would be helpful to assess whether CCK is correlated to the high tau level in the new *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mouse model.

In Figure 21, immunoperoxidase staining shows levels of CCK-expressing interneuorns in 12-16 months old WT and *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice. WT mice showed nearly 2 times more CCK cells remaining in CA1 than *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice (n=7, ***P<0.001, **P<0.01, Student's t-test). 12-16m *App*$^{NL-F}$ KI mice showed 25% more CCK-expressing cell survival than *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice in CA1 (n=7, *P<0.05, Student's t-test). Similar results were found in AD postmortem brain tissues when compared to control counterparts (Figure 22). There was a 60% increase in CCK levels in control human brain tissue than in AD postmortem brain tissue (n=7, **P<0.01, Student's t-test). The reduction of CCK levels might indicate that they are losing their inhibitory GABAergic functions during the progression of AD (Figure 21,22).



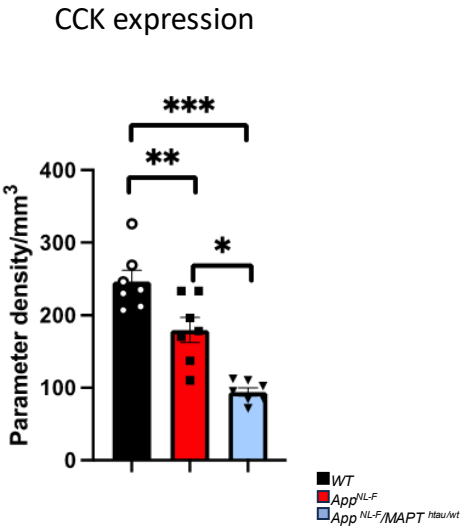
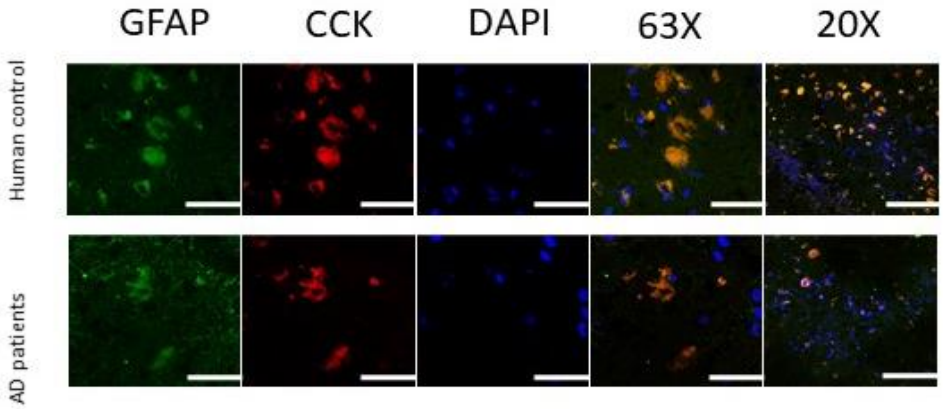


Figure 21. CCK-expressing cells decline in 12-16m transgenic mice *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ compared with WT mice. WT mice showed significantly higher CCK cell survival than *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice. *App*$^{NL-F}$ KI mice had significantly higher CCK cell amounts than *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice (n=7, *P<0.05, **P<0.01, ***P<0.001, Student's t-test).



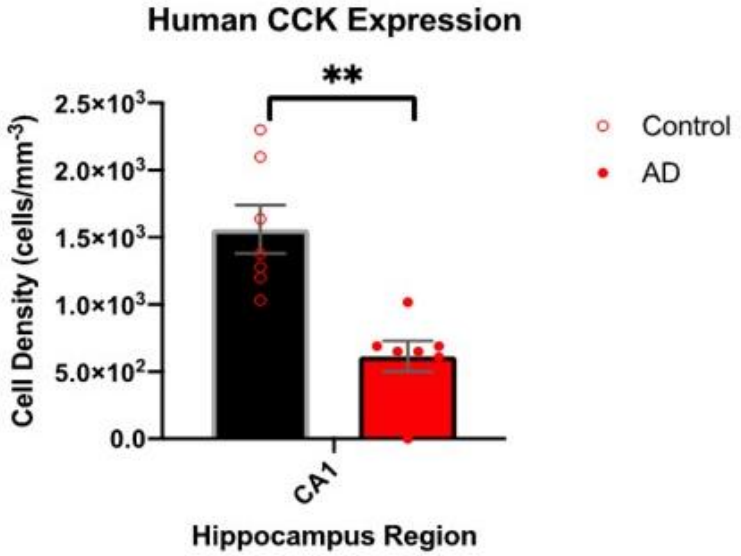


Figure 22. CCK-expressing cells declined in CA1 in AD patients than in human control brain tissue. The immunofluorescence image illustrated the amount of CCK under 63x and 20x lens in CA1 of human control and AD patients. The control group showed a markedly higher CCK-expressing cell amount than AD patients (*n*=7, ***P*<0.01, Student's t-test). GFAP was labelled with Alexa 488, while CCK was labelled with Texas Red.

3.5.2 CR-expressing interneurons exhibit resistance to AD progression

Immunohistochemical studies of post-mortem tissue from human AD patients show a preservation of CR density throughout the affected brain (Fonseca & Soriano, 1995; Hof et al., 1993; Anqi Shi et al., 2020), suggesting that they are unaffected in AD compared to healthy control. The discharge and pattern of synchronisation of inhibitory interneurons can be regulated by other interneurons specialised in making interneuron-interneuron connections, such as CR-expressing interneurons (Acsady et al., 1998). Although CR expressing interneurons were first characterised many years ago (Freund & Gulyás, 1997), there is a missing gap in the understanding of these specialised disinhibitory cells. Interestingly, post-mortem human studies in tissue with heavy amyloid deposits show that CR interneurons are preserved in AD (Hof et al., 1993). Furthermore, a previous study in our lab has suggested that the density, morphology, and function of CR-expressing interneurons studied in *App*$^{NL-F}$ KI mice were not altered, nor did they contain soluble Aβ, while CCK and SOM-expressing interneurons showed degeneration and Aβ penetration (A. Shi et al., 2020).Therefore, it was of interest to investigate this further in our new *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mouse model and compare these finding to the *App*$^{NL-F}$ KI model age-matched to healthy WT neurons. Immunoperoxidase images illustrated the levels of CR and PV-positive cells in CA1 in *App*$^{NL-F}$ KI and *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mice.

Figure 23 illustrated the morphology of CR cells under magnifications of 20x and 63x under a confocal microscope. In Figure 24, 12-16m WT mice showed similar number of CR-expressing interneurons in CA1 compared to *App*$^{NL-F}$ KI and *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mice respectively (n=5, P>0.05, Student's t-test).

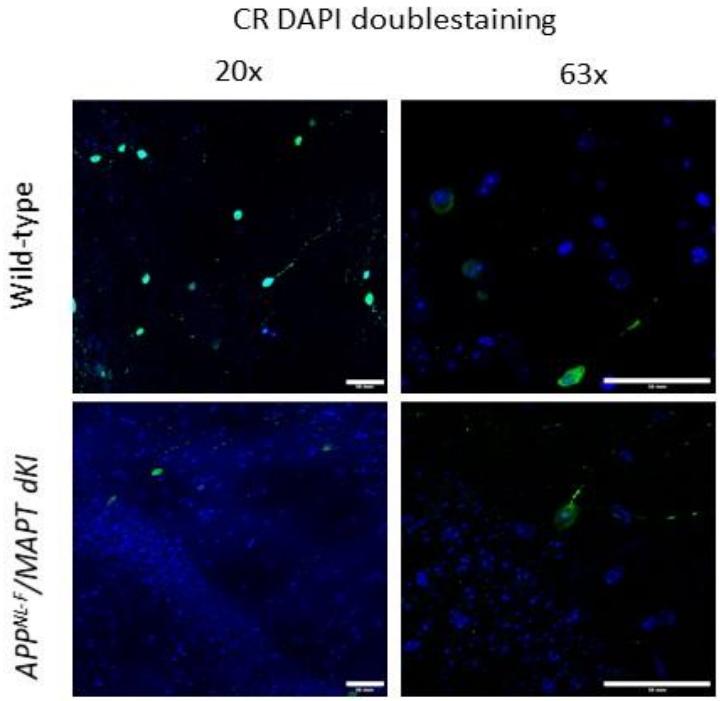


Figure 23. A significant drop in CR and PV-expressing cells was seen in transgenic mice compared with WT mice. 20X immunoperoxidase image illustrated different CR and PV- expressing cell levels in WT, *App*$^{NL-F}$ *KI* and *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mice. CR was labelled with a green Alexa488.

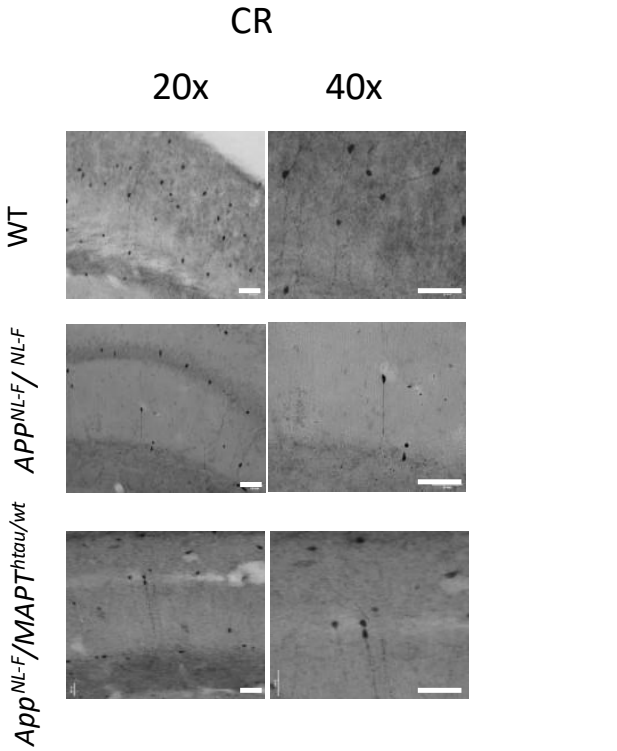


Figure 24. Immunofluorescence images illustrated the morphology and level of CR expressing interneurons in CA1 in WT, *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice. There was no difference in CR-expressing interneurons density in WT compared to *App*$^{NL-F}$ KI mice and *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice. (n=5, P>0.05, Student's t-test).

3.5.3 Reduced density of PV-expressing interneurons in AD

One of the GABAergic interneurons known as PV-expressing interneurons are distributed throughout the brain and exhibit a variety of unique anatomical and physiological traits. PV interneurons possess unique morphological characteristics that enable them to regulate microcircuits. Their extensive dendrites span multiple layers in both the hippocampus and the cortex (Hijazi et al., 2023; Hua Hu et al., 2014). They receive excitatory input from various regions, including the entorhinal cortex and medial septum in the hippocampus, as well as thalamic inputs in the cortex. Additionally, they receive excitatory signals from numerous local neurons within different hippocampal areas and cortical layers (Hafner et al., 2019; Kawaguchi et al., 2019; Swanson & Maffei, 2019; Tremblay et al., 2016). PV interneurons play a critical role in maintaining the equilibrium between excitation and inhibition (E/I) (Atallah & Scanziani, 2009; Campanac et al., 2013; Nanou et al., 2018; Poo & Isaacson, 2009; Wilent & Contreras, 2005; Wu et al., 2008). Furthermore, the accurate activation of pyramidal neurons in response to sensory stimulation relies on the feed-forward inhibition mediated by PV neurons, in addition to their role in maintaining homeostatic balance at baseline (Atallah & Scanziani, 2009; Stark et al., 2013). Hippocampal place cell activity is produced by the interplay between dendritic excitation caused by pyramidal cells and perisomatic inhibition caused by PV interneurons. When PV interneurons are optogenetically silenced, the firing rate of pyramidal neurons in their specific locations increases during behaviour. As a result, Failure to malfunction PV neurons have been linked to a number of illnesses, such as Alzheimer's disease (AD), that include network changes and cognitive impairment (Royer et al., 2012; Wehr & Zador, 2003).

In Figure 25, WT mice showed a 50% increase in the amount of PV-expressing interneurons in CA1 compared with *App*$^{NL-F}$ KI mice and *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mice at 12-16 months old (*n*=*5*, **P*<0.05, Student’s t-test). Reduced PV suggested a loss of their GABAergic inhibitory effect on modulating excitatory inputs to pyramidal cells.

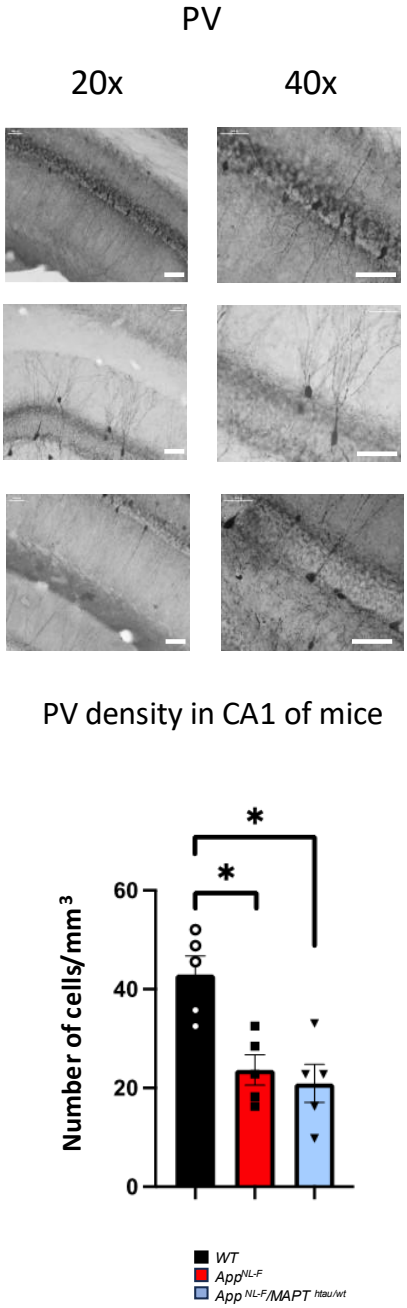


Figure 24. Immunofluorescence images illustrated the morphology and level of PV-expressing interneurons in CA1 in WT, *App*$^{NL-F}$ KI and *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice. WT mice showed a 50% increase in the number of PV-expressing interneurons in CA1 compared with *App*$^{NL-F}$ *KI* mice and *App* $^{NL-F}$/*MAPT*$^{htau/wt}$ mice at 12-16 months old (n=5, *P<0.05, Student's t-test).

3.5.4 Discussion:

In this chapter, it was the alteration of the expression of three sub-types of interneurons, CCK-CR- and PV- expressing interneurons during AD progression was investigated in the App and tau mouse models of AD and compared to the WT mice in the late stages of the disease, once the hallmarks were evident (12-16 months).

The key findings here include:

1. There was no significant difference in CR expression levels among WT and AD mouse models at 12-16m.
2. CCK and PV expression levels decreased significantly in *App^{NL-F} KI* and *App^{NL-F}/MAPT$^{htau/wt}$* mice.

3.5.3.1 Preserved number of CR cells in AD mice models

The unchanged CR expression levels in WT, *App^{NL-F} KI, and AppNL-F/MAPThtau/wt mice shown* in our results were similar to previous findings from our lab. There was no significant difference in number of CR cells in 1-3m, 4-6m and 9-18m WT and *App^{NL-F}* KI mice (Anqi Shi et al., 2020) . In addition, no significant difference was found in the expression of CR in CR-positive cells in 3-Tg AD mice at 18 months old (Zallo et al., 2018). However, not all AD mice models showed the preservation of CR cells. For example, in 3 and 12-month-old APPswe/PS1dE9 mice, there was a significant decrease in a number of CR cells in DG (Verdaguer et al., 2015). Similar decrease is also shown in 5XFAD and Tg2576 mice models (Giesers & Wirths, 2020; La Barbera et al., 2022).

Although CR modulates presynaptic signalling and Ca2+ transients to protect against excitotoxicity, CR neurons are nevertheless vulnerable to injury in some situations, such as ischemia Although CR modulates presynaptic signalling and Ca2+ transients to protect against excitotoxicity, CR neurons are nevertheless vulnerable to injury in some situations, such as ischemia (Freund & Maglóczky, 1993). Due to its quick affinity for Ca2+ binding, CR may be crucial for a number of critical processes, including nervous system development (Brandt et al., 2003; Dargan et al., 2004). It has been demonstrated that CR neurons become denser in the rat cortex as it develops. This demonstrates the critical function of CR as a neuronal growth guide during the early phases of neuronal differentiation (Brandt et al., 2003).

Given its involvement in dendritic elongation, remodelling, cytoskeletal element production, and synaptogenesis (Abraham & Meyer, 2003; Anstötz et al., 2018; Martínez-Cerdeño & Noctor, 2014), CR may be a developmental marker. Nevertheless, these roles in development remain to be established. Additionally, it has been discovered that CR is elevated in areas of the brain where neurons are destroyed (Yang et al., 2008) and serves as a marker for adult neurogenesis (Brandt et al., 2003). Adult hippocampal neurons in post-epileptic animals exhibit CR immunoreactivity, indicating that this protein is crucial for the development and differentiation of new neurons (Ueno et al., 2019). These findings suggest that CR plays a critical function in the development of neonates. However, no problems related to brain or retinal development have been reported in transgenic CR-knockout mice (Gurden et al., 1998). This result was explained by the hypothesis that either CR is not essential for development or its lack is offset by the overexpression of other CaBPs. However, more investigation is required to verify these theories. While CR-knockout mice do not exhibit developmental problems, there has been evidence of a reduction in long-term potentiation (LTP) in the dentate gyrus (DG) (Gurden et al., 1998; Schurmans et al., 1997), indicating a potential function for CR in memory formation and retrieval. Later research revealed that the development of unpleasant memory involves CR neurons in the lateral thalamus (Barsy et al., 2020). Furthermore, due to a lack of CR modulation of Ca2+ inside Purkinje cells, older mice of CR-knockout mice exhibit decreased motor control (Schiffmann et al., 1999). These findings demonstrate the significance of CR in cognitive processes. When combined, these show how crucial CR is to the brain. It's still unclear, though, what the most recent study on CR and AD. Thus, treating neurological conditions like AD requires an understanding of the role of CR in neural regulation.

3.5.3.2 Reduced number of CCK interneurons in AD mice models

Meanwhile, decreased levels of CCK have been shown in recent research. It has been demonstrated that ageing reduces the amount of binding sites in the rat hippocampus and cerebral CCK expression (Greenstein et al., 1991; Harro & Oreland, 1992; Plagman et al., 2019; Reich & Hölscher, 2024). Furthermore, compared to age-matched WT littermates, the APP/PS1 mice's hippocampal CCK mRNA levels were reported to be halved, indicating that a lack of CCK may predispose to neurodegeneration and negatively influence cognition in AD(Liu et al., 2021). In fact, more severe stages of AD have been linked to malfunction of the local source of CCK, the hippocampus CCK+ interneurons (Reid et al., 2021). Post-mortem investigations in AD patients, like those in AD animals, have shown that CCK expression in

the cerebral cortex is lower in AD patients than in healthy controls (Perry et al., 1981). In certain cortical regions, but not in others, there is a 24–38% downregulation of CCK immunoreactivity (Mazurek & Beal, 1991). Nonetheless, it has also been documented that AD patients' cerebral cortical levels of CCK derivates or CCKR binding are unaffected (Löfberg et al., 1996). Significantly, increased CSF levels of CCK were positively associated with a lower risk of AD and MCI, improved cognitive function, and increased grey matter volume in multiple brain regions, including the medial prefrontal cortex, posterior cingulate cortex, and parahippocampal gyrus (Plagman et al., 2019). Higher CCK levels were not associated with a memory-improving effect when Tau and phospho-Tau (Thr181) levels were connected with CSF CCK pools. Genetic screenings in MCI or AD patients have revealed a downregulated expression of cerebral CCK-1Rs and hippocampal CCK-2Rs in comparison to healthy controls (Hokama et al., 2014; Lin et al., 2014), which is consistent with the positive effect of CCK. Additionally, a correlation has been found between hippocampus CCK expression and cognition(Liu et al., 2021).

Recent studies have shown the neuroprotective effects of cholecystokinin in Alzheimer's disease. According to Zhang et al. (2013), CCK-8S exposure for several weeks increased the number of dendritic filopodia and spines in hippocampal CCK neurons generated WT and APP/PS1 mice in a way that was dependent on CCK-2R (L.-l. Zhang et al., 2013). In addition to altering membrane characteristics, CCK-8S treatment significantly lowered synaptic inhibition while promoting excitatory synaptic transmission, firing frequency, and postsynaptic density protein-95 (PSD-95) expression. This implies that CCK may be able to alleviate AD synaptic pathology. Another pathogenic event in the cortex (and other brain regions) in AD is glutamate-, kainate-, or NMDA-induced excitotoxicity (Akaike et al., 1991; Tamura et al., 1992). There is evidence that cortical CCK-2R activation protects against this (Chen et al., 2021; Ong et al., 2013).

In the hippocampus, Gαq/11-recruiting CCK-2Rs are expressed on excitatory pyramidal neurons as well as parvalbumin+ and CCK+ basket cells (Lee et al., 2011). Other study has shown a CCK-2R-binding, unsulphated CCK-8 homologue exhibits neuroprotective effects in an Aβ-based animal model of AD (Reich & Hölscher, 2024). A carboxyfluorescein-labelled and proteolytically resistant CCK analogue penetrated the blood-brain barrier (BBB) following injection and diffused into the cortex and hippocampal regions (Zhang et al., 2023). Long-term potentiation was restored in CCK analogue-treated APP/PS1 mice compared to untreated control, as were hippocampal dendritic spine density, synapse numbers, morphology, and several synaptic proteins (microtubule-associated protein 2, synaptophysin, and postsynaptic density protein 95 (PSD-95) (L.-l. Zhang et al., 2013; Zhang et al., 2023). Improved working

memory (Y Maze), exploratory behaviour, and spatial learning and memory (Morris Water Maze) were all the outcomes of synaptic protection. Reduction of Aβ1-42 generation and deposition was one of CCK's pro-cognitive Furthermore, CCK treatment restored the hippocampus's downregulated phosphorylation (activation) of the PI3K/Akt and PKA/cAMP response element-binding protein (CREB) pathways as well as the expression of BDNF and tyrosine kinase B (TrkB) in contrast to untreated APP/PS1 mice effects (Hao et al., 2024; Zhang et al., 2023).

In APP/PS1 mice, the CCK analogue further prevented morphological alterations in the hippocampus mitochondria (Hao et al., 2024). Reduced loss of dopaminergic neurons, their processes, and motor function in the 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (PD) as well as reduced mitochondrial damage and fragmentation, oxidative stress, and cognitive decline in the APP/PS1 mouse model) have been associated with genetic or pharmacological inhibition of Drp-1 (Baek et al., 2017; Cannavino et al., 2015; Filichia et al., 2016; Huang et al., 2019). CCK protects against pathogenic hyperfission seen in AD animal models and people by inducing the AMPK/PGC-1α pathway to restore mitochondrial fusion/fission dynamics. Improved synaptic indicators, cognition, mitochondria, and protein pathway activation—all of the previously described effects—were shown to be dependent on hippocampus CCK-2Rs by the use of a CCK antagonist and intrahippocampal receptor knockdown (Hao et al., 2024; Zhang et al., 2023).

AMPK activation inhibits mammalian target of rapamycin complex 1 (mTORC1) and, hence, induces autophagy (Li & Chen, 2019). This effect probably results in the Aβ degradation observed in APP/PS1 mice treated with CCK-8 analogue (Hao et al., 2024; Zhang et al., 2023). Another study demonstrated that CCK-8S treatments increased neural stem cell proliferation (Ki-67), while reduced apoptosis, in the rat dentate gyrus (Reisi et al., 2015). Thus, it has been demonstrated that CCK-mediated promotion of glutamate release by local astrocytes and inflammatory regulation of these astrocytes may support hippocampus regeneration (Asrican et al., 2020). Because indirect CCK-1R-signalling across vagal afferent nerves triggers hippocampal BDNF and NGF production in vivo (Tirassa & Costa, 2007; Tirassa et al., 1998), sulphated CCK-8 mimetics could show better effects regarding memory and/or neuroprotection, but at the cost of more peripheral side effects (i.e. gallballder complications) (Rehfeld, 2019).

To conclude, our studies have shown reduced CCK cells in AD mouse models compared to WT. Investigating on CCK could help to develop a therapeutic treatment for AD patients.

3.5.3.3 Reduced number of PV interneurons in AD mice models

Our data showed a decreased number of PV interneurons in AD mice models in CA1 of the hippocampus. Similar results could be found in different studies. One study showed a deficit of PV+ cells in a Tg2576 mouse model as young as 3 months of age (Cattaud et al., 2018; Huh et al., 2016). However, there are some studies which showed no changes in PV expression levels in AD mouse models (Hijazi, Heistek, Scheltens, et al., 2020; Lemmens et al., 2011; Sanchez-Mejias et al., 2020). For example, one study showed that PV-expressed interneurons were resistant to neurodegenerative in the subiculum in the AβPP/PS1 model (Trujillo-Estrada et al., 2014). The reported decrease in PV neurons number may represent a decrease in PV expression rather than a loss of neurons since restoring GABAergic transmission via chemogenetics, optogenetics, genetic manipulations, or even interneuron transplants can rescue network function and behaviour (Chen et al., 2010; Etter et al., 2019; Hijazi, Heistek, Scheltens, et al., 2020; Lu et al., 2020; Petrache et al., 2019). To find out if PV neurons are more vulnerable to neuronal death in AD, more research on the relationship between PV expression and GABAergic activity in AD, as well as PV neuron survival, is required. Though other interneurons may also be involved in this dysfunction, a consistent finding across all these studies is a decrease in inhibitory transmission and activity in AD, which is likely caused by a specific impairment of PV neurons. This dysfunction leads to a failure of inhibitory control, oscillatory changes, an overall increase in excitation and epileptic activity, and cognitive decline (Chung et al., 2020; Delorme et al., 2021; Schmid et al., 2016). Although the research mentioned above shows that PV neurons are a desirable and highly relevant target for improving memory and network function in AD, it is still unclear whether PV neuron impairment is the root cause or an effect of AD pathogenesis. It is possible that intrinsic alterations in channel characteristics or expression that are dependent on Aβ are the cause of PV neuron dysfunction. On the other hand, PV neurons may experience impairments due to altered afferent synaptic inputs, poor synaptic transmission onto PV neurons, or a general high vulnerability brought on by their special fast-spiking characteristics. More understanding of the initial changes to PV neurons in AD may assist to clarify this matter.

In recent studies, they showed that at 3 months of age, hippocampus PV neurons are initially and momentarily hyperexcitable, but not pyramidal neurons. Pyramidal neurons were hyperexcitable, while PV neurons looked to be hypoactive at a later stage of the disease, which is about 7 months of age. Crucially, Morris water maze performance in APP/PS1 mice was restored by either suppressing early PV neuron activity or increasing it subsequently. This suggests that both states—hyperexcitable initially and hypoexcitable afterwards—are causally

related to memory impairment in AD (Hijazi, Heistek, Scheltens, et al., 2020; Hijazi et al., 2023; Petrache et al., 2019). All of these results point to biphasic changes in PV neuron activity in AD mice during amyloidosis and possibly distinct mechanisms of memory impairment at various illness phases. Fascinatingly, biphasic changes in inhibitory transmission have also been documented in previous research (Hollnagel et al., 2019; Kiss et al., 2016), and patients' biphasic changes in network connection have been noted as AD progresses (Nakamura et al., 2017; Pusil et al., 2019).

In order to investigate whether early PV hyperexcitability contributes to AD progression, Hijaze and his colleagues selectively chemogenetic activate WT mice's PV neurons, causing them to become hyperexcitable artificially to mimic the lasting AD-like hippocampus network state (Hijazi, Heistek, van der Loo, et al., 2020). PV neurons underwent a permanent hyperexcitable state under these circumstances, and they also exhibited increased sensitivity to a low-dose intrahippocampal injection of Aβ. Under the condition of induced PV hyperexcitability, a low concentration of Aβ was able to cause PV neurons to become hypoexcitable, increase the frequency of pyramidal neuron firing, disrupt synaptic transmission onto pyramidal neurons, and significantly impair spatial memory. However, infusing the same concentration of Aβ into healthy mice had no effect on cells or behaviour. In addition, inhibited PV neuron activity restored synaptic transmission and intrinsic properties of both PV neurons and pyramidal neurons as well as spatial memory(Hijazi, Heistek, Scheltens, et al., 2020). These results imply that, under AD-like settings of elevated Aβ levels, early PV neuron hyperexcitability may be a major mechanism inducing network and memory deficits in AD.

Since early restoration of PV neuron activity led to a decrease in amyloid pathology and preventing Aβ generation specifically in GABAergic neurons dramatically reduced plaque pathology, PV neuron hyperexcitability may also play a role in amyloid pathology (Hijazi, Heistek, Scheltens, et al., 2020; Iaccarino et al., 2016; Rice et al., 2020). In fact, APP is highly expressed in a subset of hippocampal interneurons; in various AD animal models, hyperphosphorylated tau and Aβ were observed to accumulate in PV neurons, and 53% of PV cells in the CA1 area of the hippocampus are APP-positive (Dávila-Bouziguet et al., 2019; Höfling et al., 2019; Rice et al., 2020).

3.6 Results VI: Age-dependent alteration of extrasynaptic δ-$GABA_A$R expression in *App*$^{NL-F}$ KI, *App* $^{NL-F}$ */MAPT* $^{htau/wt}$ and *App* $^{NL-F}$ */MAPT* dKI mouse models of AD

The α1–3, β1–3 and γ2 receptors are the most often found subtype of $GABA_A$ receptors and are found synaptically. The chloride influx hyperpolarises the cell membrane and blocks the transmission of action potentials after $GABA_A$ receptor interacts with GABA for a few milliseconds. Another name for this brief fast-responding inhibition is phasic inhibition. At extrasynaptically situated GABA receptors, the γ subunit is replaced by the δ, ε, and π subunits (Amr Ghit et al., 2021; Glykys et al., 2008; Olsen, 2014). With their longer-lasting chloride currents dispersed over a wide area, such as the neuronal cell body, as opposed to currents lasting milliseconds at single synapses, extrasynaptic receptors mediate a significant portion of the total GABA-mediated inhibition (Cheng et al., 2024; Farrant & Nusser, 2005). Known also as tonic inhibition, this kind of slow continuous inhibition is triggered by ambient GABA levels. Unlike phasic inhibition, which relates to the fast synchronous opening of a relatively small number of GABA channels on the postsynaptic membrane within the synaptic cleft thus limiting the inhibition in time and space in response to an action potential at a certain synapse, tonic inhibition is constant over time and space and regulates a huge area, maybe a network of neurons rather than just a single cell (Amr Ghit et al., 2021; Wang, 2011).

Since several investigations have shown reduced GABA levels in the CSF and temporal cortex of AD patients, indicating a fundamental inhibitory malfunction, GABA is a major factor in AD pathogenesis (Czapski & Strosznajder, 2021; Li et al., 2016; Nam et al., 2023). Actually, it has been demonstrated that practically every aspect of the GABAergic system in the AD brain—including GABA levels, GABA receptor expression levels, and the GABAergic neuronal system—is adversely impacted (Ali et al., 2023; Limon et al., 2012)Changes in the composition and expression of the GABA receptor subunit may be the biological component that ties AD aetiology and GABAergic dysfunction together.

3.6.1 Reduced δ-$GABA_A$Rs in AD mouse models and AD postmortem brain tissue

Preclinical research examining the neuropsychology of AD has primarily focused on memory impairments such as declines in spatial learning and memory. Nevertheless, apart from the deterioration in cognitive function, several studies suggest that the initial phases of the ailment are characterised by neuropsychiatric manifestations, including psychological discomfort and mood disturbances. Therefore, we hypothesize that the extrasynaptic δ-subunit containing $GABA_A$ receptors (δ-$GABA_A$Rs), found on selective interneurons, known to have an emerging role in mood disorders, are altered during AD pathogenesis that manifests in the AD-associated memory loss and neuropsychiatric symptoms.

The expression of extrasynaptic δ-subunit-containing $GABA_A$Rs was analysed by immunoperoxidase labelling of CA1 and DG areas at three age windows in the three AD models compared to age-matched WT animals (Figure 26). These experiments were expanded using immunofluorescence studies to determine whether two subclasses of interneurons co-expressed these receptors (see Figures 26, 27 and 28 below).

Extrasynaptic δ-subunit-containing $GABA_A$R subunits were expressed in all layers of the CA1, including stratum oriens (SO), stratum pyramidale (SP), stratum radiatum, and stratum lacunosum moleculare (SLM). However, there was a higher expression in the SO/SP boundary and SR/SLM (Figure 26A). Neurons expressing δ-$GABA_A$ R were detected throughout the DG, with a larger abundance near the intersection of the stratum granulosum and polymorphic layer (Figure 26B). These receptors were expressed as a halo around neurons' somata and on their proximal dendrites.

Although the δ-subunit naturally declines with age in both CA1 and DG, all AD mouse models showed a significant age-dependent alteration in the expression of the δ-$GABA_A$R subunit (Figure 26 C-D) compared to the WT, with the most significant change occurring at the oldest age range of 18-22 months. In App^{NL-F} KI mice, there was a greater change in the CA1 compared to the DG. The expression of these receptors measured from immunofluorescence z-stack images (optical density, a.u.) was 1.4 ± 0.35, compared to the WT which was 3.66 ± 0.42 (a decline of 61.7 ± 5.62%, $t(10) = 4.134$, *P<0.05, WT n=5, App^{NL-F} KI n=7, Student's t-test). The expression in the DG was 1.69 ± 0.34, while the WT was 3.34 ± 0.29 (49.4 ± 3.28% reduction, $t(9) = 3.594$, *P<0.05, WT n=5, App^{NL-F} KI, n=6, Student's t-test). In human studies, post-mortem tissue from AD patients showed reductions of 55 ± 0.68% and 8.6% in the DG

and CA1 regions compared to age-matched control brains (n=5, DG: t(8) = 4.394, *P<0.05; CA1: t(8) = 2.342, *P<0.05), as shown in Figure 28.

There was no significant difference in the expression of δ-$GABA_AR$ subunits among the AD mouse models. To implement the 3Rs and reduce the number of animals used in scientific research, the rest of the study was conducted using the *App*$^{NL-F}$ KI mouse model age-matched to WT unless otherwise stated. Furthermore, this model was chosen because it had a higher probability of producing the right homozygous genes than the *App*$^{NL-F}$*/MAPT* dKI model.

Our immunofluorescence findings, shown in Figures 27 and 28, confirmed the overall decline in δ-$GABA_AR$ subunit expression in AD tissue.

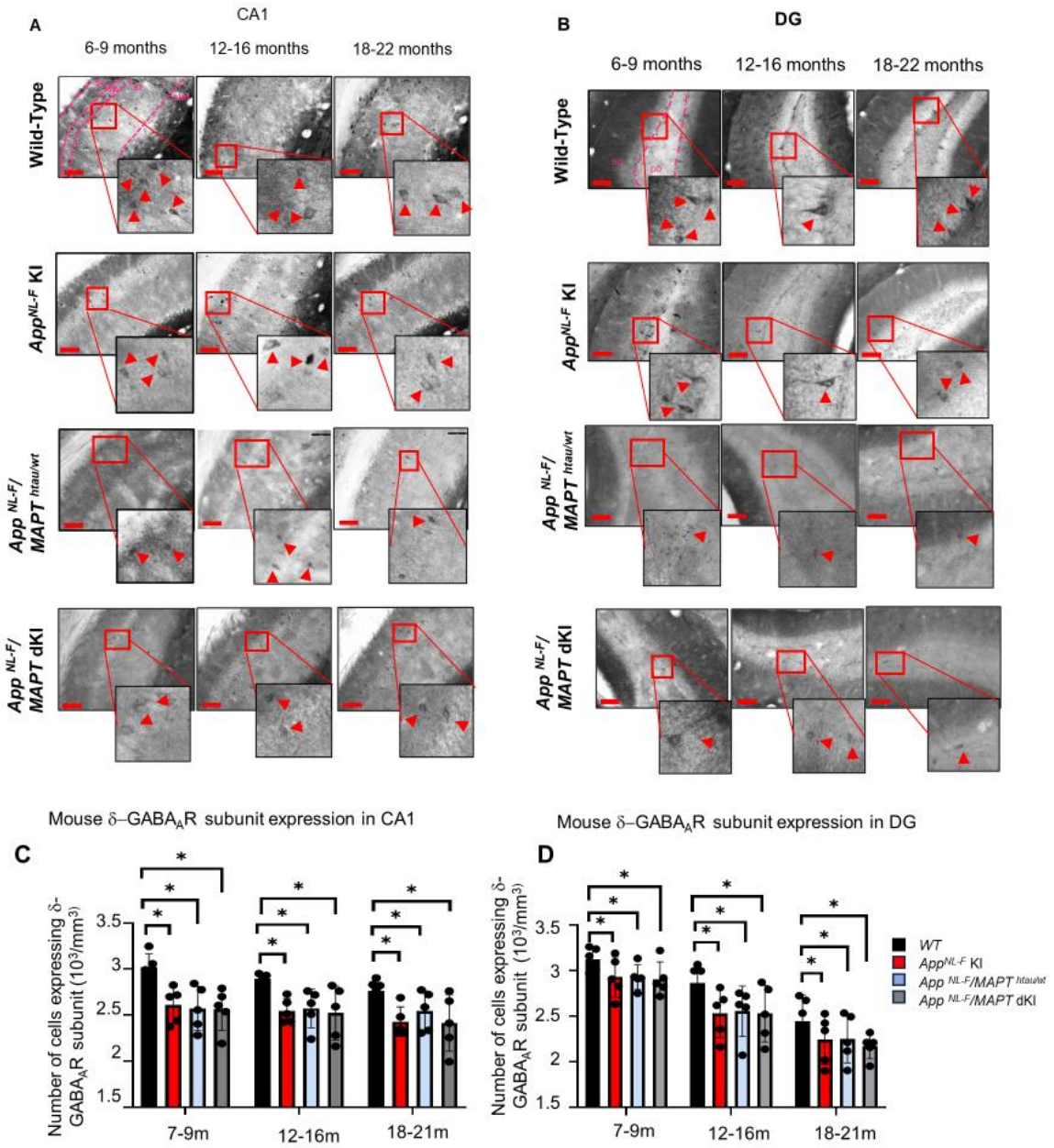


Figure 26 Alteration of the density of δ-GABA$_A$Rs in the CA1 and DG in AD. Representative immunoperoxidase staining of δ-GABA$_A$Rs with 52k δ-subunit-specific polyclonal antibody in WT, *App^{NL-F}* KI, *App $^{NL-F}$/MAPT $^{htau/wt}$ and App $^{NL-F}$/MAPT* dKI AD mouse models at 3 different age-windows in layers of CA1: *so- Stratum oriens, sp - Stratum pyramidale, sr- Stratum radiatum and slm - Stratum lacunosum*). Scale bar 10 µm (inserts scale bar 20 µm)**. (C-D)** Graphs show quantification of δ-GABA$_A$R expression levels in AD mouse models and WT mice in CA1 and DG regions. AD mouse models show a significant decline in δ-GABA$_A$R expression than age-matched WT mice in CA1 and DG. The results are represented by mean ± SEM (**P≤0.05;* Independent unpaired t-test with Shapiro-Wilks test as an outlier test)**.**

3.6.2 δ-$GABA_A$Rs are selectively expressed in sub-classes of inhibitory interneurons.

In order to study which interneurons these receptors were expressed on, we performed colocalization studies using the *App*$^{NL-F}$ KI mouse model to age-matched WT mice to investigate whether these subunits were on calretinin (CR), (shown in Figure 27), and parvalbumin (PV) interneurons (shown in Figure 28 and 29), which aligned with the expression of the stomata of these cells in SP and SLM in CA1 and expressed in the stratum granulosum polymorphic layer intersection and deep hilus of the DG.

Firstly, there was a reduction in the cell densities of PV, but not CR cells in the later stages of AD (Figures 26-29). For example, PV cells showed a reduction of 47% in CA1 and 48% in DG of *App*$^{NL-F}$ KI mice when compared with the WT group (Figure 28F) (*P<0.05, CA1: $t(10) = 5.815$, DG: $t(10) = 4.931$, n=6, Student's t-test). Overall, for WT and AD models, the δ-$GABA_A$R subunits were not colocalized with CR (Figure 27 (A-D) but were colocalized PV cells (Figure 28C-D (images shown at x63 magnification) and H). A Pearson's transformation test threshold value above 0.5 is accepted as a strong colocalization (Mukaka, 2012). For our PV and δ-$GABA_A$R colocalization, we observed a stronger colocalization of 0.6. However, there was very little colocalization of the δ-$GABA_A$R subunits with the CR cells and δ-$GABA_A$ Rs, which was below 0.2 for both genotypes studied (Figure 27D).

To address this further, we examined the colocalization of PV cells with δ-$GABA_A$ Rs using confirmed cases of AD from post-mortem human brain sections and compared these data to control human brains, where we found a significant decline in the expression of the PV cells as well as colocalization of the PV with δ-$GABA_A$ Rs, similar to the expression in our mouse model of AD (Figure 29).

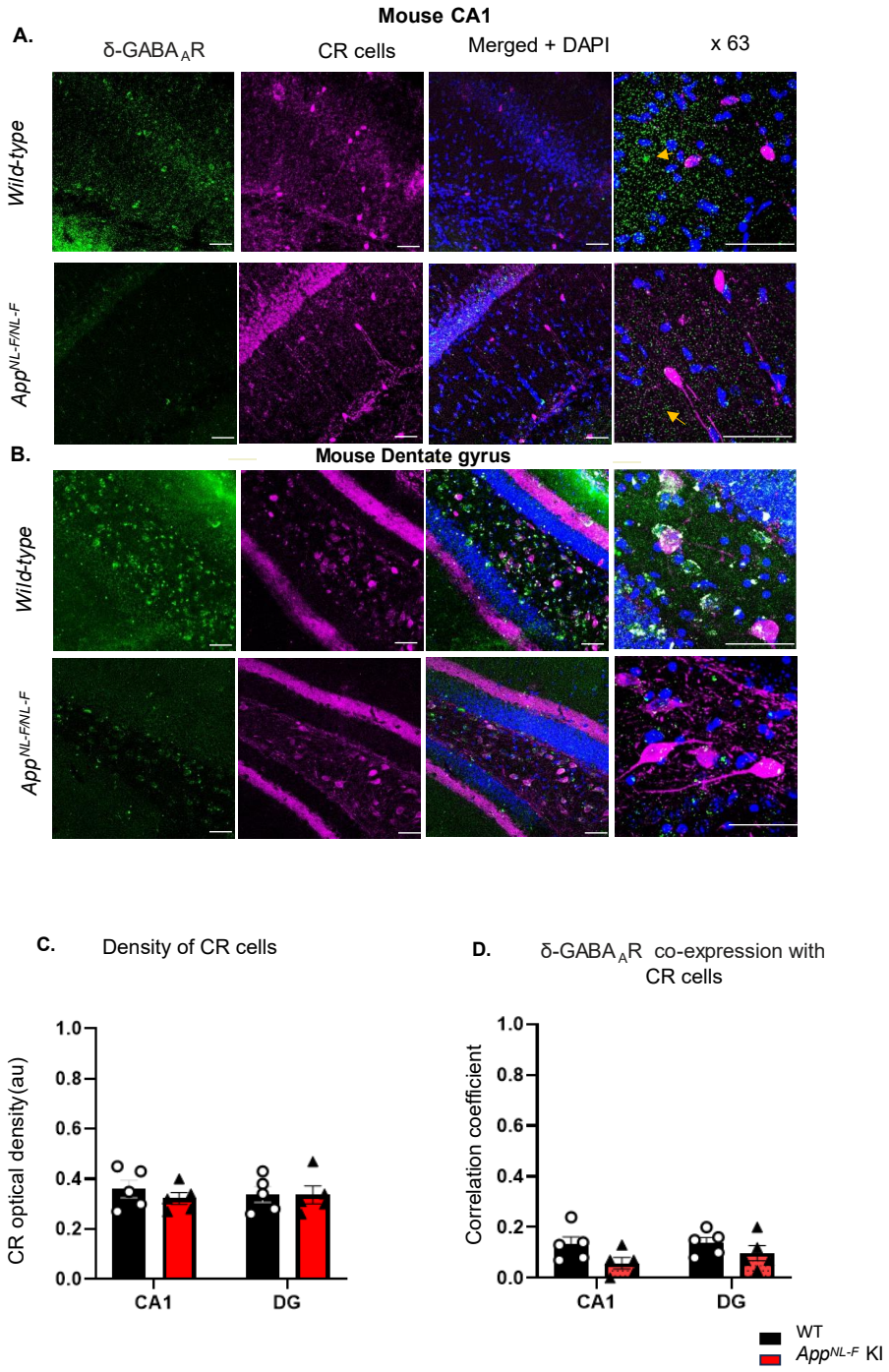


Figure 27: δ-GABA$_A$Rs are not present in CR -expressing interneurons in the hippocampus. A, B) An example of an immunofluorescence image of WT and *App^{NL-F}* KI mice with δ-GABA$_A$Rs and CR cells (magenta) in CA1 and DG regions. C) There was no significant difference between age-matched WT and *App^{NL-F}* KI mice in CA1 and DG regions (*P>0.05, n=5, Student's t-test*). D) There was low colocalisation between CR and δ-GABA$_A$Rs in CA1 and DG in WT and *App^{NL-F}* KI mice (*P>0.05,* n=5, *Student's t-test*). Yellow arrows show delta cells where there is no colocalization with CR.

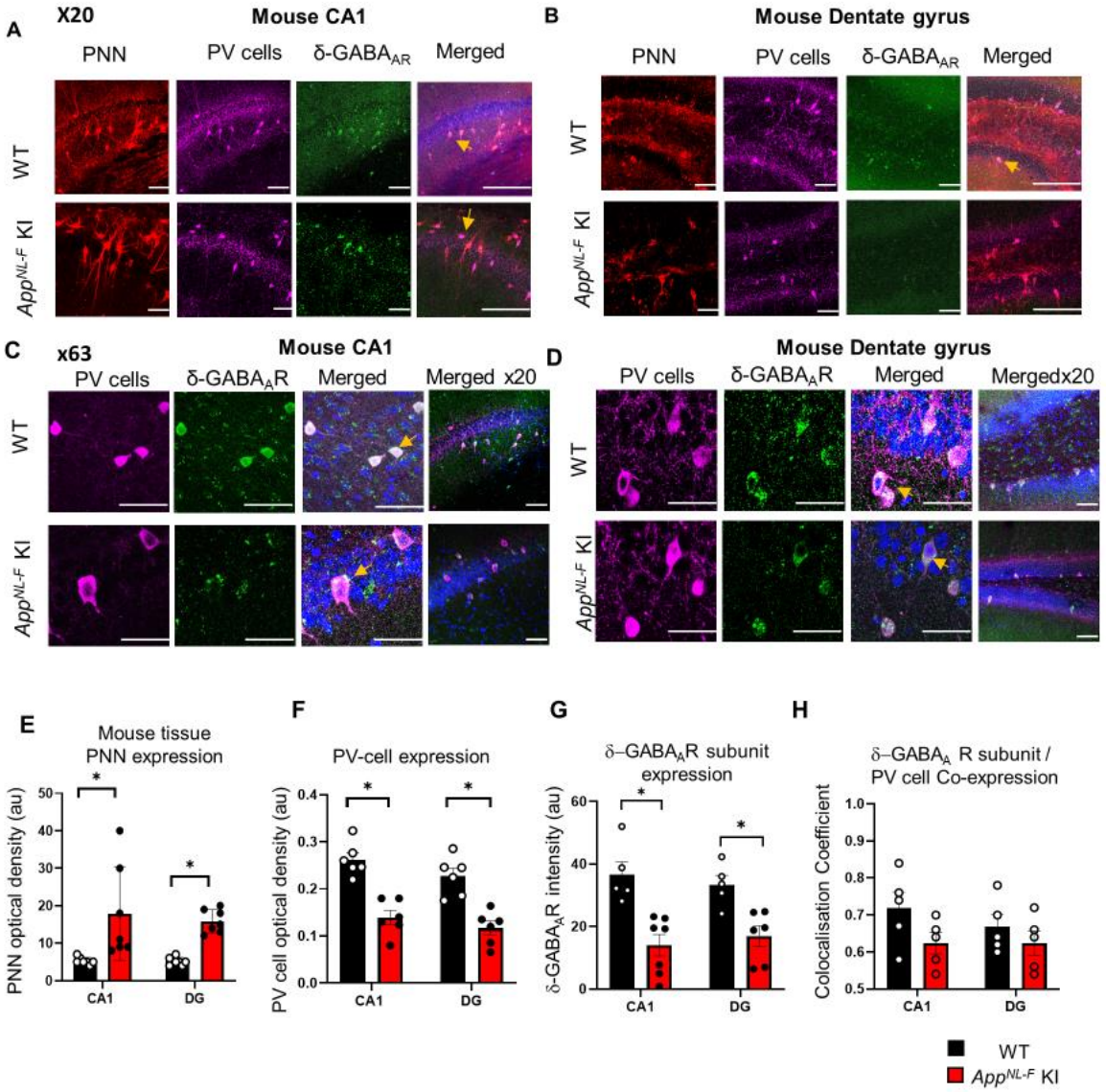


Figure 28: δ-subunit of GABA$_A$R expression analysis in old WT and App^{NL-F} KI mice. Immunohistochemistry staining of GABA$_A$R δ-subunit (GABRD) with 52k δ-subunit-specific polyclonal antibody in coronal brain sections of 12–16-month-old *App^{NL-F}* KI mice compared with age-matched WT animals. Both CA1 and dentate gyrus regions of *App^{NL-F}* KI mice showed low-intensity staining of GABA$_A$R δ-subunit compared with WT animals. Sections were co-stained with GABR(green), PV-containing cells (magenta), PNN (red) and DAPI (blue). GABA$_A$R δ-subunit is co-stained with PNN (Perineuronal nets) and PV cells under 20x and 63x magnifications. White represents colocalization between δ-GABA$_A$R and PV cells, indicated by the yellow arrowheads. Scale bar 50 µm. ***(C-D)*** GABA$_A$R δ-subunit is co-stained with PV cells only to show clarity in of co-localisation under x63 magnification. **(E- H)** Graphs show quantification of PNN, PV, δ-GABA$_A$R and colocalization between PV cells and δ-GABA$_A$R expression levels in WT and *App^{NL-F}* KI mice, a coefficient index between 0.5-1**,** represents a strong colocalization of these two proteins (**P<0.05, n =5*, *Student's t-test*).

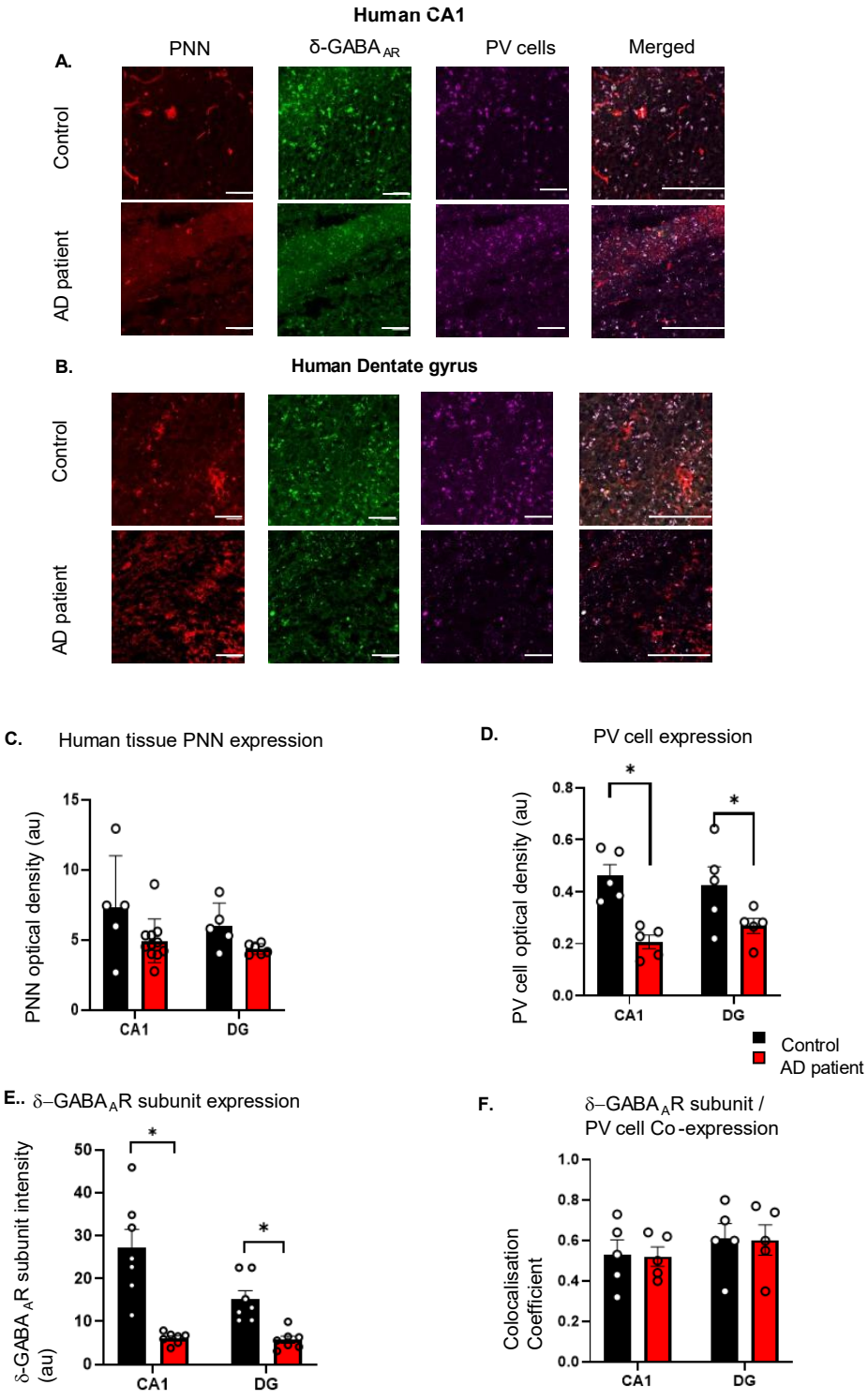


Figure 29: δ-subunit of $GABA_AR$ expression and analysis in human AD patients. (A, B) Human AD and control coronal brain sections co-stained with GABRD (green), PV (magenta) and PNN (red). Shown AD patient ID: P35/18, Control patient ID: P80/17. Scale bar 50 µm. (C- F) Graphs show quantification of PNN, PV, δ-$GABA_AR$ and colocalization between PV cells and δ-$GABA_AR$ expression levels in CA1 and DG regions from post-mortem human AD patients. Graphs show a significant reduction in the expression of PV cells and δ-$GABA_ARs$ in human post-mortem AD tissue compared to the control tissue of CA1 and DG regions (*P<0.05, n =5, Student's t-test).

3.6.3 Discussion: Age-dependent alteration of extrasynaptic δ-GABA$_A$R expression in *App^{NL-F}* KI, *App $^{NL-F}$ /MAPT $^{htau/wt}$* and *App $^{NL-F}$ /MAPT* dKI mouse models of AD

The goal of this chapter was to examine how the progression of AD is associated with δ-GABA$_A$R alterations. We have measured and compared the levels of δ-GABA$_A$R in AD mice and humans to those in a healthy control group using immunoperoxidase and immunofluorescence. Over three age windows—6–9, 12–16, and 18–21—we have seen a progressive decline in δ-GABA$_A$R in *App^{NL-F}* KI, *App^{NL-F} /MAPT* dKI, and *App^{NL-F} /MAPT $^{htau/wt}$* compared to WT. Furthermore, δ-GABA$_A$Rs expression in the hippocampal regions was found to be lower in AD patients compared to controls.

Both in this work and earlier ones, the PV and δ-GABA$_A$Rs decreased in post-mortem brain tissue of AD patients as compared to age-matched control counterparts (Govindpani et al., 2020; Limon et al., 2012). Others have demonstrated that the PV-expressing GABAergic interneurons exhibit high expression of δ-GABA$_A$Rs (Ferando & Mody, 2013). Moreover, degeneration of these interneurons has been documented in the hippocampus of the AD rodent models and proposed to be a factor in epileptiform activity and cognitive deterioration in AD (Hijazi, Heistek, Scheltens, et al., 2020; Petrache et al., 2019). Considering the role of the δ-GABA$_A$Rs, which mediate tonic inhibition and are thought to affect synaptic plasticity, memory, neuronal excitability, and anxiety, one can explain these effects resulting from the deterioration of PV-expressing interneurons (Marowsky & Vogt, 2014; Vossel et al., 2013; Wu et al., 2014). Defects in learning and memory have also been linked by others to diminished tonic inhibition brought on by decreased δ-subunit expression in the dentate gyrus granule cells (Lee et al., 2016). Functional characterisation of channels containing the δ-subunit revealed that the presence of the δ-subunit reduced the rate of acute desensitisation of GABA-evoked currents and, later, the rate of recovery in the presence of GABA as well as the maximum GABA channel open state probability, so maintaining the channels in the open state for longer (Eaton et al., 2014; Farrant & Nusser, 2005; Saxena & Macdonald, 1994).

Even though our mouse and human investigations are consistent, it is crucial to take into account for translational purposes the species variations in δ-GABA$_A$Rs expression between rodents and people. Both human and rodent brains contain δ-GABA$_A$Rs; in humans, these regions are more specific, with a high concentration in the thalamus and hindbrain (Waldvogel et al., 2010), whereas in rodents, they are expressed more abundantly throughout the hippocampus and cortex (Sperk et al., 1997). Moreover, there might be differences between

rats and humans in the heteropentameric architectures of $GABA_AR$s including the δ subunit (Sente et al., 2022). Because of these variations, medications that target δ-$GABA_AR$s may act differently in rats than in humans. As such, future research should think about testing new therapeutic ideas in human cell-based models.

3.7 Results VII: The changes of anxiety levels and AD hallmarks induced by the δ-$GABA_A$R-specific positive allosteric modulator (PAM), DS2

The 2α, 2β, and 1γ subunits are the general stochiometry of most $GABA_A$ receptors. On the other hand, 2α, 2β, and 1δ subunits make up a subpopulation of receptors, where the δ subunit takes the place of the γ subunit. Low ambient GABA concentrations can activate $GABA_A$ receptors with the δ-subunit, which are mainly located in peri- or extrasynaptic sites (Belelli et al., 2009). In fact, there is minimal desensitisation and great sensitivity to GABA in δ-$GABA_A$ receptors (Bright et al., 2011; Houston et al., 2009). According to Mody(Mody, 2005), GABA "spill-over" from the synapse is thought to play a role in the tonic activation of extrasynaptic receptors when it reaches a concentration in the low μM range. Electrophysiological experiments utilising brain slice preparations have corroborated this idea by showing that the $GABA_A$ receptor antagonists picrotoxin, gabazine, and bicuculline reduce basal current in particular neurones (Chandra et al., 2006; Jensen et al., 2013; Jia et al., 2005).

δ-containing receptors are becoming more and more significant pharmacological targets from a therapeutic standpoint. Crucially, classical $GABA_A$ receptor modulators that operate through the benzodiazepine binding site situated between the γ- and α-subunits do not affect δ-subunits receptors. However, other recognised $GABA_A$ receptor modulators, like neurosteroids, etomidate, and barbiturates, do improve the functionality of δ-$GABA_A$ receptors (Belelli et al., 2005). These latter modulators show little selectivity for δ- over γ-containing receptors, making it impossible to verify the physiological or pathophysiological role of δ-$GABA_A$ receptors pharmacologically (Belelli et al., 2002; Jensen et al., 2013; Zheleznova et al., 2008). One example is gaboxadol, a moderately δ-selective agonist that has shed light on the function of δ-$GABA_A$ receptors in the regulation of sleep. For instance, in δ0/0 mice, gaboxadol's ability to increase tonic inhibition in thalamocortical neurons and to cause hypnosis and ataxia was lessened (Herd et al., 2009).

There aren't many known selective negative allosteric modulators (NAMs) and only a few number of reported selective positive allosteric modulators (PAMs) for δ-$GABA_A$ receptors. Regarding its actions at α4β3η2 and α1β3η2 receptors, imidazopyridine DS2 is a functionally selective α4β3δ PAM. Its effects on the tonic current of thalamic ventrobasal (VB) neurons are evident, and they are mediated by α4β2δ receptors (Wafford et al., 2009). According to Hoestgaard (Hoestgaard-Jensen et al., 2010), the triamino-benzene molecule AA29504, which is an analogue of retigabine, was recently reported as a functionally selective α4β3δ

PAM. However, conclusive results are not possible due to incomplete concentration–response analysis at $GABA_A$ receptors that contain δ and γ. However, AA29504 penetrates the brain, enhances the effects of gaboxadol in cortical brain slices, and functions well in certain in vivo models. Similar to AA29504, JM-11-43A is a dihydropyrimidinone that has been described as a selective α4β3δ PAM; however, its selectivity seems to be restricted (Lewis et al., 2010).

It would be intriguing to observe what impact DS2 has on the AD mice models.

3.7.1 Normalisation of anxiety after 5 days treatment with the δ-$GABA_A$R-specific PAM, DS2

Figure 30 illustrates the findings of our (schematic depicted in Figure 29A) involving in vivo dosing of WT and *App*$^{NL-F}$ KI mouse cohorts after 1-hour treatment with vehicle (DMSO) or DS2 at three concentrations: 1, 2, and 4 mg/kg. Figure 30B depicts the impact of these various dosages in mice that had been treated with vehicle or DS2. Even after 1 hour, there was a behavioural shift from the in vivo dose at 2 mg/kg and 4 mg/kg; however, the larger dosage of DS2 was not well taken by a few of the mice, as shown by the Grimace scale. Mice began to exhibit indications of distress, including eyelid closure and reduced sensitivity to stimuli, as measured on a scale of 5 (a grimace range of 5-8 indicates concern).

We administered 2 mg/kg of the medication and vehicle to conduct additional behavioural tests. Using this optimal dose of 2 mg/kg, we treated three AD mice models with vehicle or DS2 for 5 consecutive days before assessing their anxiety levels using the dark-light chamber experimental paradigm. These findings revealed that after 5 days of DS2 treatment, anxiety levels in all three AD models returned to normal when compared to vehicle-treated mice, as depicted in Figure 30D. v KI mice showed a 20.72 ± 8.61% drop in anxiety compared to their baseline level ($t(10) = 2.814$, $P<0.05$, n=6, Student's t-test). This reduction was comparable to *the App*$^{NL-F}$ */MAPT* $^{htau/wt}$ and *App*$^{NL-F}$ /MAPT dKI mice, indicating that after DS2 treatment, anxiety levels returned to baseline control WT mice levels, regardless of the AD model's genotype. However, no significant changes in cognition were observed after 5 days of DS2 treatment, as shown in Figure 30E, although there was a minor trend of increased cognitive function.

After administering the δ-selective PAM, DS2, we observed a "normalisation" in the expression of δ-receptors in the *App*$^{NL-F}$ KI mouse model (Figures 30). *App*$^{NL-F}$ KI mice had significantly higher amounts of δ-$GABA_A$Rs in the CA1 and DG areas compared to the age-matched

vehicle-treated cohort (52.3% and 56.3%, respectively). Surprisingly, the expression of PNNs after DS2 therapy in the AD model was equivalent to WT mice. Figures 30 show that the δ-$GABA_AR$ and PNN expression profiles in the AD model are "normalised" and similar to those in control mice.

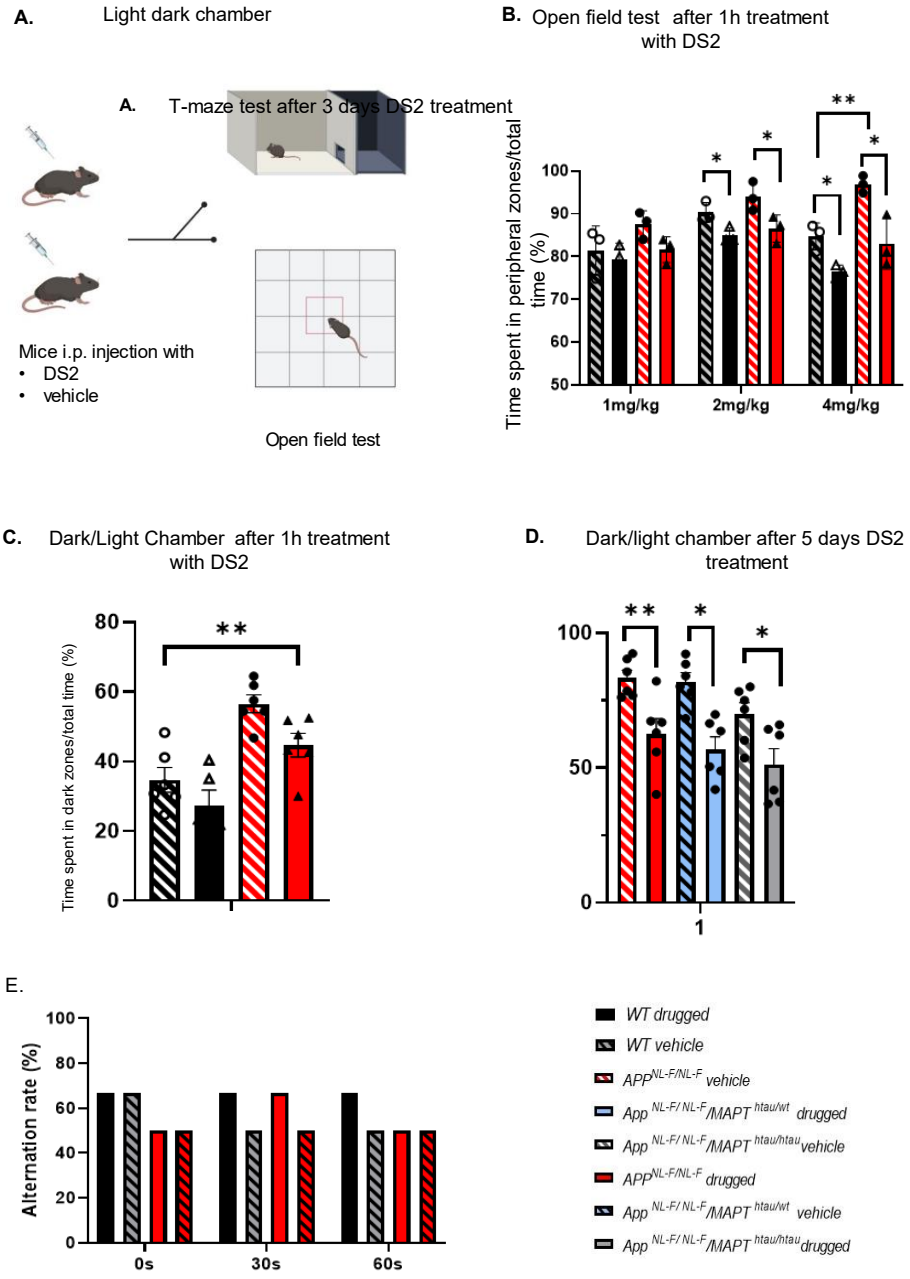


Figure 30: Treatment with a positive allosteric modulator of δ-GABA$_A$Rs reduced anxiety in *App* mouse models of AD. (A) Schematic of DS2 drug injection and light-dark chamber test. (B) Dose response curve of DS2 drug in WT and *App*$^{NL-F}$ KI mice in the open field test. Dosage range 1 mg/kg, 2 mg/kg and 4 mg/kg. A significantly lower magnitude of anxiety was seen in both WT and *App*$^{NL-F}$ KI mice in DS2-treated groups compared to the vehicle group (**P<0.05, **P<0.01, n=3, Student's t-test*). (C) Anxiety normalised in 3 AD mouse models after 1 hour of DS2 treatment in the light-dark chamber experiment. All three AD mouse models showed a significantly lower anxiety level after DS2 treatment compared to vehicle-treated groups (***P<0.01, *P<0.05, n=6, Student's t-test*). (D) Anxiety level of both WT and *App*$^{NL-F}$ KI mice was reduced in the dark-light chamber after 5 days of DS2 treatment (***P<0.01, *P<0.05, n=6,* two-way ANOVA). (E) Changes in the working memory of WT and *App*$^{NL-F}$ KI mice after 5 days of treatment of DS2 drug. There were no significant changes in the working memories of WT and *App*$^{NL-F}$ KI mice after DS2 treatment (*P > 0.05, n=6, two-way ANOVA*).

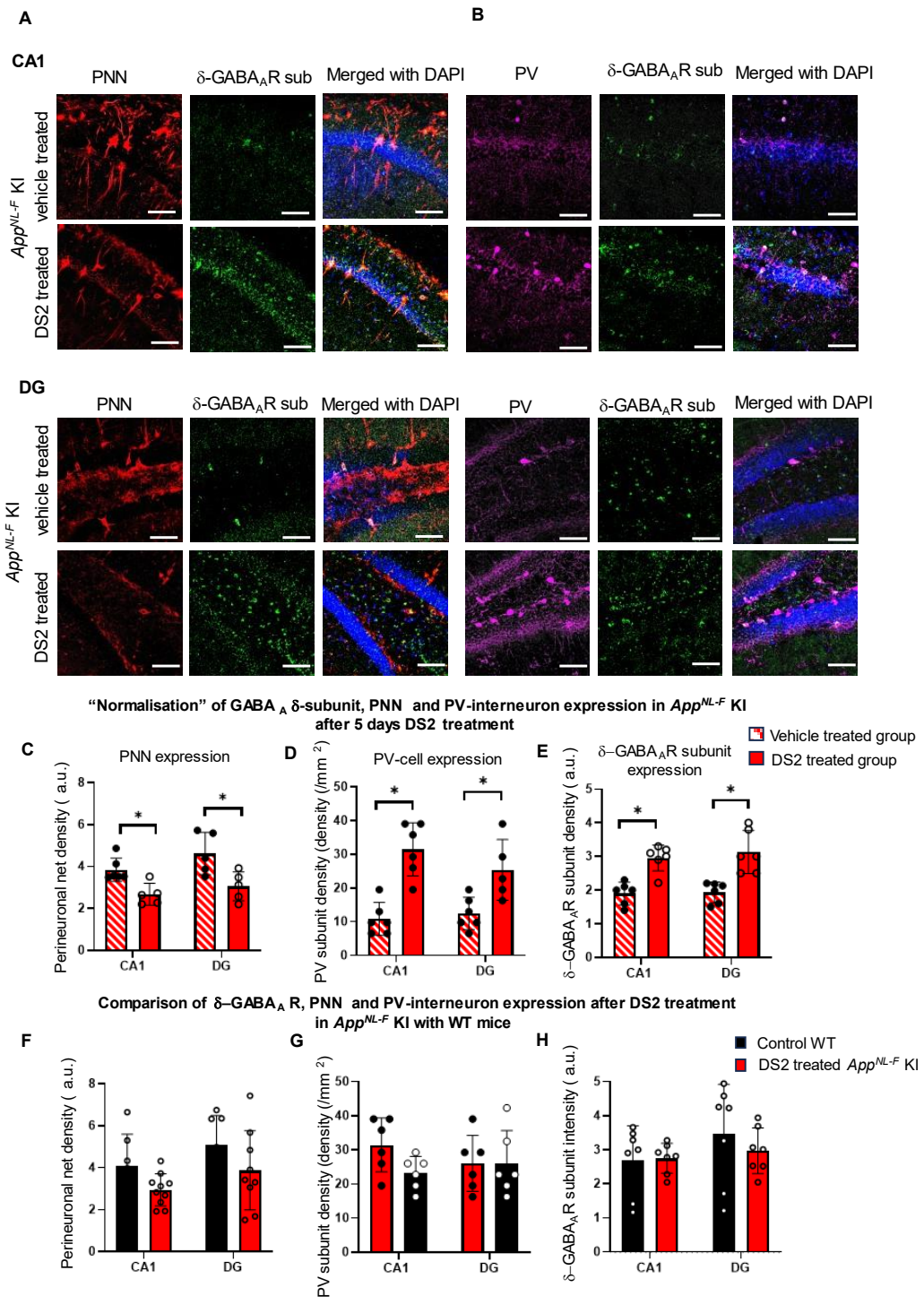


Figure 31: Recovery of the expression of δ-GABA$_A$Rs and PNNs after treatment with DS2. Immunohistochemistry staining of δ-GABA$_A$Rs, PNNs (red) and PV (magenta) in 12–16-month-old DS2-treated *App^{NL-F}* KI mice showed compared with vehicle-treated *App^{NL-F}* KI mice in CA1 and DG regions. (C-E) DS2-treated *App^{NL-F}* KI mice show significantly more expression of δ-GABA$_A$Rs and PV and less PNN expression than same-aged DMSO vehicle-treated *App^{NL-F}* mice. DS2-treated *App^{NL-F}* KI mice had similar δ-GABA$_A$R, PV and PNN expression levels compared with age-matched control WT mice in CA1 and DG regions (**P<0.05, n=6, Student's t-test*).

3.7.2 Behavioural association with anatomical changes in extrasynaptic δ-$GABA_A$Rs and AD hallmarks after treatment with the δ-$GABA_A$R-specific PAM, DS2

We investigated whether treatment with DS2 would have any effects on "normalizing" the deficit in the expression of PNN and PV-expressing cells and δ-$GABA_A$Rs, and whether neuroinflammatory markers, including GFAP, CD68, and TREM2 altered in any way. The expression of δ-$GABA_A$ Rs after 5 days of treatment with DS2 resulted in a "normalization" in the deficit in the expression of these receptor subunits in the App^{NL-F} KI mouse model. We observed 27.1% and 24.7% significant increases in the levels of δ-$GABA_A$Rs in both CA1 and DG regions of App^{NL-F} KI mice compared to the age-matched vehicle-treated cohort (CA1 $t(10) = 5.015$, DG $t(10) = 4.519$, $P<0.05$, n=6, Student's t-test) (see Figure 31E). This δ-$GABA_A$Rs expression profile was similar to that of control WT tissue.

These changes in the expression of δ-$GABA_A$ Rs were consistent with normalization of the expression of PNNs after DS2 treatment and were comparable to the values obtained from control WT mice (Figure 31 F - H). For example, for CA1 and DG, the expression of PNNs decreased by 32% and 34%, respectively (CA1: $t(8) = 3.491$, DG: $t(8) = 2.888$, $P<0.05$, n= 5, Student's t-test) after DS2 (Figure 31C) and were comparable to the WT baseline PNN values (Figure 31 F). Furthermore, after the 5-day treatment with DS2, there was a significant reduction in GFAP and CD68 compared with vehicle-treated cohorts, as shown in Figure 32. This reduction in GFAP and CD68 for App^{NL-F} KI mice was 31.3 ± 16.5% and 22 ± 4.2 % in CA1 compared to the vehicle-treated cohorts, respectively (GFAP: $t(8) = 2.783$, CD68: $t(8) = 3.31$, $P<0.05$, n=5, Student's t-test). There was a reduction in the expression of these parameters also in the WT: reduction by 29 ± 5.6 % and 16.3 ± 5.1% of GFAP, CD68, respectively, compared to the vehicle-treated cohorts, (GFAP: $t(8) = 2.511$, $P<0.05$, CD68: $t(8) = 1.789$, $P>0.05$, n=5, Student's t-test). No significant changes were observed in the expression of Aβ and PV cell expression in both genotypes (Figure 32).

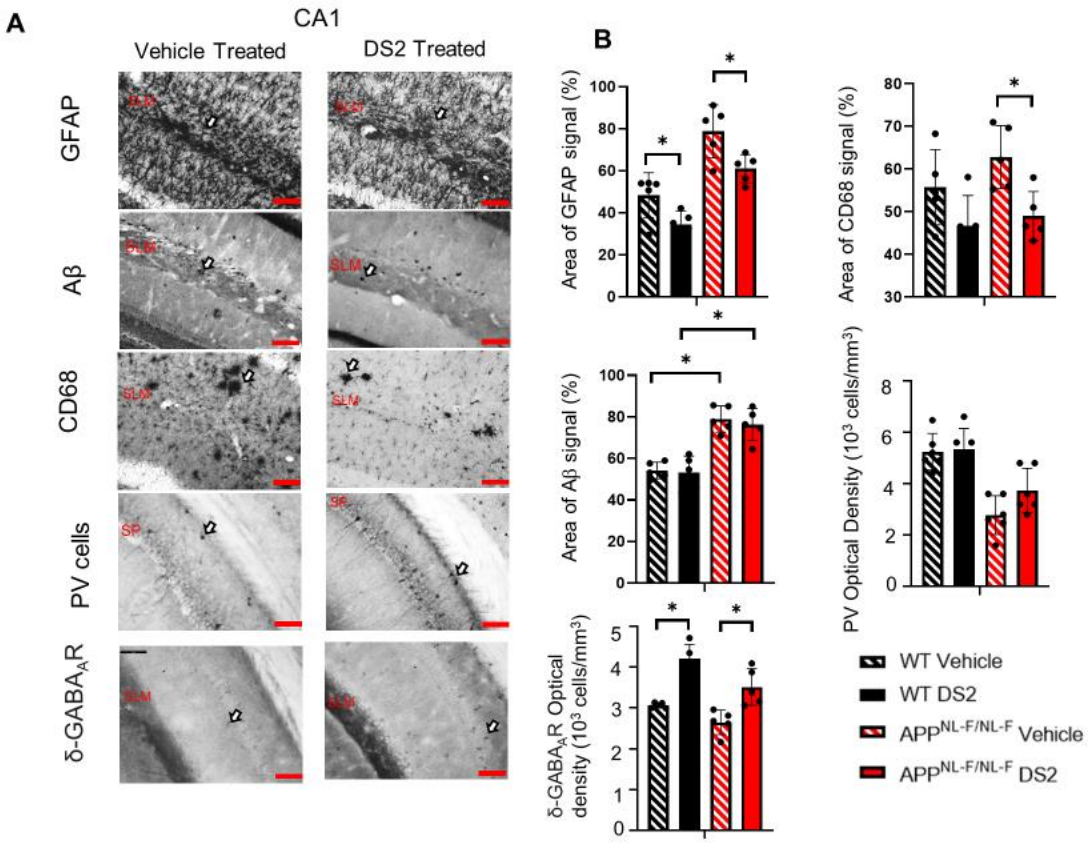


Figure 32: Recovery of neuroinflammation after treatment with DS2. (A) Representative immunoperoxidase image of WT and *App*$^{NL-F}$ KI mice stained for GFAP, Aβ, CD68, PV and δ-GABA$_A$Rs. Cells are indicated by white arrows. (B) DS2-treated *App*$^{NL-F}$ KI and WT mice showed significant increases in GFAP and δ-GABA$_A$R expression compared with DMSO vehicle-treated *App*$^{NL-F}$ KI and WT mice respectively. DS2-treated *App*$^{NL-F}$ KI mice showed a significant increase in CD68 cell density compared to vehicle-treated same genotype. There was no significant increase in Aβ level in both WT and *App*$^{NL-F}$ KI mice after DS2 treatment. In addition, there was a trend of increase in PV cell density in DS2-treated *App*$^{NL-F}$ mice compared with vehicle-treated (**P<0.05, n=5, Student's t-test*).

3.7.3 Discussion: Could δ-$GABA_A$Rs be a novel target for anxiety and memory deficits in AD?

By dosing mice with a δ-selective-compound 2 (DS2, 2 mg/kg), we investigated whether in vivo positive allosteric regulation of the δ-$GABA_A$Rs is linked with decreased anxiety. Key results from the light-dark chamber and open arena tests indicated that the anxiety displayed by the DS2-treated mice was much lower than that of the vehicle-treated mice. Previous research that found reduced excitatory/inhibitory synaptic imbalance was consistent with loss of PV- and somatostatin (SST)-expressing interneurons that correlated with high magnitude of anxiety in the 5xFAD animal model of AD, supports this. Anxiety-like behaviours were reduced and synaptic balance was restored with activation of the PV or SST interneurons in the ventral CA1 of these mice (via selectively expressing hM3Dq, an engineered form of the M3 muscarinic receptor) (Li et al., 2022). Comparably, anxiety-related behaviour in a rat with fragile X syndrome was shown to be improved by gaboxadol, a selective agonist of extrasynaptic δ-subunit-containing $GABA_A$Rs. This study revealed that Gaboxadol was effective in correcting anxiety-related behaviour, which is frequently seen in people with fragile X syndrome (Cogram et al., 2019). This suggests that treatments aimed at extrasynaptic $GABA_A$Rs may be helpful in the treatment of conditions associated to anxiety. Remarkably, the FDA has recently approved brexanolone (allopregnanolone) for the treatment of postpartum depression (Kanes et al., 2017). The neurosteroid allopregnanolone acts non-selectively on synaptic and extrasynaptic $GABA_A$Rs by enhancing inhibition at both synaptic γ2-subunit and extrasynaptic δ-subunit-containing $GABA_A$Rs. These results show promise since anxiety management requires more focused and selective treatments that target the primary systems involved in the pathophysiology of anxiety and have fewer adverse effects and a quicker start of action.

lower cellular markers of neuroinflammation shown by lower activated astrocytes was associated with decreased anxiety in the DS2-treated AD animals and WT mice. Moreover, the "normalisation" of PV- and PNN-expression and "recovery" of the downregulated δ-$GABA_A$Rs in the hippocampus were two outcomes of DS2 treatment. Future research in this area is necessary even if we did not find a discernible improvement in cognitive performance following DS2 treatment in this trial.

To conclude, investigations on δ-$GABA_A$Rs could help to produce a novel drug to treat anxiety and cognition dysfunction in AD patients.

4. General Discussion

The objective of this thesis was to further our knowledge of the pathogenic processes of AD. The study employed a top-down approach to examine the symptoms of AD, such as memory impairment and anxiety indicators, in three preclinical AD mice models: *App*$^{NL-F}$ KI*, App* $^{NL-F}$*/MAPT*$^{htau/wt}$ and *App* $^{NL-F}$*/MAPT* dKI. In addition,the study also investigated the structural changes in specific inhibitory interneurons expressing CR, CCK, and PV, which were found to be associated with neuroinflammation and the accumulation of Aβ aggregates. Furthermore, this study used *in vivo* dosing to evaluate a new therapeutic target for individuals suffering from anxiety and AD. The key findings demonstrated that:

1) The cognitive deficits observed in these 3 mouse models of AD, evidenced from the behavioural test, varied compared to the age-matched WT mice; however, there was no difference between the *App*$^{NL-F}$ KI, *App*$^{NL-F}$*/MAPT dKI* and *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ *age-matched* mice studied at three different time windows.
2) *App*$^{NL-F}$ KI, *App*$^{NL-F}$*/MAPT dKI* and *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ showed significantly higher anxiety level compared to WT at 12-16 months old, which was accompanied by increased accumulation of Aβ, microgliosis and astrocytosis.
3) An increase in GAD67, an enzyme responsible for converting glutamate to inhibitory neurotransmitter GABA levels was observed in the *App*$^{NL-F}$ KI mouse model.
4) In AD mouse models, the CR cells were maintained, however, the PV and CCK cells exhibited a notable decline in CA1 of the hippocampus compared to age-matched WT animals at 12-16 months of age
5) Enhanced activation of δ-GABAARs significantly reduced the expression levels in the CA1 region of AD mouse and human patients.
6) DS2 lowered the anxiety level in AD mice models and recovered the downregulated δ -$GABA_A$Rs, PV and other hallmarks of AD.

In 4 mouse models there was a time-dependent alternation in the pathology of AD in Aβ and tau and neuroinflammation. Overall, there was an increase in reactive astrocytes in the presence of tau while other AD hallmarks remained incomparable to *App*$^{NL-F}$ KI.

4.1 *App $^{NL-F}$ /MAPT dKI* mice: a better mouse model for AD research?

From our NOR/NOL and T-maze tests, all our AD mouse models showed proportionately more significant differences in the reduced working memory correlated with the disease hallmarks (elevation Aβ, astrocytes, and microglia) compared to WT. In addition, our open field test and light-dark chamber data showed higher anxiety levels in AD mouse models compared to WT. However, there is no significant difference in cognition function or anxiety levels between these three AD models: *App^{NL-F}* KI, *App $^{NL-F}$/MAPT $^{htau/wt}$* and *App $^{NL-F}$ /MAPT dKI*.

No significant difference was detected between Aβ, tau, and neuroinflammation markers in comparison with these three AD mice models studied. This suggested that humanised tau insertion did not exacerbate Aβ aggregation, which was consistent with data from other studies (Benskey et al., 2023; Saito et al., 2019).

The work employed the *App^{NL-F}*, *App $^{NL-F}$/MAPT $^{htau/wt}$* and *App $^{NL-F}$ /MAPT dKI* mouse model, which accurately represents late-onset familial Alzheimer's disease (FAD). This model is based on two fAD mutations that were initially discovered in Swedish and Iberian families. These mutations specifically affect the cleavage of APP (Masuda et al., 2016; Saito et al., 2014). Nevertheless, the majority of Alzheimer's disease (AD) cases are sporadic, meaning they occur randomly. Although genetic variables are involved, such as carrying APOE e4 or TREM2 mutations, it is widely agreed that a mix of genetic and environmental factors is responsible for most AD occurrences.

One further constraint is that the previous *App^{NL-F}* KI model does not consider tau pathology, which is a characteristic feature of AD. One possible solution to overcome this obstacle would be to incorporate tau pathology into the model for future investigations. There are multiple approaches available for accomplishing this task: viral injection, inducing pathology by introducing substances from experimental brain damage or breeding with a genetic mouse model that exhibits tau pathology. Humanised MAPT knock-in mice were crossbred with *App^{NL-F}* KI mice to tackle the limitation of *App^{NL-F}* KI animals in not exhibiting tau pathology on their own.

In conclusion, the absence of obvious pathogenic alterations in *App $^{NL-F}$ /MAPT dKI* mice renders it a perfect model for future explorations into malfunctions of tau protein and the course of AD in vivo.

4.2 Cell death may be the result of depleted synaptic activity as it has been established during this investigation that not only is there a loss of cell density

In various AD mouse models studied previously, amyloid plaques were characterised by highly dysmorphic neurites and spine turnover, causing a net loss of spines. Constant Aβ overproduction at dendrites or axons acts locally to reduce the number and plasticity of synapses (Griffiths & Grant, 2023; Meyer-Luehmann et al., 2008; Zhang et al., 2022). Furthermore, in hippocampal cultures, soluble Aβ causes abnormalities in spine composition, shape and abundance that are consistent with the hypothesis of soluble Aβ initiating toxic mechanisms for synaptic damage in AD progression and continuous exposure to Aβ caused abnormal spine morphology and a significant decrease in spine density (Gu & Guo, 2021; Lacor et al., 2007). Others have also shown that local dendritic and axonal abnormalities associated with Aβ deposits result in loss of synapses and dendrites and axon destruction in AD progression (Parihar & Brewer, 2010; Zhang et al., 2021).

Crossing APP transgenic mice with tau transgenic mice, the AD mouse model with both Aβ and tau have implicated tau as a major mediator of Aβ toxicity at the postsynaptic compartment and dendritic spines (Yu & Lu, 2012). Similar to previous cultured neurons results, removing endogenous tau can prevent Aβ -induced behaviour deficits in an AD mouse model showing human APP and stop excitotoxin-induced neuronal dysfunction (Rapoport et al., 2002). Synaptic physiological data shows that tau decline recovers some abnormalities in hippocampal subregions of hAPPJ20 mice (Roberson et al., 2011). In conclude, these studies support the argument that hyperphosphorylated tau proteins contribute to AD pathogenesis by promoting the Aβ accumulation (Hardy & Selkoe, 2002).

In the present study, *App*$^{NL-F}$ KI, *App*$^{NL-F}$*/MAPT* dKI and *App* $^{NL-F}$*/MAPT*$^{htau/wt}$ mouse model replicated the formation of amyloid plaques the AD progression similar to the progression in human AD patients, as amyloid plaques only can be seen in post-phenotypic mice expressing symptoms resembling the neurodegeneration diseases. Interestingly, it has been hypothesised that the PV interneuron decline present in the CA1 region is correlated with the amyloid beta ion channel hypothesis. The accumulation of external amyloid beta-senile plaques will result in the amyloid plaques integrating into the neuronal membrane (Lee et al., 2022; Shirwany et al., 2007). Once amyloid beta is integrated into the membrane, plaques allow cations to penetrate, including $Ca^{2+,}$ which affects energy metabolism and leads to oxidative stress (Mattson, 2007; Zhang et al., 2021). Our data supports certain parts of this

theory by the large amount of Aβ accumulation found in CA1. A similar study found that GABA terminals were degenerated around the Aβ plaques, which is consistent with the statement that the build-up of Aβ plaque directly leads to cellular dysfunction in AD patients (Garcia-Marin et al., 2009)Combining this finding with our studies, a valid argument can be proposed that Aβ plaques directly initiate alternation to PV+ cell neurodegeneration by likely allowing cells to be permeable to CA2+, as the hippocampal CA1 region is correlated with a loss of neuronal function and neuronal cell deficiency.

Overall, the specific alteration of the GABAergic interneurons could initiate a degeneration in GABA-mediated inhibition on primary excitatory cells, exaggerating the imbalance of excitation and inhibition in the hippocampus. In addition, the increase of GAD67 in astrocytes correlated with astrocyte-specific GAD3/4 may critically contribute to recovering the synaptic imbalance because of the essential role of healthy astrocytes in modulating the proper extracellular environment for normal neuronal function. The rise of GAD67 and GAT3/4 in astrocytes has been shown to be involved in modulating tonic background inhibition by uptaking excess extracellular glutamate in astrocytes via EAAT2 co-transporters with 3 Na^+ ions, increasing the intracellular Na^+ level, which meanwhile reverse the GAT3/4 channel mechanisms, leading to GABA expulsion from the synaptic cleft (Aldabbagh et al., 2022; A. Shi et al., 2020). Whether GAD67 and GAT3/4 level changes in the new AD mouse model *App* $^{NL\text{-}F}$*/MAPT*$^{htau/wt}$ would be of tremendous clinical significance.

4.3 Reduced expression levels of δ-$GABA_A$Rs in AD mice models

Extrasynaptic receptors mediate much of the overall GABA-mediated inhibition, in fact they are involved in over 90% of the GABA-mediated transmission (Cope et al., 2005). It has also been demonstrated that these receptors, By detecting presynaptic GABA levels in the thalamus, dynamically change cell inhibition (Brickley & Mody, 2012; Herd et al., 2013). These receptors may be active comparatively longer than phasic γ receptors because they exhibit low maximal open-state probability, acute sensitivity to GABA, and little desensitisation at saturating levels of GABA (Eaton et al., 2014; Farrant & Nusser, 2005; Hannan et al., 2020). Tonic inhibition is further linked to neurogenesis, synaptic plasticity, and cognitive functioning (Ge et al., 2006; Lee et al., 2016). Given their significance in the structure of neuronal excitability, early manipulation of extrasynaptic $GABA_A$ receptors may be seen as a useful therapy for AD-mediated hyperexcitability and excitotoxicity. Higher efficacy can be obtained with substances like THIP, gaboxadol, muscimol and neurosteroids (Meshkat et al., 2023; Sugasawa et al., 2019), even though GABA seems to be only a partial agonist of extrasynaptic receptors (as they show low efficacy in the presence of GABA with IMAX values threefold lower than the γ-containing receptors). Found only extrasynaptically, the subunits δ, α6, α5, ρ, and π influence gradual, continuous inhibition in the central nervous system (Brickley & Mody, 2012; Sente et al., 2022). The δ subunit of extrasynaptic receptors mostly forms complexes with the α6 or α4 subunit; α4βδ receptors have been localised to thalamic relay neurons, dentate gyrus, striatal medium spiny neurons, and neocortical pyramidal cells; the α6βδ complex has been mapped to cerebellar granule cells (Arslan, 2021; Sente et al., 2022). Long channel opening times combined with reduced desensitisation may result in more complex and effective inhibitory networks in rosette-like inhibitory cells, including the olfactory bulb, periglomerular cells, granule cells of the cerebellar cortex and thalamocortical neurons. Both in human patients and animal models, mutations in the δ subunit have been linked to seizures (Kienitz et al., 2022; Pressly et al., 2022). Additionally revealed to be downregulated in the middle temporal lobe tissue of AD patients investigated in vitro, this component contributes to excitatory-inhibitory imbalance and cognitive impairment (Govindpani et al., 2020). Moreover, δ receptors lower seizure susceptibility and encourage network shunting in concert with other extrasynaptic receptors. Preventing seizures requires the slow recovery of GABA currents displayed by these receptors, and it has been demonstrated that their downregulation in the dentate gyrus causes seizures-like symptoms (Bampali et al., 2023; Feng et al., 2022). Downregulation of δ subunit-containing $GABA_A$ receptors may thus be responsible for the

quiet epileptic activity or the onset of seizures in AD patients. However, more investigation is required to clarify the causes.

Furthermore, some intriguing and encouraging findings have been obtained by targeting $GABA_A$ receptors. Eight weeks of positive allosteric modulator activation of $GABA_A$ receptors in an AD animal model reduced pathogenic aspects of AD, such as Aβ synthesis, and enhanced cognitive function (S. Q. Zhang et al., 2013; Korpi & Sinkkonen, 2006). $GABA_A$ receptors are thus a major target for a number of neuropsychiatric disorders like anxiety and epilepsy as well as a symptomatic target for neurodegenerative diseases like AD.

4.4 The δ-$GABA_A$R-specific PAM, DS2 reduced anxiety and neuroinflammation in AD mouse models

Our study showed that DS2 dosing lowered the anxiety level of *App*$^{NL-F}$ KI mice back to the control WT anxiety level.

Other studies support our study that anxiety can be reduced by targeting on extrasynaptic $GABA_A$Rs in the hippocampus. In an elevated plus maze, for example, the infusion of allopregnanolone, a neurosteroid that functions as a positive allosteric modulator of $GABA_A$Rs, into the dorsal CA1 area was demonstrated to be c (Mòdol et al., 2011); however, this effect was not sustained (Engin & Treit, 2007). Allopregnanolone exhibits considerable efficacy on α4$GABA_A$Rs, despite not being selective for a single subtype of $GABA_A$Rs (Belelli et al., 2002; Chase Matthew Carver & Doodipala Samba Reddy, 2013).

Other studies also showed that DS2 reduced inflammation. Other work shows that DS2 can function by reducing inflammation and immune cell activation, providing DS2 with a novel mode of action. Functional $GABA_A$ receptors are produced by innate and adaptive immune cells (Fuks et al., 2012), and studies have shown that binding GABA reduces inflammation (Bhat et al., 2010; Reyes-García et al., 2007). This has been connected, mechanistically, to a GABA-mediated reduction in the production of IL-1β, IL-6, and IL-12 after LPS-stimulated innate immune cells, such as peritoneal macrophages.

In addition, DS2 lowered the inflammation level as well as being anxiolytic. Administration of the δ-subunit-selective drug, DS2, resulted in a reduced inflammatory response in murine macrophages and BMDCs(Neumann et al., 2019). Research indicates that a variety of murine and human innate immune cells, including peritoneal macrophages (Reyes-García et al., 2007), human peripheral blood mononuclear cells (Alam et al., 2006), monocytes, and immortalised monocytic cell lines (Wheeler et al., 2011), exhibit high levels of δ-subunit expression. Currently, there is one drug under clinical trials working on GABA receptors but not extrasynaptic δ-GABAAR. ‘Allopregnanolone’ from the University of Arizona, the allosteric modulator of GABA-A receptors, is currently at the phase 2 stage (Cummings et al., 2024).

To conclude, it is tempting to hypothesise that the δ-subunit is an essential component for understanding anti-inflammatory responses and anxiety through $GABA_A$ receptors. DS2 might has the potential to offer a new and innovative therapy for AD.

4.5 Limitations

4.5.1 Minor anxiety in mice transfer

Despite sufficient time for mice to habituate to the experiment equipment, minor anxiety unavoidably occurred during mouse transfer and handling. This would slightly alter the behaviour test results in the novel location/object recognition test. Anxiety will make mice stay still in the test environment and affect their choices. We have tried to minimum this effect as low as possible. All experimental animals in this investigation were taken care of by the same biological service unit (BSU) personnel and the same experimenter. In order to reduce the influence of environmental signals or the experimenter on behavioural trials, the mice were consistently handled by the same experimenter starting at 4 months of age in the same Biological Safety Unit (BSU). The researcher would regularly measure the weight of the mice and conduct health assessments using the Grimace Scale.

4.5.2 Human and Rodent difference

Familial AD (FAD) results in an earlier age of onset and different neuropathological and clinical features compared to sporadic late-onset (sAD)(Drummond & Wisniewski, 2017; Knopman, 2015; Tellechea et al., 2018). Since transgenic mouse models are mostly predicated on over-expression of APP and PSEN1 with FAD-linked mutations, these distinctions between sAD and FAD may affect the transferability of therapeutic findings.

Transgenic mice make up the great bulk of animal models utilised in AD research. The human APP and the wild-type mouse APP (695 isoform) share 97% of their sequences. Three amino acids in the Aβ sequence (R5G, Y10F, and H13R) are among the significant sequence changes between humans and mice (Drummond & Wisniewski, 2017; Tanzi et al., 1987; Xu et al., 2015). In wild-type mice, these variations hinder Aβ aggregation and stop amyloid plaques from forming. Consequently, the development of amyloid plaques in mice requires the expression of human APP. Despite having higher Aβ synthesis, the transgenic mice in the early transgenic models that expressed wild-type human APP did not consistently exhibit widespread AD-associated neuropathology (Boutajangout & Wisniewski, 2014; Dujardin et al., 2015; Puzzo et al., 2015). The production of human APP with mutations linked to FAD, on the other hand, produced consistent plaque pathology and variable levels of the ensuing downstream AD-associated clinical characteristics. The FAD mutation, the promoter, and the background mouse strain all have a significant impact on the

precise phenotype of each of the numerous transgenic strains that have been created. One obstacle to the translatability of success in these models is that the pathology of the great majority of AD transgenic models depends on the expression of FAD mutations, and the majority of AD clinical trials are carried out in sAD patients, whose pathogenesis differs significantly from that of FAD.

While our research on mice and humans shows similar results, it is also crucial to take into account the variations in δ-$GABA_A$R expression between these two species when considering the applicability of our findings to people. The distribution of δ-$GABA_A$Rs in the brains of rodents and humans differs. In rodents, δ-$GABA_A$Rs are more abundant in the hippocampus and cortex, as observed in a study by Sperk et al. in 1997. However, in humans, the expression of δ-$GABA_A$Rs is more specific to these regions, with a higher concentration in the thalamus and hindbrain, as reported by Waldvogel et al. in 2010. Moreover, there may be many forms of the heteropentameric structures of $GABA_A$Rs that include the δ subunit in both rats and humans (Sente et al., 2022). The variations between these two could potentially result in variations in function, meaning that medications designed to target δ-$GABA_A$R might produce different effects in rodents as opposed to humans. Future research should thus incorporate the utilisation of human cell-based models to evaluate innovative therapeutic advancements.

4.7 Future Experiments

4.7.1 Further animal experiments

There is a lack of data in App $^{NL-F}$/MAPT dKI mice which was a direct result of the COVID-19 pandemic. More NOR/NOL experiments have been conducted on these age-grouped mice when conditions allow. In addition, Power calculations or literature guidelines have guided the selection of the sample size in the experiments that are being described. But because of the difficult circumstances over the past year, the sample size was somewhat smaller than planned in one instance. The examination of anxiety in the 12–16-month WT animals was based on just 6 animals, as opposed to the typical range of 8–12. Thus, a larger sample size would be advantageous for that investigation.

4.7.2 New compound targets on δ-$GABA_AR$

App^{NL-F} KI showed a trend of improved working memory in T maze after DS2 dosing. The no significant difference in memory might be due to the low blood-brain barrier penetration of DS2 (Jensen et al., 2013; L'Estrade et al., 2019). If there is a novel drug with better penetration, it might show memory improvement in the AD mice models. Our lab is currently conducting preliminary experiments on the new MDI drugs from Prof John Atack, Cardiff University. It has been proven to show better BBB penetration compared to DS2. As a result, it could yield distinct outcomes and potentially introduce a new focus for the treatment of AD.

4.7.3 Molecular experiments

The immunohistochemistry studies conducted in this study were rigorous, duplicated, and published in peer-reviewed papers. However, in order to enhance comprehension and achieve more precision in comprehending the processes, it may be advantageous to supplement them using molecular biology approaches. Quantifying cellular mRNA using RT-PCR would provide more accurate information about the amounts of receptor expression in WT mice vs different AD mouse models. It would uncover subtle alterations in gene expression. Western Blotting methods can be used to identify the immunity and neurogenesis markers like Trem2 and wnt/B-catenin. This would contribute further comprehensive data to enhance the understanding of the course of AD.

5 Conclusion

In conclusion, we present evidence that memory impairments were associated with comparable anxiety levels displayed by mouse models carrying *App* mutations, and humanised tau associated with a decrease in δ-$GABA_AR$ expression. In terms of dementia, positive allosteric modulation of these receptors showed promise since it affected the markers of neuroinflammation (astrocytosis and gliosis,) and partially "normalised" the reduced expression.

Based on the pharmacology of extrasynaptic $GABA_ARs$ and the data presented in this work, it is possible that extrasynaptic $GABA_ARs$ could be a promising therapeutic target because of the altered expression of δ-$GABA_ARs$ in anxiety and depression (Zanettini et al., 2016). Since these receptors are linked to a lower likelihood of resistance and dependence, it can be

concluded that medicines that target them may be more effective than SSRIs and benzodiazepines (Zheleznova et al., 2009). Our main findings thus require additional research since they have significant implications for both the general public and AD patients. Future research is also necessary to examine the combined inhibitory effects of extrasynaptic $GABA_A$Rs mediating tonic inhibition and synaptic $GABA_A$Rs mediating phasic inhibition.

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